

DETECTION OF VIABLE *SALMONELLA* IN LETTUCE BY PROPIDIUM
MONOAZIDE REAL-TIME PCR AND CONTROL OF FOOD-BORNE
PATHOGENIC BACTERIA BY NONI JUICE

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ABSTRACT

Pathogenic bacteria have caused a large number of foodborne illnesses in the United States. Detection and control of these pathogens in food are two important measures to enhance the microbiological safety of food. The first objective of this study was to develop a molecular assay for specific detection of viable *Salmonella* cells in lettuce. Propidium monoazide (PMA), a DNA-modifying dye, was used to treat dead *Salmonella* cells. Real-time polymerase chain reaction (PCR) results suggest that PMA treatment effectively cross-linked with DNA from as high as 10^8 CFU/g dead *Salmonella* Typhimurium cells in lettuce. The PMA real-time PCR assay could selectively detect viable *Salmonella* at as low as 10^2 CFU/ml in pure culture and 10^3 CFU/g in lettuce. Combining a 12-hour enrichment with the assay allowed for the detection of viable *Salmonella* at 10^1 CFU/g in lettuce. The PMA real-time PCR assay provides a simple yet accurate tool for detection of *Salmonella* in food.

Moreover, this study also aimed to evaluate the antimicrobial effect of noni juice on major foodborne pathogenic bacteria, including *Escherichia coli* O157:H7, *Salmonella* Typhimurium, *Listeria monocytogenes* and *Staphylococcus aureus*, in distilled water, a laboratory medium and a food model. After 24 hours of incubation at 35 °C, 10% noni juice reduced the count of *E. coli* O157:H7, *S. Typhimurium*, *L. monocytogenes* and *S. aureus* in distilled water by 5.2, 4.8, 5.0, and 4.2 log, respectively, compared with the control. The treatment suppressed *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* in tryptic soy broth by 5.8, 8.4 and 7.9 log,

respectively, after five days of incubation. Finally, the antimicrobial activity of noni juice was evaluated in artificially contaminated mushroom soup. At 7 °C, 10% noni juice inactivated *L. monocytogenes* of 5.02 log CFU/ml in mushroom soup on day 6. At 35 °C, the treatment reduced the count of *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* in mushroom soup by 8.7, 9.7, and 8.2 log, respectively, after five days of incubation. In addition to being a functional beverage, noni juice holds great promise as a natural preservative.

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CHAPTER 1

INTRODUCTION

Consuming safe and nutritious foods is the foundation of human health. Unfortunately, pathogenic microorganisms pose a great threat to food safety. It is estimated that each year 31 major pathogens cause 9.4 million foodborne illnesses, 55,961 hospitalizations and 1,351 deaths in the United States (Scallan et al., 2011). Advanced technologies are urgently needed to ensure that food is safe to consume. Microorganisms could contaminate food during planting, harvesting, processing, storage, and delivery. Therefore, controlling the growth of microorganisms in food and screening of food for contamination by harmful microbes are two important measures to reduce the risk of foodborne illness.

Leafy greens, such as lettuce, are usually consumed raw by most Americans. Therefore, it is imperative that the lettuce in the market is free of pathogens. Traditional culture-based detection methods and nucleic acid-based detection methods for pathogenic bacteria have inherited disadvantages. For example, cultural-based detection methods take four to five days to show results; DNA-based detection methods cannot differentiate between viable and dead target cells. The first purpose of this study is aimed to establish a propidium monoazide real-time polymerase chain reaction assay for rapid and accurate detection of *Salmonella* in lettuce. Propidium monoazide is dye can selectively penetrate dead cells and react with DNA to reduce the PCR signal from DNA of dead bacterial cells (Rudi et al, 2005). Propidium monoazide real-time PCR can selectively quantify viable *Salmonella* cells.

Various preservation methods have been invented and employed by humans to enhance food quality and safety. Pasteurization, canning, freezing, refrigeration, dehydration, and preservatives are commonly used to extend the shelf-life of food.

Scientists have kept exploring new antimicrobial agents which are safe to consume and could be used as food additives. Natural preservatives have gained increasing popularity since the last decade. Plants represent a major source of natural antimicrobial agents. The rich biodiversity of plants in Hawaii provides us with a plenty of materials for this purpose. Noni (*Morinda citrifolia* L.) grows widely throughout the Pacific and has long been used in folk medicines by Polynesians. It was found that tissue fluids from ripe noni fruits could inhibit the growth of *Escherichia coli*, *Micrococcus pyogenes*, *Pseudomonas aeruginosa*, *Salmonella typhose*, *Salmonella montevideo*, *Salmonella schottmuelleri* and *Shigella paradysenteriae* (Bushnell et al., 1950). Noni juice is produced by fermenting noni fruits for several months. Many other chemical components produced after fermentation, could potentially increase the antimicrobial effect, which has, however, been unknown to scientific society. Therefore, the second purpose of this study was to investigate the potential of noni juice as a natural preservative. The antimicrobial effect of noni juice on major foodborne pathogenic bacteria was evaluated in synthetic and food models.

CHAPTER 2

LITERATURE REVIEW

2.1 Foodborne illness

Foodborne illness is any illness caused by the consumption of foods contaminated by pathogenic bacteria, viruses, parasites, or toxic substances. The symptoms of foodborne illness vary from mild gastroenteritis to serious hepatic, neurologic, renal and optical syndromes (Mead et al, 1999). For example, *Campylobacter jejuni*, a harmful bacterium mainly associated with poultry, commonly causes gastroenteritis in humans (Janssen, 2008). *Escherichia coli* O157:H7 leads to hemorrhagic colitis and occasionally kidney failure in young children and the elderly. *Toxoplasma gondii*, an important zoonotic parasite, infects about one third of the world population causing congenital infections and eye disease (Ryan and Ray, 2004). In the United States, foodborne diseases have been estimated to cause 47.8 million illnesses, 127,839 hospitalizations, and 3,037 deaths yearly (CDC, 2011). Thirty-one major pathogens account for approximately 9.4 million illnesses, 55,961 hospitalizations, and 1,351 deaths (Scallan et al., 2011). Foodborne diseases in the U.S. cost approximately 8.4 billions dollars each year in medical expenses and productivity losses. Bacterial and viral diseases account for 84% of the total cost. Among the known agents, *Salmonella* and *Staphylococcus aureus* have the largest costs at 4.0 billion and 1.5 billion dollars per year, respectively. Following them, other types of illnesses notable in expense are toxoplasmosis (445 million dollars), listeriosis (313 million dollars), *E. coli* infections including hemorrhagic colitis (223 million dollars), campylobacteriosis (156 million dollars), and *Clostridium perfringens* enteritis (123 million dollars) (Todd, 1989).

Raw foods of animal origin, including raw meat and poultry, raw eggs,

unpasteurized milk, and raw shellfish, are the most associated with foodborne illness. In addition, fresh produce are highly risky if consumed raw (Montville and Matthews, 2005). Fruits and vegetables can potentially be tainted by deadly organisms due to exposure to environmental contaminants, improper processing, and non-sanitary consumer handling. Produce-associated outbreaks account for an increasing proportion of all reported foodborne illness outbreaks associated with a known food item, rising from 0.7% in the 1970s to 6% in the 1990s (Sivapalasingam et al., 2004). Among produce-associated outbreaks, the food items accounted for high proportions included salad (mixed vegetables and fruits), lettuce, juice, melon, sprouts, and berries (Table 2.1) (Sivapalasingam et al., 2004). Among 103 produce-associated outbreaks associated with a known pathogen, 30 were caused by *Salmonella* (Sivapalasingam et al., 2004). This data suggests that lettuce is the leading single fresh produce associated with foodborne illness outbreaks and *Salmonella* is the leading pathogenic bacteria associated with produce contamination.

TABLE 2.1. Type of produce items implicated in foodborne illness outbreaks in the United States, 1973 through 1997 (Sivapalasingam et al., 2004).

Produce items	No. of outbreaks
Multiple produce items	105
Salad	76
Mixed fruit	22
Mixed vegetables	7
Single produce items	85
Lettuce	25
Melon	13
Seed sprout	11
Apple or orange juice	11
Berry	9
Tomato	3
Green onion	3
Carrot	2
Apple	1
Pear	1
Pineapple	1
Basil	1
Celery	1
Cucumber	1
Fresh elderberry juice	1
Fresh-squeezed lemonade	1

2.2 Pathogenic bacteria

Despite various causes of foodborne illness, pathogenic bacteria remain the predominant agents. Twenty-one major food-related bacteria account for approximately 3.6 million illnesses, 35,796 hospitalizations, and 861 deaths per year in the U.S. (Scallan et al., 2011). According to the U.S. Department of Agriculture/Economic Research Service (USDA/ERS) (1996), six bacterial pathogens present in food products, including *Salmonella*, *E. coli* O157:H7, *L. monocytogenes*, *S. aureus*, *Campylobacter jejuni*, and *C. perfringens*, cost 2.9 billion to 6.7 billion dollars in human illness annually in the United States.

2.2.1 *Salmonella*

Salmonella is one of the leading causes of foodborne illness throughout the world. Estimates indicate that *Salmonella* accounts for approximately 1.03 million illnesses, 19,533 hospitalizations, and 378 deaths per year in the U.S. (Scallan et al., 2011). This results in a cost of around 2.8 billion dollars annually in medical expenses and lost productivity (Bishwa et al., 2004). There are thousands of serotypes in this genus. *Salmonella* serotype Typhimurium is the most common in the U.S. This pathogen can induce a strong host innate immune response. The symptoms of salmonellosis include gastroenteritis, typhoid fever, and bacteremia (Coburn et al., 2007). There have been several high-profile *Salmonella* outbreaks involving leafy greens. A total of 361 cases of *Salmonella* Typhimurium infections associated with lettuce occurred in England and Wales between August and September in 2000 (Horby and others 2003). In the fall of 2009, 124 people were sickened across the U.S. due to *S. Typhimurium*-contaminated lettuce (Falkenstein 2009). There were large outbreaks of *Salmonella* Senftenberg infection linked to basil (Pezzoli et al., 2008).

