CPS 2019
FINAL PROJECT REPORT

Project Title
Detection, validation, and assessment of risks implied by the viable but non-culturable (VBNC) state of enteric bacterial pathogens in fresh produce

Project Period
January 1, 2017 – December 31, 2018

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Objectives

The central research objective is to modify loop-mediated isothermal amplification (LAMP) methods by incorporation of intercalating dye to enable rapid and sensitive detection of both viable and VBNC Salmonella and Shiga toxigenic E. coli (STEC). Assay performance will be validated and field/processing trials conducted to provide data to support an analysis of the risk implied by the survival of VBNC Salmonella and STEC in lettuce. Specific objectives are:

1. *Induce the VBNC state in relevant bacterial pathogens. The effect of low nutrient and temperature on VBNC induction will be examined in Salmonella and STEC by measurement of total culturable and VBNC cells over time.*
2. *Assess intercalating dye performance and optimize treatment condition (dye concentration, treatment time, etc.) of intercalating dye. The ability of ethidium monoazide and propidium monoazide to penetrate viable cells will be compared to select the dye less likely to cause consequent false positive results.*
3. *Develop a LAMP assay, optimize assay conditions for both Salmonella and STEC, and verify assay performance in vitro.*
4. *Develop and optimize a PCR assay for both Salmonella and STEC and verify assay performance in vitro.*
5. *Optimize conditions for the detection of pathogens in spiked produce (lettuce, tomato, cantaloupe and spinach) with the intercalating dye-LAMP assay and verify assay performance in planta.*
6. *Determine the survival of VBNC Salmonella and STEC on lettuce in the field and during simulated processing.*
7. *Risk assessment. Perform semi-quantitative assessment of the risk implied by Salmonella and STEC in the VBNC state using data from field trials and simulated processing.*

Funding for this project provided by the Center for Produce Safety through:
CPS Campaign for Research

Abstract

Escherichia coli O157:H7 and *Salmonella* have been identified as the leading cause of foodborne outbreaks linked to fresh produce. These enteric bacterial pathogens pose potential risks because they can enter into a viable but non-culturable (VBNC) state under environmental stresses, which cannot be detected by conventional laboratory tests but can resuscitate under optimum condition and cause illness. We developed a method of coupling propidium monoazide (PMA) with loop-mediated isothermal amplification (LAMP) that can detect VBNC *E. coli* O157:H7 and *Salmonella*, followed by the validation for an in-field study. *E. coli* O157:H7 EDL933 and *S.* Enteritidis 43353 were used for VBNC induction in 7% NaCl. PMA-LAMP and PMA-qPCR assays along with the plating assay were developed and applied to quantify both *E. coli* O157:H7 and *Salmonella* in the VBNC state in pure culture. The field study and risk assessment were performed to analyze the risks related to the VBNC state of pathogens in field-grown produce. The PMA-LAMP assay had a detection limit of 1.02–10.02 CFU/reaction for the VBNC state of *E. coli* O157:H7 and 2.07–20.7 CFU/reaction for the VBNC state of *S.* Enteritidis in pure culture, which was comparable to that of the PMA-qPCR assay. Furthermore, the PMA-LAMP assay was about 1 h faster than the PMA-qPCR assay. In the field study, we observed that *E. coli* O157:H7 and *Salmonella* could be induced to the VBNC state on field-grown lettuce. Our risk assessment demonstrated that the overall risk of human illnesses derived from the consumption of contaminated romaine lettuce due to VBNC *E. coli* O157:H7 and *Salmonella* was serious and moderate to serious, respectively. The PMA-LAMP assay improves the detection of VBNC *E. coli* O157:H7 and *Salmonella* in fresh produce and provides new insights into the ecology of both bacterial pathogens on field-grown lettuce.

Background

E. coli O157:H7 and *Salmonella* are leading pathogens and a continuous threat to public health. *E. coli* O157:H7 is one of the most foodborne outbreak-associated strains in the group of Shiga toxin-producing *E. coli* (STEC). It has been estimated that *E. coli* O157:H7 caused 73,000 illnesses and 250 deaths annually [1]. The life-threatening Hemolytic Uremic Syndrome (HUS) occurs in 10% of *E. coli* O157:H7-infected people, which can result in severe damage of the kidneys [2]. In the United States, a 2018 multistate outbreak caused by *E. coli* O157:H7 contaminated romaine lettuce resulted in 210 illnesses, 96 hospitalizations, and included 27 HUS cases and 5 deaths across 36 states [3]. *Salmonella* has been identified as the leading cause of foodborne illness according to the Centers for Disease Control and Prevention (CDC). It was estimated that over 23,000 hospitalizations and 450 deaths occur annually due to the infection of *Salmonella* in the United States [4]. The overall reported *Salmonella* infection incidences continuously increased from 2014 to 2016 [5]. Therefore, there is a critical need to prevent and control *E. coli* O157:H7 and *Salmonella*. Although ground beef, poultry and eggs are common vehicles for *E. coli* O157:H7 and *Salmonella* infections, fresh produce has become an increasingly effective vehicle of infection due to rising consumption, large-scale production and widespread distribution [6]. According to CDC, fresh produce–related outbreaks can be largely attributed to *E. coli* O157:H7 and *Salmonella* infections. Nearly 75% of illnesses related to *E. coli* O157 were caused by the consumption of contaminated leafy green vegetables [7]. In 2015, an outbreak of *Salmonella* Poona linked to cucumbers infected 907 people in 40 states and 6 deaths were reported [8]. Another recent (2018) reported outbreak of *E. coli* O157:H7 in romaine lettuce has caused 25 hospitalizations and 62 cases among 16 states [9]. Since the microbiological quality of fresh produce can be influenced at every step from pre-harvest to post-harvest, it is of great importance to understand the bacterial risk in fresh produce.

Salmonella and *E. coli* O157:H7 have been shown to be able to survive on produce surfaces for a relatively long period and their ability to enter into the dormant physiological state complicates

efforts for the detection of pathogens on fresh produce [10]. Pathogens in a dormant state, notably the viable but non-culturable (VBNC) state, can preclude the detection by routine diagnostic procedure due to their inability to grow in microbiological media commonly used for food microbiological analysis [11]. The VBNC state is considered a survival strategy for bacteria under stressful conditions, characterized by the loss of ability of bacteria to form colonies on microbiological routine media. Despite the failure to grow or be cultivated on media, VBNC cells still maintain a low metabolic and respiratory rate and retain infectivity and pathogenicity under certain conditions [12]. Entering into the VBNC state is regarded as a survival strategy and protection of cells against unfavorable environments for non-sporulating bacteria. In the VBNC state, bacteria are identified to be more resistant than their counterparts in various adverse conditions, including starvation, extreme high or low temperature, high salinity, stressful pH or exposure to heavy metals [13-17]. It has been reported that VBNC cells can be resuscitated from the non-culturable state to the culturable state under certain conditions or when the stress is removed, and regain virulence and eventually cause infections. For example, *Salmonella* is shown to be resuscitated by a temperature upshift with the supplementation of catalase [18], incubation with pyruvate [19] or with resuscitation-promoting factor (Rpf) proteins [20]. Because of the potential risk posed by the presence of enteric bacterial pathogens in the VBNC state, it is necessary to develop an effective detection method and validate the method in fresh produce.

To successfully detect VBNC cells, two characteristics of bacteria in the VBNC state should be verified for identification and detection, namely cell culturability and cell viability. Conventional plating method in combination with other methods to detect cell viability is usually used to detect VBNC cells. The commonly used viability assays include flow cytometry, quantitative polymerase chain reaction (qPCR), or reverse transcription quantitative PCR (RT-qPCR). Flow cytometry requires an expensive instrument and well-trained personnel, and has complexity of performance. The qPCR requires a complicated thermal cycler, which limits its use for in-field applications. Recently, a nucleic acid–based method named loop-mediated isothermal amplification (LAMP) has emerged as a promising alternative to detect foodborne pathogenic microorganisms. Compared to qPCR that requires thermal cycles, LAMP can be conducted at a constant temperature ranging from 50 to 72°C and the reaction can be performed in 5–10 min. The reaction has high amplification efficiency as it amplifies DNA 10^9 to 10^{10} times in only 15–60 min. The results can be easily seen by fluorescence or a turbidity signal in real time or by endpoint visualization. Since the LAMP primers consist of four to six primers targeting six to eight regions of the target sequence, the assay is highly specific to targeted microorganism(s). Currently, LAMP has been reported to detect different pathogens in foods, such as STEC, *Salmonella*, *Campylobacter* and norovirus [21-25]. However, the inability to differentiate between live and dead cells limits its application. Propidium monoazide (PMA) and ethidium monoazide (EMA) are intercalating dyes that selectively enter dead-cell membranes and bind to the DNA inside of the dead cells, making it unable to be amplified in the following amplification reaction. However, the permeability of EMA into the live cells of some bacterial species hinders its wide use in the DNA amplification assay as it leads to the loss of DNA. Therefore, PMA was used in our current study in combination with the nucleic acid–based molecular methods, *i.e.,* LAMP and qPCR, for the detection of VBNC enteric pathogens in fresh produce.

The aim of this study was to develop a rapid and sensitive method for the detection of both viable and VBNC states of *E. coli* O157:H7 and *Salmonella* in fresh produce. The effectiveness of the PMA-LAMP assay was compared with the PMA-qPCR assay, and the efficacy of the assay was determined by using fresh produce spiked with VBNC cells. To further investigate the efficacy of the PMA-LAMP assay, a field study and risk assessment were also performed.

Research Methods and Materials

Objective1. Induce the VBNC state in relevant bacterial pathogens.

Bacterial strains and culture conditions

E. coli O157:H7 strain EDL933 and *Salmonella enterica* Enteritidis strain 43353 were used in this study for assay optimization and sensitivity testing. An additional 29 strains, as listed in **Table 1,** were used for specificity testing. *S.* Enteritidis and *E. coli* O157:H7 were preserved in 20% (v/v) glycerol at −80°C, and then streaked onto tryptic soy agar (TSA) (BD Diagnostic Systems, Sparks, MD) at incubated at 37°C for 16 h. The other strains (for specificity testing) were grown on TSA (*Salmonella*, *E. coli*, *Pseudomonas*, *Listeria*, *Staphylococcus*) or Mueller Hinton agar (BD Diagnostic Systems) supplemented with 5% sheep blood (*Campylobacter*). *Campylobacter* strains were incubated under a microaerophilic condition (i.e., 10% CO_2).

Table 1. Strains used in this study for LAMP specificity test.

Strain group	Serovar	Strain	LAMP result
Salmonella			
S. enterica subsp. *Enterica*	Enteritidis	ATCC 43353	+
	Enteritidis	0EA2669	+
	Enteritidis	3512H	+
	Enteritidis	ME13	+
	Enteritidis	ME14	+
	Enteritidis	PT30	+
	Heidelberg	Summerland	+
	Typhimurium	SL1344	+
	Typhimurium	Summerland	+
Escherichia coli	O157:H7	EDL 933	+
	O157:H7	Summerland	+
	O157:H7	ATCC43895	+
	O157:H7	ATCC43888	+
	O157:H7	ATCC43889	+
	O157:H7	ATCC43890	+
	O157:H7	ATCC35150	+
	O8:H16	ATCC43888	-
	O26:H11	M38539	-
	O73:H2	T53317	-
	O103:H2	W20260	-
	O111:Hnon-motile	M21374	-
	O121:H19	H32130	-
	O118:H16	T53809	-
	O165:Hnon-motile	T71841	-
Other bacteria			
Pseudomonas	*aeruginosa*	ATCC 9721	-
	aeruginosa	PA14	-
Listeria	*monocytogenes*	15B88	-
	monocytogenes	15B98	-
Staphylococcus	*aureus*	MRSA-10	-
Campylobacter	*jejuni*	ATCC 33560	-
Campylobacter	*coli*	RM1875	-

Preparation of VBNC *E. coli* O157:H7 and *S.* Enteritidis cultures
Both *E. coli* O157:H7 and *Salmonella* Enteritidis strains were grown in TSB at 37°C for 16 h. Bacterial cells were later harvested by centrifugation at 15,000 ×*g* for 3 min and washed three times with PBS. The cell pellets were re-suspended in 7% NaCl to a concentration of 10^8 CFU/mL. These cultures were then incubated at 37°C with constant shaking at 180 *rpm*. The culturability of the cells was tested by the conventional plating assay at 37°C overnight. When the cell counts on TSA plates dropped below the detection limit (1 CFU/mL), cell viability was analyzed by using the fluorescent LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's protocol. Briefly, the fluorescent SYTO 9 stains all the cells in the population, including the ones with a damaged membrane. In contrast, the membrane-permeable dye propidium iodide (PI) only penetrates the damaged bacterial cell membrane. As a result, when both stains are present, the viable cells are stained with green fluorescence and the dead cells are stained with red fluorescence. The formation of VBNC cells was confirmed when the plating counts decreased to an undetectable level, while the viable cells were still observed under the fluorescence microscope.