2.2.2 *Escherichia coli* O157:H7

Escherichia coli is Gram-negative rod-shaped bacterium that originally inhabits the intestines of humans and animals. Most *E. coli* strains are harmless, but some serotypes can make humans sick. The most notorious is *E. coli* O157:H7. This serotype belongs to the enterohemorrhagic *E. coli* (EHEC), which has the ability to produce Shiga toxins (Griffin, 1995). *E. coli* O157 infections cause 73,000 illnesses, more than 2,000 hospitalizations and 60 deaths annually in the U.S. The recent estimated annual cost of illness due to *E. coli* O157 infection reached \$405 million, among which \$370 million for premature deaths, \$30 million for medical care, and \$5 million in lost productivity (Frenzen et al., 2005). *E. coli* O157:H7 can cause hemorrhagic colitis which is characterized by abdominal pain, bloody diarrhea and fever. In some cases, around 3-7% of patients develop deadly hemolytic uremic syndrome (HUS). HUS occurs in all ages but most often in children under 10. The associated complications are pallor, intravascular destruction of red blood cells, depressed platelet counts, lack of urine formation, and acute renal failure. Cattle are the primary reservoir of this pathogen. Common vehicles for *E. coli* O157:H7 foodborne infections include ground beef and other bovine products. In addition, outbreaks caused by *E. coli* O157:H7 have also been associated with leafy vegetables. There were several *E. coli* O157:H7 outbreaks linked to baby spinach and lettuce (CDC, 2006).

2.2.3 *Listeria monocytogenes*

Listeria monocytogenes is a Gram-positive rod, 1-2 micrometer in length and 0.5 micrometer wide, facultative anaerobe bacterium. It can grow between 3 °C and 45 °C but the optimum temperature range is 30 °C to 37 °C (Murray et al., 1926). The disease caused by *Listeria* is called listeriosis, which is commonly manifested as

septicemia, meningitis, and still birth in pregnant women (Armstrong and Fung, 1993; Gray and Killinger, 1966). The risk populations for listeriosis include pregnant women, newborn babies and people with weak immune systems. Although *Listeria monocytogenes* infection is relatively uncommon, the fatality rate among risk populations is around 30% (Ramaswamy et al., 2007). *Listeria monocytogenes* outbreaks are highly associated with ready-to-eat foods. This is due to the fact that most ready-to-eat foods are stored in the refrigerator. Under this condition, *Listeria monocytogenes* can grow with a generation time of about 24 h whereas many other bacteria cannot (Montville and Matthews, 2005).

2.2.4 *Staphylococcus aureus*

Staphylococcus aureus is a species of Gram-positive, coccus, facultative anaerobic bacteria. *S. aureus* produces a wide variety of exoproteins which are called staphylococcal enterotoxins and contribute to its ability to colonize and cause disease in mammalian hosts (Dinges et al, 2000). As staphylococcal enterotoxins are highly heat-stable, they may be present in food when *S. aureus* cells are killed by heating (Balaban and Rasooly, 2000). Staphylococcal food intoxication is characterized by vomiting, nausea, cramps, diarrhea and headache that occur shortly after ingestion of staphylococcal enterotoxin-contained foods (Montville and Matthews, 2005). Human beings are the main reservoir of *S. aureus*. The pathogen is commonly colonized in the nose or on the skin. It may spread to foods due to improper handling and poor sanitation (Montville and Matthews, 2005).

2.3 Detections methods for foodborne pathogenic bacteria

2.3.1 Conventional detection methods

Detection of pathogenic bacteria in foods is of utmost importance in ensuring the

safety of food supplies as well as for recognizing and intervening in food-related outbreaks. Traditionally, microbial detection is based on cultural enrichment and isolation on non-selective and selective media. For example, the protocol for detection of *Salmonella* in food samples is composed of pre-enrichment in buffered peptone water for 24 hours, enrichment in Pappaport-Vassiliadis broth for 24 hours, isolation on xylose lysine deoxycholate agar, screening of suspect colonies on triple sugar iron agar slant, and confirmation by serological tests (Andrews et al., 1998). The cultural-based detection methods have high sensitivity and specificity. However, these methods are time-consuming and labor-intensive.

2.3.2 Polymerase Chain Reaction

Polymerase chain reaction (PCR) is an analytical technique for *in vitro* DNA amplification developed by Kary Mullis in 1983 (Mullis et al., 1986). Each cycle of PCR consists of three steps: denaturation in which double stranded DNA separates into two single strands at 94-95 °C, annealing in which *Taq* DNA polymerase binds to the primer-template hybrid and begins DNA synthesis, and extension in which two new DNA strands synthesize. Then, the cycle is repeated with double newly generated DNA as the template. PCR is highly sensitive in that millions copies of target DNA in a sample can be generated after 30 cycles in just a couple of hours. The specificity of PCR relies on two primers involved that are designed to flank a fragment of nucleic acid present in the target organism. PCR assays have been established for detection of bacterial pathogens commonly found in food (Hanna et al., 2005; McKillip and Drake, 2004). While PCR methods are sensitive, specific and rapid, they have certain disadvantages. Amplification products need to be verified via laborious gel electrophoresis. Moreover, conventional PCR methods lack quantitative capabilities.

2.3.3 Real-time PCR

Real-time PCR was developed by Saiki in 1985 (Saiki, 1985). This technique not only amplifies a specific target DNA sequence in a sample but also monitors the amplification progress using fluorescence technologies. During amplification, how quickly the fluorescent signal reaches a threshold level correlates with the amount of target DNA, allowing quantification of original target molecules. After amplification, the final product can be further characterized by running melting curve analysis, which helps confirm the melting temperature (GC content) of amplicons. Two fluorescence technologies, SYBR Green and TaqMan probe, are commonly used in real-time PCR. SYBR Green dye can bind to double-stranded DNA generated from PCR amplification and emit light to indicate the concentration of double-strand DNA. The drawback of SYBR Green real-time PCR is its non-specific binding to any double-stranded DNA. TaqMan probe real-time PCR has relatively higher specificity than SYBR Green real-time PCR. The principle relies on the 5' to 3' exonuclease activity of *Taq* DNA polymerase to cleave a dual-labeled oligonucleotide called TaqMan probe during hybridization to the complementary target DNA sequence (Holland et al., 1991). Since real-time PCR has quantitative capability and high sensitivity, it has been increasingly used for pathogen detection and quantification in foods (Norton, 2002). For example, real-time PCR has been successfully applied to detect *Listeria monocytogenes* in cheeses (Rudi et al., 2005) and *Salmonella* in raw milk (Van-Kessel et al., 2003). Like other DNA-based detection methods, however, real-time PCR cannot discriminate between live and dead target cells. Bacterial DNA may remain stable and amplifiable after the cells are dead (Josephson et al., 1993). Therefore, real-time PCR may generate false-positive results in food analysis.

2.3.4 Propidium monoazide real-time PCR

Propidium monoazide (PMA) is a membrane-impermeant dye that selectively penetrates cells with compromised membranes, which can be generally considered dead. The chemical structure of PMA is shown in Figure 2.1. PMA is designed based on ethidium monoazide (EMA), a chemical reducing the PCR signal from DNA of dead bacterial cells (Rudi et al, 2005). EMA has been used in combination with real-time PCR for rapid quantification of viable bacteria in foods and food processing environment (Guy et al, 2006; Rudi et al, 2005). Nevertheless, the limitation of EMA has been found in some studies. For example, EMA treatment prior to PCR results in a significant loss of genomic DNA of viable *Campylobacter jejuni* and *Listeria monocytogenes* cells (Flekna et al, 2007). Nocker et al. (2006) developed PMA from the DNA-binding dye propidium iodide. It has been demonstrated that PMA does not penetrate membranes of viable cells though it is equally efficient as EMA in excluding DNA from dead cells (Nocker et al., 2006).

Upon intercalation in the DNA of dead cells, the photoinducible azide group allows PMA to be covalently cross-linked by exposure to bright light (Figure 2.2) (Nocker et al., 2006). The cross-linking makes double stranded DNA lose the capability to separate into two single strands to be amplified during the following PCR reaction. Combined with PMA treatment, the PCR methods can selectively detect viable cells of target microorganisms (Nocker et al., 2006). Recently, PMA real-time PCR has been used to distinguish viable from dead *Bacillus subtilis* spores (Rawsthorne et al., 2009) as well as detect viable lactic acid bacteria and bifidobacteria in fermented milk (García-Cayuela et al., 2009).

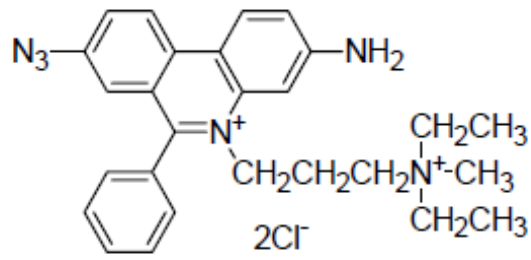


FIGURE 2.1 Chemical structure of propidium monoazide (Biotium, 2009).

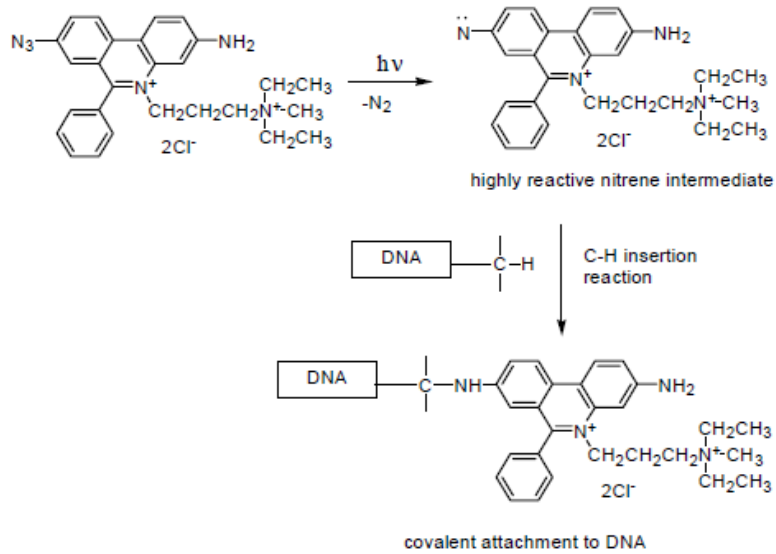


FIGURE 2.2 Cross-linkage reactions between propidium monoazide and DNA under exposure to high frequency wavelength light (Biotium, 2009).

2.4 Food preservatives

2.4.1 History and background

With the increasing demand for convenient foods, food preservatives have played a more important role in modern food technology (Saad et al., 2005). Food preservatives are widely used in preventing the growth of undesirable microorganisms and delaying enzymatic or chemical reactions to extend the shelf life and enhance the quality of food. Food preservatives may be either synthetic compounds intentionally added into foods or naturally occurring biologically derived substances. The most traditional food preservative is sodium chloride, which has long been used in vegetables and meats to extend storage. Furthermore, organic acids (such as vinegar), alcohol, and sugars have been applied to preserve foods. In modern times, other chemicals such as sodium benzoate, nitrite and sulfite have been approved for use as food preservatives (Sofos et al, 1998).

2.4.2 Natural food preservatives

As natural food ingredients become popular, interest in antimicrobial agents originated from natural sources has increased. In contrast, most consumers hesitate to accept synthetic preservatives because some of them may potentially cause adverse effects on human health (Urbain and Campbell, 1987). As a result, a number of antimicrobial agents have been isolated from plants, animals and microorganisms (Beuchat and Golden, 1989; Lopez-Malo et al., 2000). Shan et al. investigated the antibacterial efficiency of five spices and herb extracts (cinnamon stick, oregano, clove, pomegranate peel, and grape seed) against *Listeria monocytogenes*, *Staphylococcus aureus*, and *Salmonella enteric* in cheese. The results indicate that the clove extract possesses the highest antibacterial and antioxidant activity in cheese and holds potential as a natural food preservative (Shan et al., 2010). Another recent

study evaluated the inhibitory effect of essential oils of mint, rosemary, orange and other plants against *Arcobacter butzleri*. The study showed that 0.5% (vol/wt) essential oil of rosemary completely inactivated *A. butzleri* in cooked minced beef stored at 4 °C (Irkin et al., 2010). Other examples of natural antimicrobials that have been successfully applied in foods include egg-white lysozyme, natamycin, and nisin (Sofos et al., 1998).

2.4.3 Noni juice

Noni (*Morinda citrifolia* L.) is a tropical plant widely distributed in Hawaii, Southeast Asian and Australia. The plant is noted for its medical functions and has been used in folk medicines by Polynesians for over 2000 years (Whistler, 1985). Noni is primarily used to stimulate the immune system and fight bacterial, parasitic and fungal infections (Dixon et al., 1999). Scientific studies suggest that noni may have analgesic effect and anti-oxidant, anti-inflammatory, and anti-tumor properties (Akihisa et al., 2007; Basar et al., 2010; Booncha et al., 2006; Hirazumi and Furusawa, 1999; Younos et al., 2009). Noni juice is made by fermenting the fruits in sealed containers for up to two months, recovering the juice through lixiviation and/or mechanical pressure, pasteurizing, and conditioning (Nelson, 2002). The juice has become a popular functional beverage. Constituents in noni fruits may have antimicrobial activity (Locher et al., 1995; Saludes et al., 2002). It was found that tissue fluids from ripe noni fruits could inhibit the growth of *Escherichia coli*, *Micrococcus pyogenes*, *Pseudomonas aeruginosa*, *Salmonella typhose*, *Salmonella montevideo*, *Salmonella schottmuelleri* and *Shigella paradysenteriae* (Bushnell et al., 1950). This indicates that noni juice could potentially be used as a natural preservative to control pathogenic and spoilage bacteria in food.

CHAPTER 3

DETECTION OF VIABLE *SALMONELLA* IN LETTUCE BY PROPIDIUM MONOAZIDE REAL-TIME PCR

3.1 Abstract

Contamination of lettuce by *Salmonella* has caused serious public health problems. Polymerase chain reaction (PCR) allows rapid detection of pathogenic bacteria in food, but it is inaccurate as it might amplify DNA from dead target cells as well. The objective of this research was to investigate the stability of DNA of dead *Salmonella* cells in lettuce and to develop an approach to detecting viable *Salmonella* in lettuce. *Salmonella*-free lettuce was inoculated with heat-killed *Salmonella* Typhimurium cells and stored at 4 °C. Bacterial DNA extracted from the sample was amplified by real-time PCR targeting the *invA* gene. The results indicate that DNA from the dead cells remained stable in lettuce for at least 8 days. To overcome this limitation, propidium monoazide (PMA), a dye that can selectively penetrate dead bacterial cells and cross-link their DNA upon light exposure, was combined with real-time PCR. Lettuce samples inoculated with different levels of dead or viable *S. Typhimurium* cells were treated or untreated with PMA before DNA extraction. Real-time PCR suggests that PMA treatment effectively prevented PCR amplification from as high as 10^8 CFU/g dead *S. Typhimurium* cells in lettuce. The PMA real-time PCR assay could detect viable *Salmonella* at as low as 10^2 CFU/ml in pure culture and 10^3 CFU/g in lettuce. With 12-h enrichment, *S. Typhimurium* of 10^1 CFU/g in lettuce was detectable. In conclusion, the PMA real-time PCR assay provides an alternative to real-time PCR assay for accurate detection of *Salmonella* in some foods.

3.2 Introduction

As one of the most notorious foodborne pathogens, *Salmonella* is estimated to account for 1,027,561 illnesses, 19,533 hospitalizations, and 378 deaths annually in the United States (Scallan et al., 2011). The primary habitat of *Salmonella* is the intestinal tract of animals. Although eggs, poultry and meat products are common foods linked to salmonellosis outbreaks, the infections have also been traced to consumption of leafy green vegetables (Sivapalasingam et al., 2004). There have been several high-profile outbreaks involving lettuce contaminated by the pathogen. For example, 361 cases of *Salmonella* Typhimurium infections occurred in England and Wales between August and September in 2000. Lettuce was identified as the culprit (Horby et al., 2003). In fall 2009, 124 people were sickened across the U.S. due to *S. Typhimurium*-contaminated lettuce (Falkenstein 2009).

Accurate and efficient methods are urgently needed for detection of *Salmonella* in food. While standard culture-based methods are sensitive, four to five days are required to show results (Andrews et al., 1995). Nucleic acid-based techniques, such as polymerase chain reaction (PCR), have been widely employed for rapid screening of foods for pathogenic bacteria such as *Salmonella* (Gallegos-Robles et al., 2009; Hanna et al., 2005). Nevertheless, bacterial DNA may persist in the environment for a long time after cell death (Josephson et al., 1993; Masters et al., 1994). Intact DNA from dead cells may yield false-positive results in PCR, causing unnecessary product recalls and economic losses. Reverse transcription-PCR theoretically can overcome the drawback of PCR because it amplifies bacterial mRNA that has short half-life (Kushner, 1996). Yet the technique suffers from its poor sensitivity due to the instability of target mRNA (Wang and Mustapha 2010).

Ethidium monoazide (EMA), a DNA-modifying dye, can penetrate dead

bacterial cells without intact membrane. Upon exposure to light, the dye can cross link the DNA in dead cells, thus preventing DNA denaturation during subsequent PCR (Nogva et al., 2002). EMA has been used in combination with real-time PCR for rapid quantification of viable bacteria in foods and food processing environment (Guy et al., 2006; Rudi et al., 2005). Recently, Wang and Mustapha (2010) established an EMA real-time PCR assay for detection of viable *Salmonella* in chicken and eggs. The assay could detect the pathogen in a range of 10^3 to 10^9 CFU/ml for pure culture and 10^5 to 10^9 CFU/ml for food samples. Following 12 h of enrichment, it could detect *Salmonella* at as low as 10 CFU/ml in chicken rinse and egg broth (Wang and Mustapha 2010).

It seems that EMA is still inadequate for the differentiation between DNA from viable and dead cells. Nocker and others (2006) showed that EMA could also penetrate live cells of some bacterial species and cause partial DNA loss. In order to improve EMA, Nocker and others (2006) developed a new molecule, propidium monoazide (PMA), from the DNA-binding dye propidium iodide. It has been demonstrated that PMA does not penetrate membranes of viable cells though it is equally efficient as EMA in excluding DNA from dead cells (Nocker et al., 2006). Further, Pan and Breidt (2007) reported that EMA has lethal effect on viable *Listeria monocytogenes* whereas PMA does not. The difference may be due in part to the higher positive charge of the PMA molecule compared to the EMA molecule (Nocker et al., 2006). Combining PMA with real-time PCR offers a promising alternative for enumerating viable bacterial cells in food (Garcia 2009). This study aimed to investigate the stability of DNA of dead *Salmonella* cells in lettuce and to use PMA real-time PCR for rapid detection of viable *Salmonella* in lettuce.