Objectives 2 – 4. Assess intercalating dye performance and optimize treatment condition of intercalating dye. Develop a LAMP and qPCR assay, optimize assay conditions for both Salmonella and STEC, and verify assay performance in vitro.

LAMP primers and reaction conditions
The genes *wzy* and *agfA* were selected as specific targets for detection of *E. coli* O157:H7 and *S*. Enteritidis, respectively. LAMP primers were designed using the online PrimerExploer program (version4; Fujitsu Ltd., Japan). Each set of primers includes two outer primers (F3, B3), two inner primers (FIP, BIP), and one or two loop primers (LF, LB) targeting 7 to 8 specific gene sequences. The sequences of the primers are listed in **Table 2**.

Table 2. LAMP and qPCR primers used in the current study to detect *Salmonella* Enteritidis and *E. coli* O157:H7 by targeting *agfA* and *wzy* genes.

Primer name	Sequence (5'-3')	Position
LAMP primers		
Salmonella-agfA (U43280.1)		
F3	GGTCACATTAAACGAACCCT	1619-1638
B3	TGTCCGTCCGAAACAGTA	1796-1813
FIP	TGTGTATGGCTTTGTCCCCC-CGTCTGTCAGGTGCAAAT	1680-1699, 1640-1657
BIP	ACAAACGCTATCTTTACCTGCTAA-TTAACCGAGTCCTCTGAAG	1706-1729, 1768-1786
LF	CGTCGCGCACAGAGAGA	1658-1674
LB	CGATTGAGTTGTCTCGTCTTAGTG	1735-1758
O157-wzy (AF061251)		
F3	GTTCTGCTCCATACGTAGT	288-306
B3	GGTTAACAATAGAGCAAGTTGA	488-509
FIP	ACAGGGAATAAAGCATCAAGACTTA-TGATAAAAAATTTGCTCCCATG	370-394, 310-331
BIP	AATTCCTTTTCTAACTCTGGTGTCG-AAGTGTTCCATATGTTGTTTCT	416-440, 466-487
LF	TGGAGAACGGTTACATAAACAAGT	341-364

Primer name	Sequence (5'-3')	Position
PCR and qPCR primers		
Salmonella-*agfA*-F	TTACTGTCGGCCAATACGGC	1530-1549
Salmonella-*agfA*-R	CAAAACCAACCTGACGCACC	1420-1439
O157-*wzy*-F	TGCTCCCATGTCTCCAAATACT	843-864
O157-*wzy*-R	ACGATTTCTTTCCGACACCAGA	734-755

The LAMP conditions were optimized according to the New England Biolabs' protocol. The LAMP reaction mix (25 µL) for the detection of *E. coli* O157:H7 contained 1× Thermopol reaction buffer (New England Biolabs, Ipswich, MA), 8 mM $MgSO_4$, 1.2 mM each deoxynucleoside triphosphate (dNTP), 1.6 µM FIP/BIP, 1.6 µM LF/LB and 0.8 µM F3/B3, 0.4 U/µl *Bst* 2.0 DNA Polymerase (New England Biolabs), and 2 µL DNA template. The LAMP reaction mix (25 µL) for the detection of *S.* Enteritidis consisted of 1× Thermopol reaction buffer (New England Biolabs), 8 mM $MgSO_4$, 1.2 mM each deoxynucleoside triphosphate (dNTP), 1.6 µM FIP/BIP, 0.8 µM LF/LB and 0.2 µM F3/B3, 0.4 U/µl *Bst* 2.0 DNA Polymerase (New England Biolabs), and 2 µL DNA template. One negative control of 2 µL sterile distilled water, instead of targeted DNA template, was included in each run. The LAMP reactions were carried out at 65°C for 30–60 min and terminated at 80°C for 5 min in the LA-320C Loopamp Realtime Turbidimeter (Eiken Chemical Co., Ltd., Tokyo, Japan). The turbidity derived from the white precipitation of magnesium pyrophosphate, a by-product of the LAMP reaction, was monitored every 6 seconds. The time threshold value (T_t) was measured when differential value of the turbidity increase exceeded 0.1.

qPCR assays
As comparisons, qPCR testing was conducted in parallel. The qPCR mix consisted of 1× SensiFAST SYBR Lo-ROX mix (Bioline USA Ltd., MA), 0.4 µM each reverse and forward primer and 2 µL DNA template. The qPCR reaction was carried out in a CFX96™ real time PCR thermocycler (Biorad, Hercules, CA) that included an initial denaturation at 95°C for 10 min, followed by denaturation at 95°C for 15 seconds and annealing and extension at 60°C for 1 min for 39 cycles. In addition, a melting curve was conducted from 65 to 95°C with an increment of 0.5°C per 0.05 seconds. Fluorescence signals were collected using the SYBR/FAM channel, and one negative control without target DNA was included in each run.

Objective 5. Optimize conditions for the detection of pathogens in spiked produce (lettuce, tomato, cantaloupe and spinach) with the intercalating dye-LAMP assay and verify assay performance in planta.

Assay evaluation for the detection of VBNC enteric pathogens in romaine lettuce, spinach and cherry tomato
Samples of fresh produce (romaine lettuce, spinach and cherry tomatoes) were purchased from local supermarkets and analyzed within 6 h of purchase. All the fresh produce samples were confirmed as *E. coli* O157:H7- or *Salmonella*- negative prior to spiking. Romaine lettuce and spinach samples were sorted and cut into 10-g portions, with care taken to avoid the torn edge of the leaf. Cherry tomato samples were cut and weighed into ~10-g portions. To determine the sensitivity of LAMP and qPCR, 10-fold serial dilutions of VBNC cells were prepared by centrifugation and then washed twice in PBS. Then, a spot inoculation method was applied on the surface of each fresh produce type. Briefly, 1 mL of VBNC cell culture was spot inoculated on the surface of romaine lettuce and spinach samples, resulting in samples with VBNC cell inoculation levels ranging from 10^8 to 10^3 CFU/mL. For cherry tomatoes, 100 µL of inoculum

was spot-inoculated onto the surface of the sample. Three samples without inoculation were included as negative controls. All samples were left in a laminar flow biosafety cabinet for 2 h to air dry. When the samples had dried, they were added separately to a Stomacher bag (VWR International, Radnor, PA) and 90 mL of buffered peptone water (BD Diagnostic Systems) was added, and then each bag was sealed tightly. The sample in the Stomacher bag was manually massaged for 1 min and then put on a horizontal shaker for shaking at 50 *rpm* for 30 min to improve the recovery of VBNC cells from the sample. After recovery, 10 mL of the homogenate was taken from the sample wash and centrifuged at 1,000 ×*g* for 5 min to remove the large plant and soil particles, followed by another centrifuge step at 5,000 ×*g* for 10 min to collect bacterial cells. The pellets were re-suspended in 100 µL of PrepMan Ultra sample preparation reagent (Applied Biosystems, Foster City, CA) and transferred to a 1.5-mL microcentrifuge tube. The cell suspensions were then heated at 95°C for 10 min for gDNA extraction, and centrifuged at 15,000 ×*g* for 2 min. The supernatant was kept for further LAMP and qPCR analysis. The experiments were independently repeated three times.