3.3 Materials and methods

Preparation of viable and dead *Salmonella* cells

In this study, *Salmonella* Typhimurium ATCC 14028 was used as a representative of *Salmonella* serotypes. The strain was grown in tryptic soy broth (TSB, BD Diagnostic Systems, Sparks, Md.) at 35 °C for 12 h and serially diluted with 0.1% peptone water to generate cell suspensions of 10¹ to 10⁹ CFU/mL. To obtain dead *S.* Typhimurium cells, the cell suspensions were heated at 100 °C for 10 min. The viability of cells was checked by incubating three 1 mL heat-treated cell suspensions separately in 9 mL TSB at 35 °C for 48 h.

Inoculation of dead *Salmonella* cells into lettuce

Bagged, shredded lettuce was purchased from a local food store. It was determined to be *Salmonella*-free using standard culture methods (Andrews and other 1995). Two and half milliliters dead *S.* Typhimurium cell suspension of 10⁷ CFU/mL prior to heat inactivation were inoculated individually into four sterile Whirl-Pak[®] sample bags (Fisher Scientific, St. Louis, Mo.), which contained 25 g lettuce. Samples were stored at 4 °C for 8 d. On days 0, 1, 4, and 8, the sample was mixed with 225 mL peptone water and homogenized with Stomacher 400 Circulator (Seward, Worthing, United Kingdom) at 260 rpm for 1 min. One milliliter homogenate was centrifuged at 12000×g for 2 min to obtain cell pellets.

DNA extraction

Cell pellets were resuspended in 100 µL PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, Calif.). Cell suspensions were boiled for 10 min and centrifuged at 12000×g for 2 min. The supernatant was used as template DNA for real-time PCR.

Real-time PCR

The 7300 Real-Time PCR System (Applied Biosystems) was used. A PCR reaction of 25 μ L contained 12.5 μ L 2 \times Real-time PCR SYBR Green Mix (Applied Biosystems), 0.5 μ L 50 μ M *invA*-F (5'-gattctgtactaatggtgatgatc-3'), 0.5 μ L 50 μ M *invA*-R (5'-gccaggctatcgccaataac-3') (Fey and others 2004), and 5 μ L DNA extract. The real-time PCR program consisted of 95 $^{\circ}$ C for 10 min and 40 cycles of 95 $^{\circ}$ C for 15 sec and 60 $^{\circ}$ C for 60 sec. Each DNA sample was analyzed in triplicate. Cycle threshold (Ct) values, describing the PCR cycle number at which fluorescence rises above the base line, were determined using the software package provided by the Applied Biosystems.

PMA treatment

The procedure of PMA treatment was adopted from Nocker and others (2006). Briefly, PMA was dissolved in 20% dimethyl sulfoxide to yield a stock solution of 20 mM and stored in the dark at -20 $^{\circ}$ C. 1.25 μ L PMA stock solution was added into 500 μ L *S. Typhimurium* cell suspensions of different concentrations. Following 10-min incubation in the dark at room temperature with occasional mixing to allow PMA to penetrate the dead cells and bind to the DNA, the sample was incubated in ice for 1 min (Wang and others 2009) and then exposed to 650-W halogen light for 2 min. The sample was placed about 20 cm from the light source and laid horizontally on ice to avoid excessive heating. After photo-induced cross-linking, cells were collected by centrifugation at 12000 \times g for 2 min and washed with 500 μ L sterile distilled water.

Efficacy of PMA in removing DNA of dead *Salmonella* cells in lettuce

Dead *S. Typhimurium* cell suspensions (2.5 mL) were inoculated into 25 g lettuce to reach high final concentrations of 10⁸, 10⁷, and 10⁶ CFU/g. These samples were individually mixed with 225 mL peptone water and homogenized at 260 rpm for 1 min. Then, two tubes of 500 μ L homogenate were collected from each sample.

One set was treated with PMA before DNA extraction; the other set was directly applied to DNA extraction. DNA extracts were subjected to the real-time PCR assay described above.

Quantification of viable *Salmonella* cells by PMA real-time PCR

Viable *S. Typhimurium* cell suspensions of 10^1 to 10^9 CFU/mL in 0.1% peptone water were treated or untreated with PMA. Bacterial DNA was extracted from cell pellets from both sets of samples and subjected to the real-time PCR assay described above. Standard curves were constructed by plotting Ct values generated from real-time PCR against *S. Typhimurium* cell concentrations (Log CFU/ml).

Quantification of viable *Salmonella* cells in lettuce by PMA real-time PCR

Viable *S. Typhimurium* cell suspensions (2.5 mL) in 0.1% peptone water were inoculated into 25 g lettuce to reach final concentrations of 10^1 to 10^8 CFU/g. These samples were individually mixed with 225 mL peptone water and homogenized at 260 rpm for 1 min. Then, two tubes of 500 μ L homogenate were collected from each sample. One set was treated with PMA before DNA extraction; the other set was directly applied to DNA extraction. DNA extracts were subjected to the real-time PCR assay described above. Standard curves were constructed by plotting Ct values generated from real-time PCR against *S. Typhimurium* cell concentrations in lettuce (Log CFU/g).

Detection of low concentrations of viable *Salmonella* cells in lettuce by PMA real-time PCR

Viable *S. Typhimurium* cell suspensions (2.5 mL) were inoculated into 25 g lettuce to reach low final concentrations of 10^0 to 10^2 CFU/g. These samples were individually mixed with 225 mL TSB and homogenized at 260 rpm for 1 min. The homogenates were incubated at 35 $^{\circ}$ C for 24 h. At 4, 8, 12, and 24 h, two tubes of

500 μ L homogenate were collected from each sample. One set was treated with PMA before DNA extraction; the other set was directly applied to DNA extraction. DNA extracts were subjected to the real-time PCR assay described above.

3.4 Results and discussion

Since there was little information on the stability of DNA of dead bacterial cells in food ecosystems, this study started with assessing the persistence of DNA of dead *S. Typhimurium* cells in lettuce. *Salmonella*-free lettuce was inoculated with heat-killed *S. Typhimurium* cells to reach 10^6 CFU/g. During refrigeration, bacterial DNA was extracted from inoculated lettuce and subjected to the real-time PCR assay targeting the *invA* gene (Fey and others 2004). On day 0, the Ct value generated from the sample was 23.6. Over time, the Ct value increased slightly and reached 26.3 on day 8 (Figure 3.1 a). Based on the real-time PCR standard curve shown in Figure 3.5, the derived concentrations of *S. Typhimurium* in lettuce ranged from 6.37 Log CFU/g on day 0 to 5.59 Log CFU/g on day 8 (Figure 3.1 b). This agrees with previous reports that PCR cannot differentiate between DNA from dead and living cells (Josephson and others 1993; Masters and others 1994). While the cells are dead, *S. Typhimurium* DNA can remain stable in lettuce for a long time and cause false-positive results in PCR.