PMA sample treatment and DNA extraction
PMA solution (20 mM in H_2O; Biotium Inc., Hayward, CA) was added to 100 µL of bacterial culture to obtain a final concentration of 10 µM. After 10 min of incubation in the dark with constant shaking, the mixtures were put on ice and exposed to a 300-W halogen light, with constant shaking for 15 min, at a distance of 20 cm from the light source. After washing with sterile distilled water three times, the mixtures were then boiled at 95°C for 10 min to release the genomic DNA (gDNA). The aliquots (2 µL) were used for both LAMP and qPCR assays.

Objective 6. Determine the survival of VBNC Salmonella and STEC on lettuce in the field and during simulated processing.

Preparation of field plots
Two independent field study trials were performed in two plots located approximately 1.5 m apart. Each plot was 9 m long and divided into 8 separate rows 150 cm apart (**Figure 1**). Romaine lettuce was double planted in 8 rows. Two outer guard rows did not receive inoculum and served as the control rows, whereas the other rows received the inocula of viable and VBNC cells and served as the treatment rows. Plastic fencing (36" high) was installed between the rows to reduce lateral transfer by dust or rain splash, and water was supplied ad libitum through a perforated irrigation hose. Wide black plastic mulch (1.2 m wide) was placed over the rows to cover both sides of the water drip line equally.

Romaine lettuce plants (cv. Parris Island Cos; West Coast Seeds, Delta, Canada) were grown from seed in a greenhouse for 3 weeks and hardened off in the shadow house for 1 week before planting. The seedlings were transplanted by hand in 8 rows per plot, at a density of 100 plants per row. One week prior to the inoculation, each of the 8 rows was thinned to 50 plants.

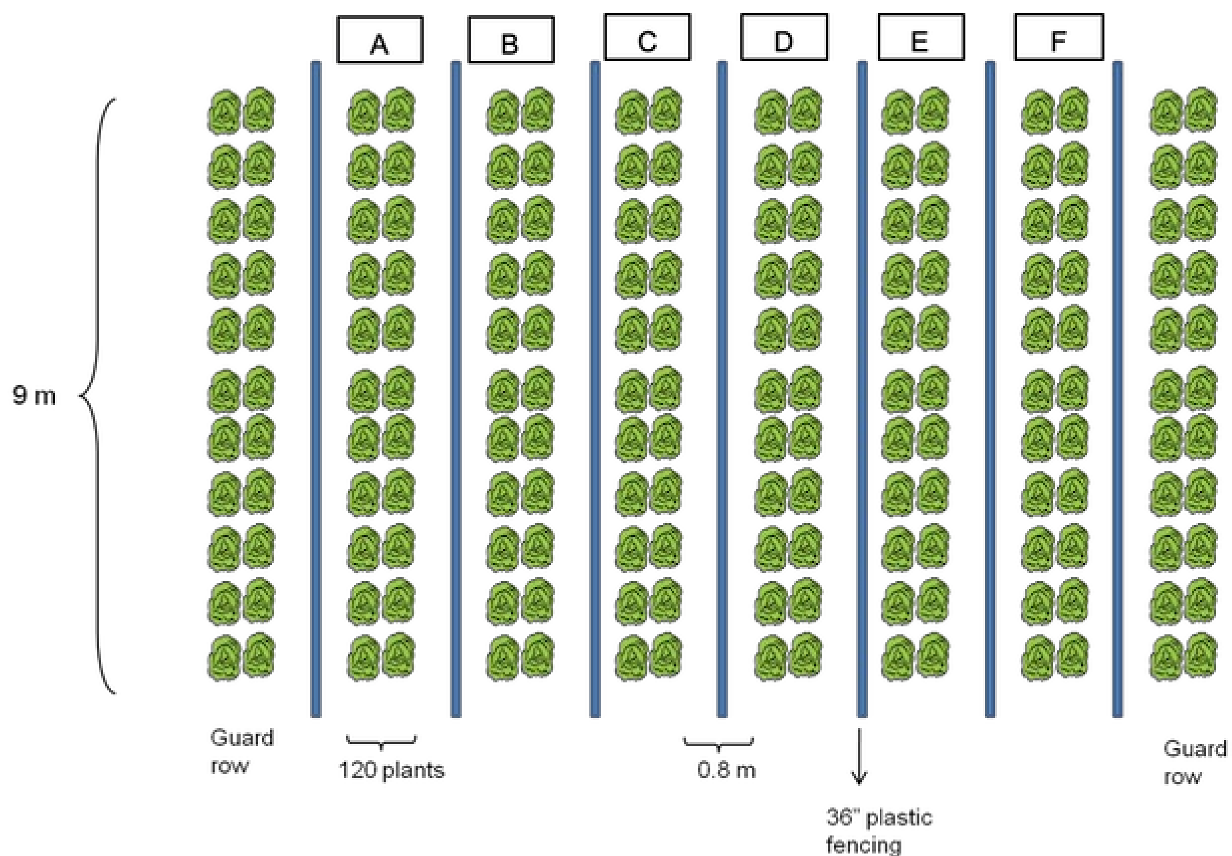

Figure 1. Layout of one representative experimental plot. Two plots located 1.5 m apart were used to carry out two independent trials.

Lettuce inoculation

The inocula were prepared in three treatments of viable and VBNC cells for both *E. coli* O157:H7 and *Salmonella* Typhimurium. Each treatment contained viable cells and VBNC cells at the ratios of 100:0, 0:100 and 50:50 respectively. Specifically, the treatments consisted of: romaine lettuce in individual rows inoculated with (A) an overnight culture of *E. coli* O157:H7; (B) a mixture (50:50) of cells from an overnight culture of *E. coli* O157:H7 and cells in the VBNC state induced by exposure to high NaCl concentration; (C) *E. coli* O157:H7 cells the VBNC state; (D) an overnight culture of *Salmonella* Typhimurium; (E) a mixture (50:50) of cells from an overnight culture *Salmonella* Typhimurium and cells in the VBNC state induced by exposure to high NaCl concentration; and (F) *Salmonella* Typhimurium cells in the VBNC state (**Figure 1**).