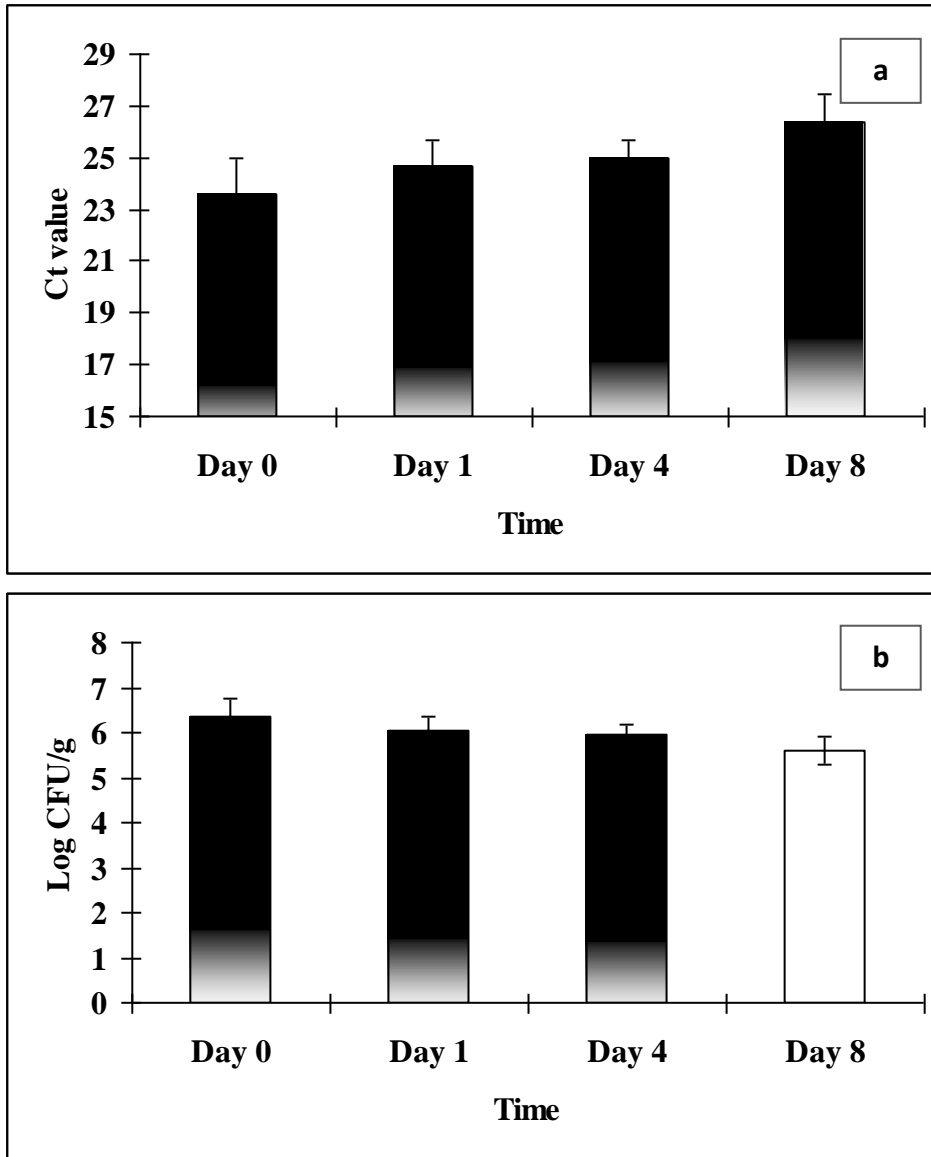


FIGURE 3.1a-Real time PCR Ct value change of DNA from dead *Salmonella* Typhimurium at 10⁶ CFU/g in lettuce during storage at 4 °C. FIGURE 3.1b-Derived concentration of *Salmonella* Typhimurium in lettuce based on the real-time PCR standard curve shown in Figure 3.5. Results were averaged from two repeated experiments.

In order to overcome the limitation of real-time PCR, PMA was used to treat bacterial cells before DNA extraction. The efficiency of PMA in excluding the DNA of dead *S. Typhimurium* cells in lettuce was evaluated. Without prior PMA treatment, the Ct value generated from the sample containing 10^8 CFU/g dead *S. Typhimurium* cells was 18.4. As the concentration of *S. Typhimurium* in lettuce decreased, the Ct value gradually increased. In contrast, the DNA extracted from all three PMA-treated samples, ranging from 10^6 to 10^8 CFU/g, did not yield any amplicons within 40 real-time PCR cycles. The fluorescence signal remained low as the background signal of the system (Figure 3.2). This demonstrates that 50 μ M PMA is sufficient to exclude DNA of as high as 10^8 CFU/g dead *Salmonella* cells in lettuce.

The effect of PMA treatment on real-time PCR-based detection of culturable *Salmonella* cells was evaluated. Viable *S. Typhimurium* cell suspensions of different concentrations were treated or untreated with PMA prior to DNA extraction. Without prior PMA treatment, the detection range of the real-time PCR assay was from 10^2 to 10^8 CFU/ml, with good quantitative accuracy ($R^2=0.9589$). Higher concentration (10^9 CFU/ml) was detectable but not quantifiable (Figure 3.3). PMA treatment did not influence the detection range of the real-time PCR assay. While it resulted in approximately 1.3 increases in the Ct values across different concentrations, a better fit of the linear regression line was achieved ($R^2=0.992$) (Figure 3.3). Nocker and others (2006) demonstrated that PMA did not penetrate viable cells of *Salmonella Typhimurium*. A slight increase in the Ct values from samples treated by PMA was probably due to the procedures of PMA treatment. A small number of *S. Typhimurium* cells might be lost during the washing step after PMA treatment. These results indicate that PMA is highly selective in penetrating

only the dead cells of *S. Typhimurium*. The treatment has no adverse effect on detection of viable *Salmonella* by real-time PCR. The specificity of real-time PCR amplification was confirmed by running dissociation curves. The melting temperature (T_m) of the PCR products was around 79 °C; no unspecific products were formed (Figure 3.4).

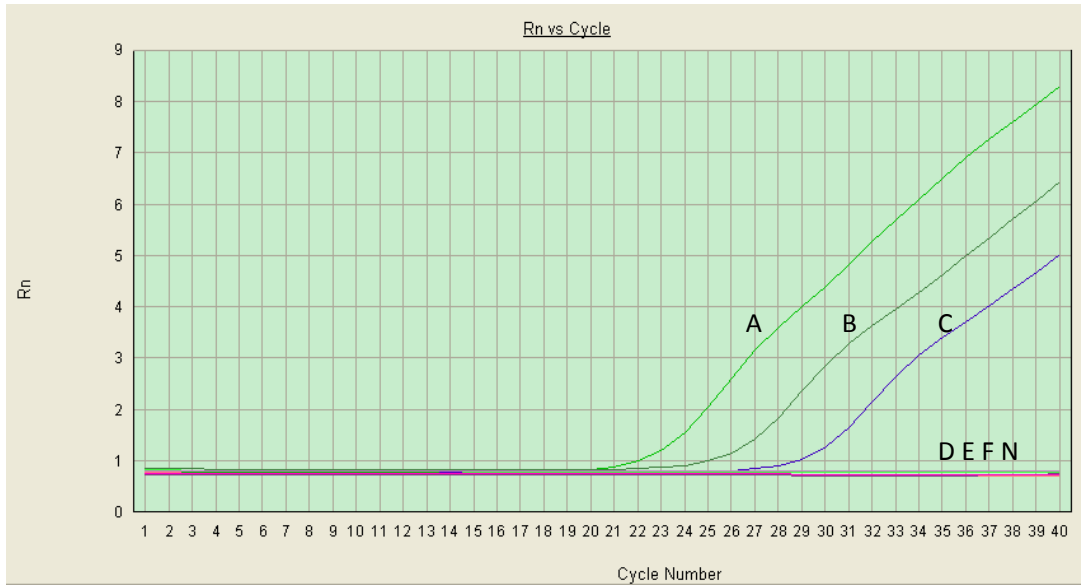


FIGURE 3.2-Real-time PCR amplification of DNA from PMA-untreated (A: 10⁸ CFU/g, B: 10⁷ CFU/g, C: 10⁶ CFU/g) and PMA-treated (D: 10⁸ CFU/g, E: 10⁷ CFU/g, F: 10⁶ CFU/g) dead *Salmonella* Typhimurium in lettuce. N: negative control (water).

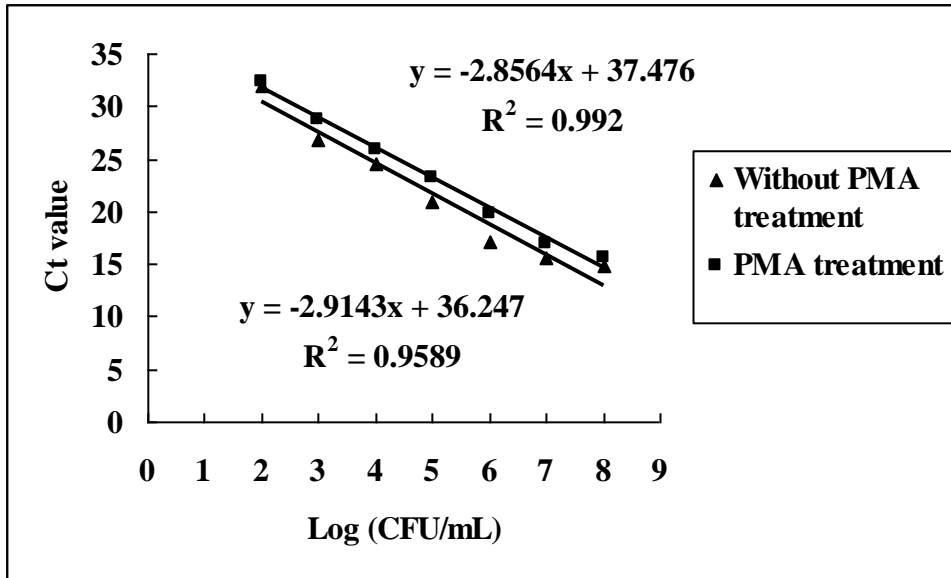


FIGURE 3.3-Standard curves for detection of viable *Salmonella* Typhimurium cells by real-time PCR and PMA real-time PCR. Results were from two repeated experiments.

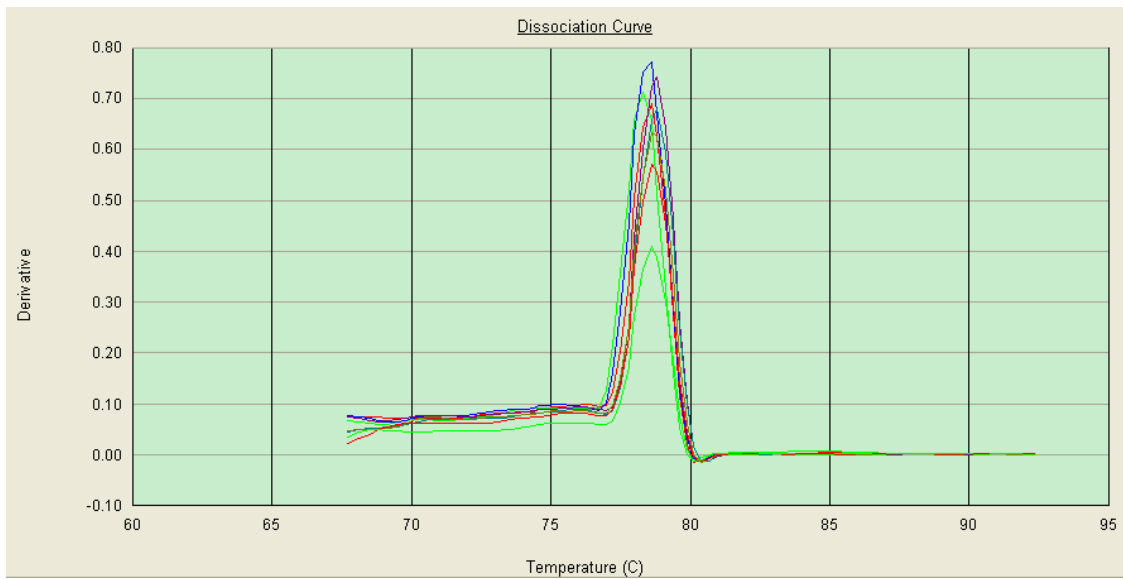


FIGURE 3.4-Dissociation curves of the PMA real-time PCR products from viable *Salmonella* Typhimurium cells.

Moreover, the PMA real-time PCR assay was applied to lettuce inoculated with viable *S. Typhimurium* cells at 10^1 to 10^8 CFU/g. The real-time PCR results from PMA-untreated and PMA-treated samples are shown in Figure 3.5. Two standard curves exhibited very similar linear relationship between the Ct value and the concentration of *S. Typhimurium* in lettuce, both with high coefficients of determination (R^2 , 0.9921 vs. 0.9982). The real-time PCR assay had a linear quantitative detection range of 6 logs and a detection limit of 10^3 CFU/g, regardless of the sample was treated with PMA. The EMA real-time PCR assay established by Wang and Mustapha (2010) could detect *Salmonella* at as low as 10^5 CFU/ml in chicken rinse and egg broth. The higher detection limit might be due in part to the capability of EMA penetrating viable bacterial cells (Nocker and others 2006). Further, the chemical composition of food matrices may affect the detection sensitivity of real-time PCR. Compared with lettuce, chicken and eggs contain more protein and fat, which might interfere with the amplification of DNA by PCR (Wilson 1997).

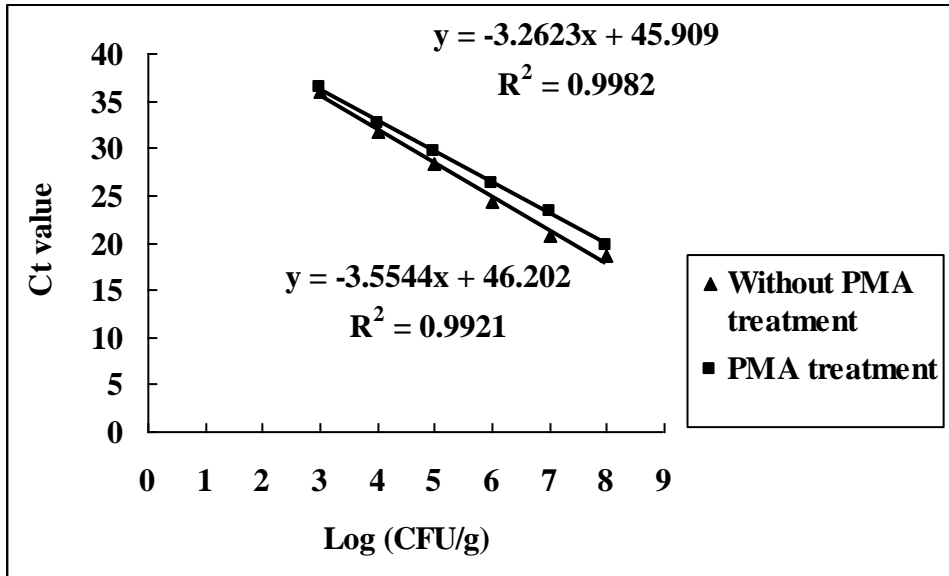


FIGURE 3.5 Standard curves for detection of viable *Salmonella* Typhimurium in lettuce by real-time PCR and PMA real-time PCR. Results were from two repeated experiments.

To detect low concentrations of viable *Salmonella* cells in lettuce, enrichment was conducted prior to the PMA real-time PCR assay. Lettuce was inoculated with viable *S. Typhimurium* cells to reach 10^0 to 10^2 CFU/g and enriched in TSB at 35 °C for varying time. Enriched samples yielded similar results from the PMA real-time PCR assay and from the real-time PCR assay. There is no improved detection time or sensitivity with the use of PMA. With a 4-h enrichment, *S. Typhimurium* of 10^2 CFU/g in lettuce was detectable by both assays. A lower concentration, 10^1 CFU/g, could be detected following enrichment for 12 h. However, *S. Typhimurium* of 10^0 CFU/g in lettuce was not detectable even after a 24-h enrichment (Table 3.1). In this study, the aerobic plate count of lettuce was 2.9×10^5 CFU/g. It is likely that high level of background microflora in lettuce outcompeted very low level of inoculated *S. Typhimurium* during enrichment in nonselective TSB. Therefore, selective media favoring *Salmonella* could be used for enrichment in order to reduce competition from indigenous microbes in lettuce and improve the performance of the PMA real-time PCR assay.

TABLE 3.1-Detection of low concentrations of viable *Salmonella* Typhimurium in lettuce by real-time PCR and PMA real-time PCR after enrichment ^a.

Enrichment time (h)	<i>S. Typhimurium</i> concentration before inoculation(CFU/g)	Real-time PCR result ^b	PMA real-time PCR result ^b
4 h	10 ²	30.6±0.5	31.2±0.5
	10 ¹	-/-	-/-
	10 ⁰	-/-	-/-
8 h	10 ²	30.1±0.2	30.6±0.4
	10 ¹	-/-	-/-
	10 ⁰	-/-	-/-
12 h	10 ²	29.3±0.1	30.0±0.1
	10 ¹	30.6±0.6	31.1±0.5
	10 ⁰	-/-	-/-
24 h	10 ²	28.8±0.5	30.1±0.4
	10 ¹	30.6±0.1	30.5±0.9
	10 ⁰	-/-	-/-

^a Results were from two repeated experiments.

^b Presented as average Ct value ± standard deviation. -/- means undetectable in two repeated experiments.

3.5 Conclusions

Prior PMA treatment can effectively prevent PCR amplification from dead *Salmonella* cells in lettuce. The PMA real-time PCR assay can selectively detect viable *Salmonella* at as low as 10^2 CFU/ml in pure culture and 10^3 CFU/g in lettuce. Combining a 12-h enrichment with the assay allows for the detection of viable *Salmonella* at 10^1 CFU/g in lettuce. The whole process can readily be completed within 14 h. The PMA real-time PCR assay provides an alternative to real-time PCR assay for accurate detection of *Salmonella* in food.

CHAPTER 4

ANTIMICROBIAL EFFECT OF NONI (*MORINDA CITRIFOLIA* L.) JUICE ON FOODBORNE PATHOGENIC BACTERIA IN SYNTHETIC AND FOOD MODEL SYSTEMS

4.1 Abstract

Noni (*Morinda citrifolia* L.) juice is considered to be a natural health drink. The purpose of this study was to evaluate the antimicrobial effect of noni juice on major foodborne pathogenic bacteria in synthetic and food model systems. *Escherichia coli* O157:H7, *Salmonella* Typhimurium, *Listeria monocytogenes*, and *Staphylococcus aureus* were individually inoculated at *ca* 5.0 log CFU/ml into distilled water containing 2.5%, 5% or 10% noni juice. Specific selective agar media were used to enumerate the pathogenic bacteria in the water during incubation at 35 °C. 2.5% noni juice inactivated *S. Typhimurium* and *L. monocytogenes* at 24 h. *L. monocytogenes* and *S. aureus* in 5% noni juice became undetectable at 8 h and 24 h, respectively. 10% noni juice inactivated *E. coli* O157:H7 at 24 h. The antimicrobial activity of 10% noni juice was further investigated in tryptic soy broth incubated at 35 °C. Compared with the control, the treatment suppressed *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* by 5.83, 8.41 and 7.85 log, respectively, after five days of incubation. However, the inhibitive effect of noni juice on *S. aureus* in tryptic soy broth was limited. Finally, the antimicrobial activity of noni juice was evaluated in artificially contaminated mushroom soup. At 7 °C, *L. monocytogenes* of 5.02 log CFU/ml in mushroom soup containing 10% noni juice was inactivated on day 6. At 35 °C, 10% noni juice suppressed *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* in mushroom soup by 8.71, 9.67, and 8.24 log,

respectively, after five days of incubation. To sum up, noni juice holds great promise as a natural antimicrobial.

4.2 Introduction

Preservatives are commonly used in food processing to enhance the microbiological safety and extend the shelf life of food products. Despite being effective, synthetic preservatives are not readily accepted by the consumer in that some of them may potentially cause health problems (Urbain and Campbell, 1987). There has been an increasing interest in antimicrobial agents originated from natural sources. Research shows that various plants, such as fruits, herbs, spices and teas, may contain compounds that can inhibit or even kill microorganisms (Beuchat et al., 1989; Brul and Coote, 1999; Lopez-Malo et al.). Extracts from these plants may be added into foods as natural preservatives.

Noni (*Morinda citrifolia* L.) is a tropical plant widely grown in the Pacific islands. Roots, leaves and fruits of the plant have long been used by Polynesians as a folk medicine to treat wounds, colds, fever, and skin infections (Wang et al., 2002). Noni contains various bioactive compounds such as glycosides, polysaccharides, iridoids, alkaloids, lignans, trisaccharide fatty acid esters, anthraquinones, scopoletin, morindin, vitamins, and minerals (Yang et al., 2007). Previous studies suggest that noni may have analgesic effect and anti-oxidant, anti-inflammatory, and anti-tumor properties (Chan-Blanco et al., 2007; Wang et al., 2002). Traditionally, noni juice is made by fermenting the fruits in sealed containers for up to two months, recovering the juice through lixiviation and/or mechanical pressure, pasteurizing, and conditioning (Nelson, 2002). Due to its potential health benefits, noni juice has gained increasing popularity in the functional beverages market.