Research Results

Objective1. Induce the VBNC state in relevant bacterial pathogens.

We applied 7% NaCl at 37°C as the osmotic pressure to induce *E. coli* O157:H7 and *Salmonella* into the VBNC state. *E. coli* O157:H7 and *Salmonella* cells at 10^8 CFU/mL were treated under this osmotic stress condition, and the survival of both bacteria was observed. When exposed to high osmotic pressure, the culturability of *E. coli* O157:H7 declined to the undetectable level (<3 CFU/mL) after 5 days in 7% NaCl, a faster induction rate than that observed for *S.* Enteritidis, which dropped to an undetectable level after 14 days ($P < 0.05$) (**Figure 2**).

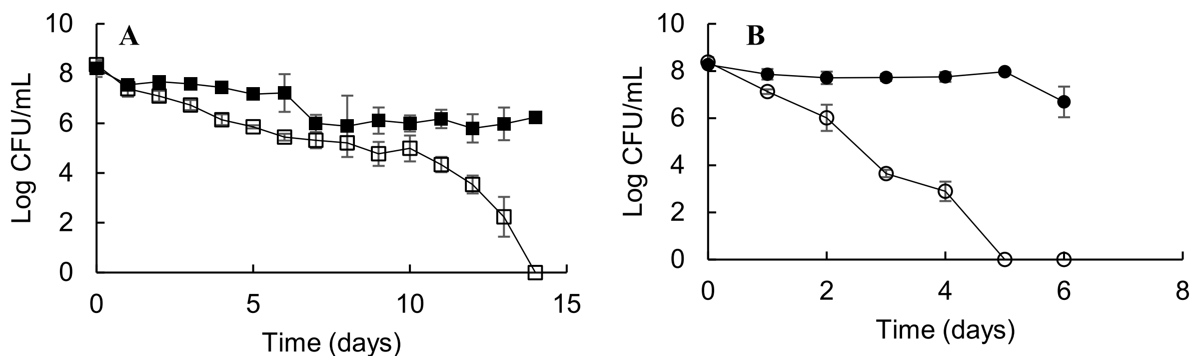

Figure 2. Survival curves of *Salmonella* (A) and *E. coli* (B) incubated in 7% NaCl for up to 2 weeks. Open squares (□) represent the culturable cell counts of *Salmonella* using TSA plating assay. Closed squares (■) represent the viable *Salmonella* counts using the PMA-LAMP assay. Open circles (o) represent the culturable cell counts of *E. coli* O157:H7 using TSA plating assay. Closed circles (●) represent viable *E. coli* O157:H7 counts using the PMA-LAMP assay.

After 5 days, the viable cell counts of *E. coli* O157:H7 decreased slightly and then remained at 10^7 CFU/mL. The loss of culturability of *E. coli* O157:H7 and the presence of viable cells collectively suggested that *E. coli* O157:H7 cells in 7% NaCl entered into the VBNC state. In contrast, *S.* Enteritidis declined to an undetectable level after 14 days of treatment and the viable cell counts decreased slowly to a population of 10^6 CFU/mL at the end of incubation (**Figure 2**). Approximately 1% of the *Salmonella* population was induced into the VBNC state, while 10% of the *E. coli* O157:H7 population was induced into the VBNC state.

Objectives 2 – 5. Assess intercalating dye performance and optimize treatment condition of intercalating dye. Develop a LAMP and qPCR assay, optimize assay conditions for both Salmonella and STEC, and verify assay performance in vitro. Optimize conditions for the detection of pathogens in spiked produce.

For *Salmonella* spiked fresh produce samples, similar results were observed from three independent experiments. Both tomato and spinach showed a detection limit of 10^4 CFU/g by PMA-LAMP, whereas the romaine lettuce showed a 10-fold higher detection limit with 10^5 CFU/g. In comparison, the PMA-qPCR assay could consistently detect *Salmonella* at 10^5 CFU/g while in spiked tomato samples the detection limit was 10 times more sensitive than in the other varieties of fresh produce.

Table 3 summarizes the sensitivity of the PMA-LAMP and PMA-qPCR assays. For the uninoculated control samples, all the targeted genes tested negative for both strains. For *E. coli* O157:H7-spiked romaine lettuce samples, the PMA-LAMP assay consistently gave positive results with spiking levels from 5.13×10^3 to 5.13×10^8 CFU/g, resulting in a detection limit of 5.13×10^3 to 5.13×10^4 CFU/g. For tomato and spinach, the detection limits were down to 5.13×10^4 CFU/g. The PMA-qPCR assay showed similar sensitivity to the PMA-LAMP assay in all the varieties of fresh produce.

Table 3. Comparison of sensitivity of PMA-LAMP and PMA-qPCR when testing serially diluted VBNC cells in pure culture and in spiked produce samples.

Sample type	Method	Detection limit (CFU/reaction or CFU/g)	Quantification equation	Linear R^2
E. coli O157:H7-pure culture	PMA-LAMP	1.02-10.02	y = -2.71x + 31.332	0.954
	PMA-qPCR	1.02-10.02	y = -3.3366x + 32.407	0.996
E. coli O157:H7-spiked lettuce	PMA-LAMP	5.13×10^3-5.13×10^4	y = -1.2x + 26.345	0.966
	PMA-qPCR	5.13×10^4	y = -3.1697x + 39.842	0.985
E. coli O157:H7-spiked tomato	PMA-LAMP	5.13×10^4	y = -1.9683x + 37.876	0.960
	PMA-qPCR	5.13×10^4	y = -3.1534x + 37.789	0.992
E. coli O157:H7-spiked spinach	PMA-LAMP	5.13×10^4	y = -3.6348x + 51.768	0.996
	PMA-qPCR	5.13×10^4	y = -3.549x + 38.148	0.997
Salmonella-pure culture	PMA-LAMP	2.07-20.7	y = -3.55x + 31.159	0.896
	PMA-qPCR	2.07-20.7	y = -3.3558x + 35.8	0.998
Salmonella-spiked lettuce	PMA-LAMP	1.05×10^5	y = -2.05x + 31.558	0.905
	PMA-qPCR	1.05×10^4	y = -3.0322x + 48.434	0.995
Salmonella-spiked tomato	PMA-LAMP	1.05×10^4-1.05×10^5	y = -4.0933x + 52.839	0.962
	PMA-qPCR	1.05×10^4	y = -3.1104x + 47.167	0.981
Salmonella-spiked spinach	PMA-LAMP	1.05×10^5	y = -3.1967x + 40.567	0.933
	PMA-qPCR	1.05×10^4	y = -3.5318x + 48.599	0.997

Objective 6. Determine the survival of VBNC Salmonella and STEC on lettuce in the field and during simulated processing.

Populations of *E. coli* O157:H7 recovered from lettuce were determined by selective and overlay plating assays, and are shown in **Figure 3A–3C.** Populations of culturable cells for treatment A and B determined by using both plating assays declined over the course of the trial. *E. coli* O157:H7 could only be recovered by enrichment after 21 days. Differences in the bacterial population after 1 week in the field determined by using the two plating assays provided evidence of increasing levels of injury of the survived cells (**Figure 3A, B**). Moreover, total viable bacterial population determined by using qPCR suggested that some of the culturable cells entered into a non-culturable state *in planta*. As expected, recovery of culturable *E. coli* O157:H7 from lettuce inoculated with VBNC cells (treatment C) was limited (**Figure 3C**). However, total viable bacterial population determined by using qPCR was also indicative of a gradual decrease and an overall loss of viability in both culturable and VBNC cells over the course of the trial.

The behaviour of *S.* Typhimurium on romaine lettuce was different from that of *E. coli* O157:H7 from several perspectives. The rate of decline in the population of culturable cells determined by using selective and overlay plating assays was less than that of *E. coli* O157:H7. In addition, *S.* Typhimurium could be recovered without enrichment three weeks after the inoculation (**Figure 3D–3F**). The difference between total viable populations determined by using qPCR and total culturable populations determined by using the plating assay was indicative of the induction into VBNC state *in planta.* In contrast with the results of *E. coli* O157:H7, *S.* Typhimurium cells in the VBNC state (treatment E) did not decrease over time.

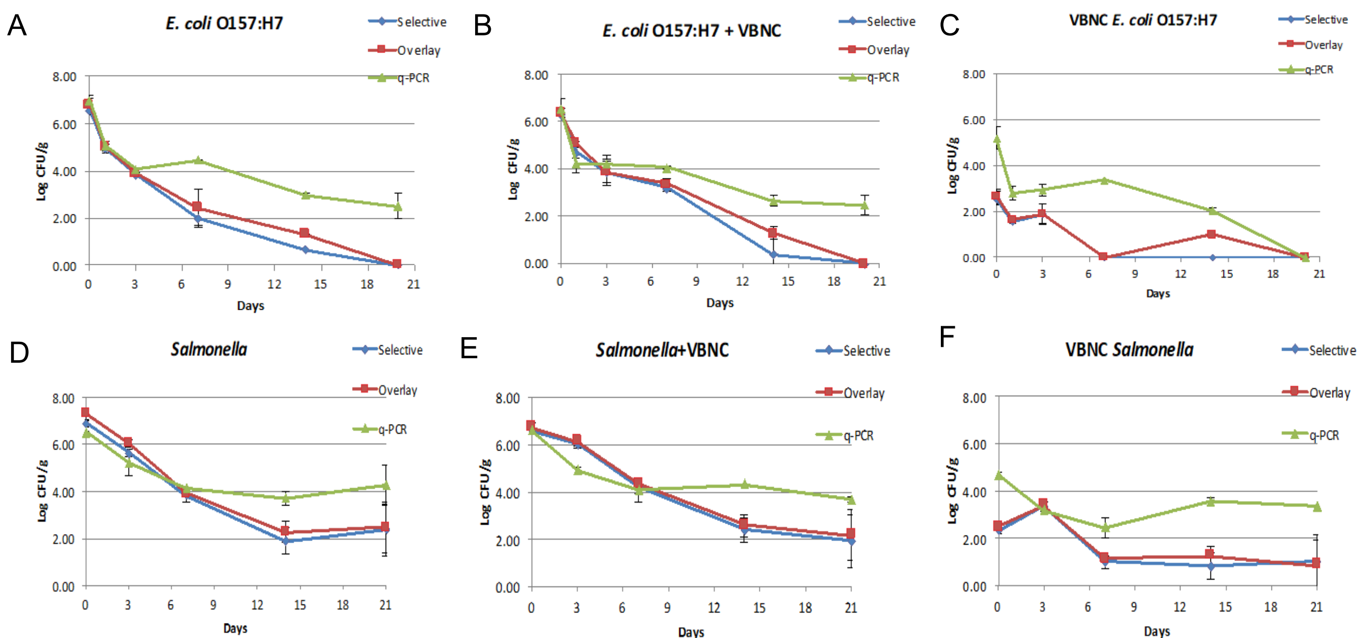

Figure 3. Fate of *E. coli* O157:H7 and *S.* Typhimurium on field-grown romaine lettuce. Romaine lettuce was separately inoculated with (A) an overnight culture of *E. coli* O157:H7; (B) a mixture (50:50) of cells from an overnight culture and *E. coli* O157:H7 cells in the VBNC state; (C) *E. coli* O157:H7 cells in the VBNC state; (D) overnight culture of *S.* Typhimurium; (E) a mixture (50:50) of cells from an overnight culture and *S.* Typhimurium cells in the VBNC state; and (F) *S.* Typhimurium cells in the VBNC state. Each point is the mean population per gram of lettuce from two samples, which includes three mature heads of romaine lettuce taken randomly from the treatment rows.

As for the simulated processing conditions, 100 ppm of chlorine was used to inactivate pathogens in romaine lettuce. *E. coli* O157:H7 was detected in 5/6 and 4/6 bags of unwashed cut lettuce derived from plots that were inoculated with culturable (treatment A) or a mixture of culturable and VBNC (treatment B) cells, irrespective of storage temperature (**Table 4**). *E. coli* O157:H7 was also recovered from 1/2 and 2/6 bags of cut lettuce derived from plants inoculated with VBNC cells only (C) after storage at 4 and 12°C, respectively. Washing in chlorinated water prior to storage reduced but did not eliminate the number of positive samples in cut lettuce from field treatments (A) and (B), but *E. coli* O157:H7 was not detected in any of the cut lettuce derived from lettuce inoculated with VBNC cells (treatment C). Hence, washing in chlorinated

water reduced the frequency of detection in cut lettuce after 1 week of storage at both temperatures.