Research indicates that organic solvent extracts from noni fruits possess an antimicrobial activity (Locher et al., 1995; Saludes et al., 2002). Bushnell *et al.* reported that tissue fluids from ripe noni fruits could inhibit the growth of *Escherichia*

coli, *Micrococcus pyogenes*, *Pseudomonas aeruginosa*, *Salmonella typhose*, *Salmonella montevideo*, *Salmonella schottmuelleri* and *Shigella paradysenteriae* (Bushnell et al., 1950). Noni juice is produced by fermenting noni fluid in seal container up to two months. There is little information on the antimicrobial potential of fermented noni juice. The objectives of this study were to evaluate the effect of noni juice on the fate of major foodborne pathogenic bacteria in distilled water, tryptic soy broth and to determine the antimicrobial activity of noni juice in mushroom soup stored at different temperatures.

4.3 Materials and methods

Bacterial strains and media.

E. coli O157:H7 strain C7927, *Salmonella* Typhimurium ATCC 14028, *Staphylococcus aureus* ATCC 25923 and *Listeria monocytogenes* strain F2365 were obtained from the Food Microbiology Laboratory, University of Hawaii at Manoa. Each strain was cultivated by two successive transfers in tryptic soy broth (TSB) at 35 °C for 16 h. One milliliter of the broth culture was serially diluted with 9 ml of 0.1 M phosphate-buffered saline (PBS). Selective agar media were used to enumerate the pathogenic bacteria: MacConkey sorbitol agar for *E. coli* O157:H7, xylose lysine desoxycholate agar for *S. Typhimurium*, Baird-Parker agar for *S. aureus*, and modified Oxford agar for *L. monocytogenes*. All microbiological media were obtained from BD Diagnostic Systems (Sparks, Md.).

Antimicrobial effect of noni juice on foodborne pathogenic bacteria in distilled water.

Noni juice was purchased from a grocery store in Honolulu, Hawaii. Noni juice, 0.25, 0.5, and 1.0 ml, was added into 9.75, 9.5, and 9.0 ml of sterile distilled water,

respectively. The samples were mixed thoroughly to make 2.5, 5, and 10% noni juice. Appropriate dilutions of *E. coli* O157:H7, *S. Typhimurium*, *S. aureus* and *L. monocytogenes* were added separately into each noni juice sample to achieve final concentrations of *ca* 5.0 log CFU/ml. After being thoroughly mixed, the samples were incubated at 35 °C for 0, 4, 8, and 24 h. At each time, one milliliter of the samples was serially diluted with PBS and spread plated onto corresponding pathogen-specific agar. Typical colonies on each selective agar were counted after incubation at 35 °C for 24 to 48 h.

Antimicrobial effect of noni juice on foodborne pathogenic bacteria in TSB.

One milliliter of noni juice was added into 9 ml of TSB and mixed thoroughly. Appropriate dilutions of the four bacterial cultures were added separately into TSB containing 10% noni juice to achieve final concentrations of *ca* 5.0 log CFU/ml. The samples were thoroughly mixed and incubated at 35 °C. On days 0, 1, 3 and 5, one milliliter of the samples was serially diluted with PBS and spread plated onto corresponding pathogen-specific agar. Typical colonies on each selective agar were counted after incubation at 35 °C for 24 to 48 h.

Antimicrobial effect of noni juice on foodborne pathogenic bacteria in mushroom soup.

Condensed cream of mushroom soup was purchased from a local grocery store and diluted 3-fold with sterile distilled water. One milliliter of noni juice was added into 9 ml of mushroom soup and mixed thoroughly. Appropriate dilutions of *E. coli* O157:H7, *S. Typhimurium* and *L. monocytogenes* were added separately into mushroom soup containing 10% noni juice to achieve final concentrations of *ca* 5.0 log CFU/ml. Two sets of samples were prepared. One set was stored at 35 °C for 0, 1, 3 and 5 days whereas the other set was stored at 7 °C for 0, 1, 3, 6 and 9 days. On

each sampling day, one milliliter of the samples was serially diluted with PBS and spread plated onto corresponding pathogen-specific agar. Typical colonies on each selective agar were counted after incubation at 35 °C for 24 to 48 h.

Statistical analysis.

The experiments were repeated twice and conducted as a randomized complete block design with treatments, storage times, and replicates. The counts of each pathogenic bacterium were log-transformed and subjected to analysis of variance. The least significant difference (LSD) was used to compare means of different treatments at different times at $P < 0.05$.

4.4 Results and Discussion

The antimicrobial activities of different concentrations of noni juice against *E. coli* O157:H7, *S. Typhimurium*, *L. monocytogenes* and *S. aureus* were first evaluated in distilled water (Table 4.1). Compared with the control, 2.5% noni juice significantly reduced the numbers of *S. Typhimurium*, *L. monocytogenes* and *S. aureus* after incubation for 4h, 8 h and 24 h, respectively. It completely inactivated *S. Typhimurium* and *L. monocytogenes* at 24 h. Moreover, 5 and 10% noni juice showed significant antibacterial effect against *S. Typhimurium* since 4 h. These treatments completely inactivated *L. monocytogenes* and *S. aureus* at 8 h and 24 h, respectively. Finally, 5% noni juice reduced the number of *E. coli* O157:H7 by 1.43 log whereas 10% noni juice completely inactivated the pathogen at 24 h.

The antimicrobial effect of 10% noni juice on the four pathogenic bacteria was further assessed in TSB at 35 °C (Table 4.2). All the strains grew from 3.6-5.2 log CFU/ml to 8.5-9.2 log CFU/ml within 24 h, regardless of the medium was supplemented with noni juice. Compared with the controls, the counts of all

pathogenic bacteria in TSB containing 10% noni juice were significantly reduced on day 3. The treatment reduced the number of *E. coli* O157:H7 and *S. Typhimurium* by 5.83 and 8.41 log, respectively, on day 5. The count of *L. monocytogenes* decreased from 9.08 log CFU/ml on day 1 to 2.73 log CFU/ml and undetectable on days 3 and 5, respectively. In contrast, the effect of noni juice on *S. aureus* in TSB was limited. 10% noni juice suppressed the pathogen by approximately 1.3 log, compared with the control, on days 3 and 5.

Finally, noni juice was evaluated for its antimicrobial activity against *E. coli* O157:H7, *S. Typhimurium* and *L. monocytogenes* in a food model, mushroom soup. At 7 °C, the levels of *E. coli* O157:H7 and *S. Typhimurium* remained stable during nine days of storage, regardless of the soup was supplemented with 10% noni juice (Table 4.3). Due to its psychrotrophic nature, *L. monocytogenes* grew slowly in control sample and increased from 5.12 log CFU/ml on day 0 to 7.94 log CFU/ml on day 9. In contrast, the count of this pathogen in noni juice sample was reduced from 5.02 log CFU/ml on day 1 to 2.89 log CFU/ml and undetectable on days 3 and 6, respectively (Table 4.3). Table 4.4 shows the antibacterial effect of noni juice on *E. coli* O157:H7, *S. Typhimurium* and *L. monocytogenes* in mushroom soup stored at 35 °C. In control samples, the numbers of all three pathogens increased from 4.9-5.2 log CFU/ml to 8.2-8.9 log CFU/ml in 24 h and then remained stable through the rest of incubation. 10% noni juice significantly suppressed the pathogens in mushroom soup. *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* were completely inactivated on days 5, 5, and 3, respectively.

TABLE 4.1. Effect of noni juice on *Escherichia coli* O 157: H7, *Salmonella* Typhimurium, *Listeria monocytogenes*, and *Staphylococcus aureus* in distilled water at 35 °C for 0, 4, 8, and 24 hours

Target bacterium	Noni juice concentration	Viable cell count (log CFU/ml)			
		0 h	4 h	8 h	24 h
<i>E. coli</i> O157:H7	0%	5.17±0.02 A	5.22±0.02 A	4.82±0.06 A	4.00±0.03 A
	2.5%	5.17±0.00 A	5.12±0.05 AB	4.70±0.14 A	3.77±0.05 A
	5%	5.12±0.01 A	4.84±0.04 BC	4.74±0.13 A	2.57±0.59 B
	10%	4.85±0.02 A	4.73±0.05 C	4.08±0.02 B	- C
<i>S. Typhimurium</i>	0%	4.71±0.12 A	4.65±0.04 A	4.16±0.09 A	4.15±0.17 A
	2.5%	4.69±0.02 A	4.47±0.09 A	4.14±0.01 A	- B
	5%	4.76±0.01 A	4.19±0.04 B	3.18±0.07 B	- B
	10%	4.77±0.05 A	3.09±0.08 C	1.15±0.21 C	- B
<i>L. monocytogenes</i>	0%	5.02±0.04 A	4.22±0.02 A	5.12±0.05 A	3.92±0.07 A
	2.5%	4.44±0.82 A	3.18±0.05 B	0.50±0.71 B	- B

	5%	4.91±0.11 A	2.38±0.11 C	- B	- B
	10%	4.85±0.02 A	2.08±0.05 C	- B	- B
<i>S. aureus</i>	0%	4.20±0.05 A	4.17±0.06 AB	4.11±0.08 A	3.45±0.12 A
	2.5%	4.15±0.08 A	4.05±0.05 B	4.05±0.05 AB	2.49±0.15 B
	5%	4.23±0.12 A	4.23±0.01 A	3.94±0.02 BC	- C
	10%	4.20±0.05 A	4.07±0.07 AB	3.78±0.07 C	- C

“-” means undetectable. Means with different letters (A through C) within a column for each bacterium are significantly different at $P < 0.05$.