Analyses for *S.* Typhimurium in bagged lettuce derived from all the field treatments are also shown in **Table 4**. *S.* Typhimurium was recovered in all bags of cut lettuce from treatments (C) and (D), irrespective of washing or storage temperature. *S.* Typhimurium was also recovered from 5/6 bags of cut lettuce derived from treatment (E), irrespective of storage temperature, but not from lettuce washed in chlorinated water prior to storage at 4°C; in contrast, 3/6 samples from this treatment combination were positive after one week of storage at 12°C. The latter is a significant observation, as it signals the potential survival and temperature dependent resuscitation of VBNC cells in cut lettuce made from a contaminated crop.

Table 4. Survival of *E. coli* O157:H7 and *S.* Typhimurium in unwashed and washed (100 ppm chlorine) romaine lettuce stored at 4°C or 12°C for 1 week. Values are the number of samples in which *E. coli* O157:H7 was detected per 6 samples analyzed.

	No. of samples (/6) positive by enrichment after 1 week			
	No treatment at 4°C	No treatment at 12°C	100 ppm chlorine at 4°C	100 ppm chlorine at 12°C
E. coli 100%	5	5	1	2
E. coli 50:50	4	4	4	6
E. coli VBNC	0	0	0	0
Salmonella 100%	6	6	6	6
Salmonella 50:50	6	6	6	6
Salmonella VBNC	5	5	0	3

Objective 7. Risk assessment. Perform semi-quantitative assessment of the risk implied by Salmonella and STEC in the VBNC state using data from field trials and simulated processing.

The risk assessment and assumptions, as stated in points 1 to 10 below, take into consideration i) the experimental results presented by the pre- and post-harvest study of VBNC *E. coli* O157:H7 and *S.* Typhimurium in the current study, ii) the fact that the lettuce heads were cut before sanitizing with 100 ppm chlorine, iii) outbreak data for the hazard identification and characterization, and iv) the movement of lettuce from farm-to-harvest-to-retail.

Assumptions:

1) Romaine lettuce was grown using clean cold water with minimal contamination from wild animals (i.e., rodents/birds).
2) Romaine lettuce was harvested using clean and sanitized equipment and was stored in clean and sanitized containers at 4°C prior to washing.
3) Romaine lettuce was contaminated by both non-VBNC and VBNC *E. coli* O157:H7 after harvest.
4) Romaine lettuce was contaminated by both non-VBNC and VBNC *Salmonella* after harvest.
5) After harvest, the cut romaine lettuce (all leaves remained intact and undamaged) was not mixed and not placed in bags prior to 100-ppm chlorine sanitization.
6) Prior to packaging and/or delivery, the contaminated romaine lettuce was washed in potable cold water for 5 min, followed by a 100-ppm cold chlorinated wash for 5 min.

7) The 100-ppm cold chlorinated water was considered as the sanitizing “kill-step” in which pathogenic bacteria were killed
8) The washed and cleaned romaine lettuce was stored loosely in clean and sanitized containers and refrigerated at 4°C.
9) Romaine lettuce will be consumed by various age groups from 10–70+ years of age. Given the outbreaks that have been associated with leafy vegetables, everyone, but especially those who are pregnant and/or immunocompromised, is told to avoid consumption, yet consumption still occurs.
10) Potential modes of contamination include: irrigation water, which can be contaminated from animal production facilities that leach feces/manure into soil and water; dust from large feedlots blowing in the wind and settling on leafy greens; wild and domestic ruminant and non-ruminant animals; lack of field sanitation and contamination from workers; harvesting equipment and how the lettuce is harvested, such as creating cut surfaces on the leaves; wash/rinse water and ice used for transport/storage; improper storage (i.e., high temperature) and transportation; and cross-contamination during bagging with other foods or other types of leafy greens.

Based on the assumptions and potential modes of contamination as listed above, the following is the overall risk of illness derived from the consumption of romaine lettuce contaminated by VBNC *E. coli* O157:H7 and *Salmonella* and which has not been subjected to a final sanitizing step (**Table 5**): in brief, (i) for *E. coli* O157:H7 the risk of illness is moderate to serious and (ii) for *Salmonella* the risk of illness is serious.

The overall risk of illness from the consumption of romaine lettuce contaminated by VBNC *E. coli* O157:H7 and *Salmonella* and which has been subjected to a final sanitizing wash with 100 ppm free chlorine for 5 min is moderate to serious for *E. coli* O157:H7 and *Salmonella* (**Table 6**).

The findings from this risk assessment show that despite using a final sanitizing step of 100 ppm free chlorinated wash and storing romaine lettuce at 4°C in clean sanitized containers, the risk to illness from *E. coli* O157:H7 and *Salmonella* is moderate to serious. It is possible that avoiding harsh stressful growing conditions, using warm potable water of 40–45°C for rinsing, and increasing the concentration and/or time used for the chlorine wash step with or without UV light would aid in reducing the potential risk of illness. Further work is needed to determine the effect of these additional factors.

Table 5. Overall qualitative health hazards risk assessment for romaine lettuce contaminated by VBNC *E. coli* O157:H7 or *Salmonella*, and is harvested and rinsed without a final sanitizing kill step, and consumed by people 10 to 70 years of age who may also be pregnant and immunocompromised.

Microorganism	Sanitizing Step (100 ppm free chlorinated wash for 5 min)	Likelihood of Survival	Likelihood of Multiplication Needed for Infection to Occur**	Severity or Consequence	Risk Rating
E. coli O157:H7	No	Very Low to Low	No	Critical	Moderate to Serious Risk
Salmonella	No	Medium to High	No	Major	Serious Risk

** This takes into consideration whether multiplication of the microorganisms can occur and if enough time exists (before consumption) for the microorganisms to reach potentially hazardous levels.

Table 6. Overall qualitative health hazards risk assessment for romaine lettuce contaminated by VBNC *E. coli* O157:H7 and *Salmonella*, and is harvested and rinsed with a final sanitizing kill step of 100 ppm free chlorinated wash, and consumed by people 10 to 70 years of age who may also be pregnant and immunocompromised.

Microorganism	Sanitizing Step (100 ppm free chlorinated wash for 5 min)	Likelihood of Survival	Likelihood of Multiplication Needed for Infection to Occur**	Severity or Consequence	Risk Rating
E. coli O157:H7	Yes	Very Low to Low	No	Critical	Moderate to Serious Risk
Salmonella	Yes	Low to Medium	No	Major	Moderate to Serious Risk

** This takes into consideration whether multiplication of the microorganism can occur and if enough time exists (before consumption) for the microorganisms to reach potentially hazardous levels.

Outcomes and Accomplishments

Our *goal* was to develop an innovative PMA-LAMP assay that can provide an easy-to-use, rapid, and sensitive way to detect fresh produce contamination by the VBNC state of *Salmonella* and *E. coli* O157:H7.

In the current study, we have successfully induced *Salmonella* and *E. coli* O157:H7 into the VBNC state by incubation of the cells in 7% NaCl for 5 and 14 days, respectively. The analysis showed that the PMA-LAMP assay had a detection limit of 1.02–10.02 CFU/reaction for *E. coli* O157:H7 and 2.07–20.7 CFU/reaction for *S.* Enteritidis, which were comparable to that of the PMA-qPCR assay. However, the PMA-LAMP assay only took 15 min to show the amplification of the target, which was about 1 h faster than the PMA-qPCR assay. The efficacy of the PMA-LAMP assay was evaluated by using lettuce, spinach and tomato inoculated with the VBNC state of *E. coli* O157:H7 and *S.* Enteritidis, and it was demonstrated to be accurate and robust with the lowest detection limit of 5.13×10^3 CFU/g occurring with romaine lettuce without bacterial enrichment. In this project, we have successfully developed a fast, highly sensitive, and easily interpreted assay for the detection of the VBNC state of *Salmonella* and *E. coli* O157:H7 in fresh produce.

Summary of Findings and Recommendations

In the current study, the PMA-LAMP assay was validated to be rapid (from 15 to 45 min), specific (100% inclusivity and 100% exclusivity), sensitive (1–20 CFU/reaction in pure bacterial culture), quantitative (R^2 from 0.896 to 0.996), and robust. Qualitative risk assessment was performed based upon the field study conducted in Summerland, BC, Canada. There were relatively high risks related to the consumption of romaine lettuce contaminated with non-VBNC and VBNC *E. coli* O157:H7 and *Salmonella.* The major findings in the current study were summarized as follows:

1. The VBNC state of *E. coli* O157:H7 and *S.* Enteritidis was successfully induced by using 7% NaCl, and the cells lost culturability in 5 and 14 days, respectively.
2. PMA-LAMP assay was validated to be a rapid (from 15 to 45 min), sensitive (1–20 CFU/reaction in pure bacterial culture), specific (100% inclusivity and 100% exclusivity) and quantitative (R^2 from 0.896 to 0.996) technique to determine the viable and VBNC state of *E. coli* O157:H7 and *Salmonella.*
3. In the field study, the behaviour of *S.* Typhimurium on romaine lettuce was different from that observed by using *E. coli* O157:H7; *S.* Typhimurium showed a slower decline rate within 3 weeks of inoculation than that of *E. coli* O157:H7, indicating that *S.* Typhimurium is more resistant to the environmental conditions used as compared to *E. coli* O157:H7. Furthermore, the significant difference between viable cell counts and culturable cell counts recovered from lettuce leaves suggested the successful induction of both bacteria into the VBNC state.
4. In the simulated sanitization process, we identified that there was potential survival and temperature-dependent resuscitation of VBNC bacterial cells in cut lettuce harvested from the contaminated crop. The appropriate storage temperature and effective sanitization step were essential to reduce the risks related to the viable and VBNC state of *E. coli* O157:H7 and *Salmonella*.
5. In the qualitative risk assessment study, we have demonstrated that the risk associated with the consumption of romaine lettuce contaminated by VBNC *E. coli* O157:H7 and *Salmonella*, which has been subjected to a final sanitizing step of 100-ppm free chlorinated wash for 5 min, is serious for *E. coli* O157:H7 and moderate to serious for *Salmonella*.

Our distinctive, quantifiable and measurable outcomes provide the first comprehensive data on the extent and fate of the VBNC state of *Salmonella* and *E. coli* O157:H7 in fresh produce, which supports realistic assessment of potential risks and a critical first step in the development of future mitigation strategies to address the consequence of produce contaminated by pathogens in physiological states leading to variable survival characteristics. In addition, the availability of the PMA-LAMP assay provides a reliable method to verify the efficacy of post-harvest treatments meant to reduce contamination by both viable and VBNC pathogens in fresh produce.

Considering all the findings in the current project, we recommend using the PMA-LAMP assay to determine the viable and VBNC state of enteric bacterial pathogens in pure culture and sanitized fresh produce samples. However, PMA-qPCR is more feasible to quantify viable and VBNC bacteria in environmental samples such as field-grown lettuce. When growing romaine lettuce, one should avoid harsh and stressful growing conditions and use warm chlorinated water along with UV light. After an effective sanitization step, using an appropriate storage temperature is essential for post-harvest processing to reduce the risks derived from the viable and resuscitated VBNC state of pathogens in fresh produce. Accidental consumption of romaine lettuce contaminated by non-VBNC and VBNC *E. coli* O157:H7 and *Salmonella* can lead to moderate to serious consequences.

APPENDICES

Publications and Presentations

Presentations:

L Han and X Lu (2018). *Detection, validation, and assessment of risks implied by the viable but non-culturable (VBNC) state of enteric bacterial pathogens in fresh produce*. International Association for Food Protection, Salt Lake City, Utah, USA.

L Han and X Lu (2018). *Detection, validation, and assessment of risks implied by the viable but non-culturable (VBNC) state of enteric bacterial pathogens in fresh produce*. BC Food Processors Association Annual Conference, Vancouver, BC, Canada.

Manuscripts in preparation:

L Han, S Orban, P Delaquis, S Bach, X Lu. Assessment of viable and viable but non-culturable (VBNC) *Escherichia coli* O157:H7 and *Salmonella* Typhimurium on field grown lettuce. (*In preparation*)

L Han, L Ma, X Lu. Rapid and specific detection of viable but non-culturable *Escherichia coli* O157:H7 and *Salmonella* Enteritidis in fresh produce by coupling propidium monoazide with loop-mediated isothermal amplification. (*In preparation*)

Budget Summary

Funds from CPS were used to support personnel salaries ($88,965), fringe benefits ($12,303), supplies and materials ($56,497), equipment and technical services ($646), contractual ($31,484) and other costs ($701), as well as travel ($5,642) for Dr. Xiaonan Lu to attend the annual CPS Research Symposium in 2017 and 2018. Indirect costs totaled $6,044. The total amount spent was $202,282 out of the total award of $204,368. The remaining $2,086 will be used for publication fees ($450) and for Dr. Lu’s travel expenses to the 2019 CPS Research Symposium to present final results.

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