TABLE 4.2. Effect of 10% noni juice on *Escherichia coli* O 157: H7, *Salmonella* Typhimurium, *Listeria monocytogenes*, and *Staphylococcus aureus* in tryptic soy broth at 35 °C for 0, 1, 3, and 5 days

Target bacterium	Noni juice concentration	Viable cell counts (log CFU/ml)			
		0 day	1 day	3 day	5 day
<i>E. coli</i> O157:H7	0%	3.81±1.15 A	9.02±0.01 A	8.95±0.13 A	8.98±0.22 A
	10%	3.69±0.98 A	8.73±0.01 A	7.19±0.73 B	3.15±0.22 B
<i>S. Typhimurium</i>	0%	5.00±0.14 A	9.06±0.00 A	9.23±0.16 A	9.15±0.09 A
	10%	5.13±0.05 A	8.50±0.19 A	4.29±1.07 B	0.74±1.05 B
<i>L. monocytogenes</i>	0%	5.00±0.14 A	9.19±0.04 A	7.97±0.69 A	7.85±0.10 A
	10%	5.16±0.22 A	9.08±0.01 A	2.73±0.52 B	- B
<i>S. aureus</i>	0%	4.37±0.23 A	8.56±0.59 A	8.79±0.16 A	8.41±0.32 A
	10%	4.49±0.06 A	8.58±0.07 A	7.39±0.13 B	7.21±0.18 B

“-” means undetectable. Means with different letters (A through B) within a column for each bacterium are significantly different at $P < 0.05$.

TABLE 4.3. Effect of 10% noni juice on *Escherichia coli* O 157: H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* in mushroom soup at 7 °C for 0, 1, 3, 6, and 9 days

Target bacterium	Noni juice concentration	Viable cell counts (log CFU/ml)				
		0 day	1 day	3 day	6 day	9 day
<i>E. coli</i> O157:H7	0%	5.18±0.09 A	5.21±0.14 A	5.06±0.08 A	5.27±0.08 A	5.18±0.19 A
	10%	5.08±0.04 A	5.04±0.07 A	4.50±0.74 A	5.06±0.02 A	5.02±0.22 A
<i>S. Typhimurium</i>	0%	5.14±0.23 A	4.73±0.60 A	5.15±0.03 A	5.03±0.10 A	5.04±0.02 A
	10%	4.96±0.23 A	5.15±0.00 A	5.07±0.02 A	4.91±0.12 A	4.61±0.33 A
<i>L. monocytogenes</i>	0%	5.12±0.03 A	5.19±0.12 A	5.11±0.01 A	6.10±0.08 A	7.94±0.05 A
	10%	5.02±0.12 A	4.72±0.38 A	2.89±0.08 B	- B	- B

“-” means undetectable. Means with different letters (A through B) within a column for each bacterium are significantly different at $P < 0.05$.

TABLE 4.4. Effect of 10% noni juice on *Escherichia coli* O 157: H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* in mushroom soup at 35 °C for 0, 1, 3, and 5 days

Target bacterium	Noni juice concentration	Viable cell counts (log CFU/ml)			
		0 day	1 day	3 day	5 day
<i>E. coli</i> O157:H7	0%	5.00±0.21 A	8.37±0.09 A	8.71±0.07 A	8.71±0.08 A
	10%	4.85±0.21 A	4.87±0.09 B	3.56±0.79 B	- B
<i>S. Typhimurium</i>	0%	4.93±0.18 A	8.84±0.23 A	8.81±0.25 A	9.67±0.02 A
	10%	4.97±0.05 A	3.78±0.31 B	0.65±0.92 B	- B
<i>L. monocytogenes</i>	0%	5.17±0.09 A	8.27±0.01 A	7.80±0.28 A	8.24±0.16 A
	10%	5.09±0.12 A	4.22±0.48 B	- B	- B

“-” means undetectable. Means with different letters (A through B) within a column for each bacterium are significantly different at $P < 0.05$.

The antimicrobial activity of plant extracts is mainly related to their content of phenolic compounds (Davidson and Naidu, 2000). Chan-Blanco *et al.* identified rutin, scopoletin, and three other unknown phenolic compounds in noni juice (Chan-Blanco *et al.*, 2007). Rutin was claimed to have antimicrobial properties (Cushnie and Lamb, 2005). Scopoletin could inhibit the growth of *E. coli* O157:H7 under both aerobic and anaerobic conditions (Sylvia *et al.*, 1998). Additionally, depending on the degree of fermentation, noni juice may contain various amounts of ethanol (8813 to 13066 mg L⁻¹) and lactic acid (396 to 2936 mg L⁻¹). The pH of noni juice lies between 3.6 and 3.9 (Chan-Blanco *et al.*, 2007). Both the acidity and ethanol content of noni juice may also contribute to its antimicrobial effect.

This study demonstrated the antimicrobial effect of noni juice on major foodborne pathogenic bacteria. In distilled water, 10% noni juice completely killed *E. coli* O157:H7, *S. aureus* and *L. monocytogenes* after 24 hours incubation at 35 °C (Table 4.1). Kim *et al.* (2008) evaluated the antimicrobial effect of muscadine seed extracts on *E. coli* O157:H7. Depending on the type of muscadine seeds and the extraction method, water-soluble extracts reduced the level of *E. coli* O157:H7 by 2.6 to 7.6 log within 2 hours (Kim *et al.*, 2008). Noni juice exhibited the antimicrobial effect more slowly than muscadine seed extracts. This may be explained by the fact that muscadine seed extracts were more concentrated than 10% noni juice.

Compared with the control, noni juice exhibited approximately 8 log suppression of *S. Typhimurium* and *L. monocytogenes* in TSB and 5 log suppression of *E. coli* O157:H7 after 5 days incubation at 35 °C (table 4.2). It is interesting that the inhibitive effect of noni juice on the three pathogenic bacteria was not evident until their counts reached the same high level (9.0 log₁₀ CFU ml⁻¹) as the control on day 1. Kim and Fung (2004) observed similar cell death patterns of *E. coli* O157:H7, *S.*

Typhimurium, *S. aureus* and *L. monocytogenes* when they evaluated the antibacterial property of arrowroot tea extracts in brain heart infusion. It was speculated that the cells lost their resistance to natural antimicrobials when they entered the stationary phase, probably due to the exhaustion of nutrients and/or the accumulation of waste products (Kim and Fung, 2004).

In mushroom soup stored at 7 °C, 10% noni juice suppressed *L. monocytogenes* by 7.0 log on day 9, compared with the control (Table 4.3). Its antibacterial effect on *E. coli* O157:H7, *S. Typhimurium* and *L. monocytogenes* in mushroom soup was more significant at 35 °C (Table 4.4). The juice exhibited 8.2 to 9.7 log suppression of the three pathogens on day 5. Greater survival rates of foodborne pathogenic bacteria in foods supplemented with natural antimicrobials at lower temperatures were reported previously (Kim et al., 2001; Yuste and Fung, 2003). This could be a result of reduced cell membrane fluidity and permeability. Under refrigeration, antimicrobial compounds may not enter and thus inhibit bacterial cells effectively (Nair et al., 2005). Also, cold-shock proteins (CSPs) might be induced in bacterial cells during incubation at low temperatures. Research indicates that CSPs can inhibit ribosomal translation and decrease the rate of biochemical reactions in cells (Ermolenko and Makhatadze, 2002). This hibernation in the cold might increase the anti-stress ability of bacteria. Therefore, bacteria showed strong survive ability under solution with noni juice at refrigeration temperature.

4.5 Conclusions

In conclusion, noni juice can inhibit *E. coli* O157:H7, *S. Typhimurium* and *L. monocytogenes* in distilled water, TSB and mushroom soup, especially at elevated temperatures. In addition to being a functional beverage, noni juice could potentially be used as a natural preservative to enhance the microbiological safety of food. Studies are being undertaken to explore the antimicrobial activity of noni juice in other food ecosystems. Further research is needed to assess the influence of noni juice on the organoleptic properties of supplemented foods.

CHAPTER 5

CONCLUSIONS

A new real-time PCR assay was successfully developed to quantify *Salmonella* cells in lettuce. This method overcame the disadvantage of previous nucleic-acid based detection methods by treating the sample with the novel DNA-modifying dye PMA before DNA extraction and real-time PCR. It was demonstrated that PMA treatment could effectively exclude DNA from dead *Salmonella* cells and did not affect the detection of the viable cells. The treatment enhanced the accuracy but not the sensitivity of real-time PCR. The PMA real-time PCR assay could selectively detect viable *Salmonella* at as low as 10^3 CFU/g in lettuce. The sensitivity of the method could be improved with selective enrichment of the sample. The PMA real-time PCR assay can be extended to quantify *Salmonella* in other food matrices. Since foods differ in chemical compositions and indigenous microflora, the activities of DNA polymerase and oligonucleotide probe may be affected markedly in real-time PCR. A new standard curve representing the relationship between Ct value and the number of *Salmonella* cells needs to be created for each food matrix.

Noni juice showed significant inhibitory effects on *E. coli* O157:H7, *S. Typhimurium* and *L. monocytogenes* in distilled water, TSB and mushroom soup. This suggests that noni juice has potential to be used as a natural preservative to enhance the microbiological safety and extend the shelf-life of food. The mode of action of noni juice on these pathogenic bacteria could be investigated in the future. The juice might affect membrane permeability, enzyme activity, and/or cellular metabolism. Understanding this mechanism would help us further explore which chemical components in noni juice are responsible for the antimicrobial effect.

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