CA LEAFY GREEN RESEARCH PROGRAM

2011-12 FINAL REPORT

Proposal Title: Validation of rapid pathogen detection methods and kits applied to pre-harvest operations for leafy greens production.

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General description:

This study was developed to better understand the factors that influence the detection of *Escherichia coli* O157:H7 and *Salmonella enterica* on leafy greens, particularly lettuce. Environmental stress, pathogen population size, incubation time and type of enrichment, competition with other leaf microbiota as well as sample mass (leaf tissue amount per test) were considered to assess the capabilities of selected molecular methods to detect these two most common enteric pathogens associated with industry routine monitoring, surveillance-based recalls, and outbreaks.

Field trials were performed during the summer and fall of 2011 at UC Davis Plant Sciences Research Farm. A total of 4 types of lettuce were planted including Green Oak, Green Romaine, Red Oak, and Lolla Rosa in order to have green and red-pigmented varieties. Each lettuce variety was planted in a 100 ft bed which represented a plot-block, and each block/variety was replicated in triplicate in the field, utilizing a randomized complete block distribution. The field was inoculated with two doses of attenuated pathogens; log 3 and log 5 CFU/mL (2L per block), and samples were collected after 3 and 10 days of inoculation. Both attenuated bacterial isolates, PTVS155 (*E. coli* O157:H7) and PTVS177 (*Salmonella enterica* sv Typhimurium), are rifampicin resistant strains, which facilitates their recovery and identification. Both rifampicin-resistant isolates retain the genetic and somatic determinants that are reactive with commercial platforms for PCR and serological detection of each pathogen type; the exception is the attenuated *E. coli* O157:H7 strains which do not have shigatoxin conferring DNA sequences and therefore give a negative result when screened for *stx*1 and *stx*2. Inoculated material was harvested and processed within 12 hours of collection. The size of the lettuce leaves was that consistent with mini-greens not further cut, as in use for spring mix salad blends. Collection of the material consisted of excising the intact leaf material above soil-line with sterile scissors and depositing in a sterile plastic sample bag. In total, one bag of approximately 1.5 Kg of plant material was collected from each block; a subsample of 25, 125 or 375 g was collected in triplicate. Thus, for each cultivar a replicated triplicate with 3 separate sub-replicates each was analyzed.

The experiments performed during the summer of 2011 were divided in two parts. In one, survival of both pathogens during the leafy greens production under prevailing weather conditions was determined. In this part of the study, recovery and colony confirmation was performed using direct selective-plating and/or selective enrichment (all media supplemented with 80 mg/L of rifampicin). Rifampicin is a commonly used selective agent to facilitate the recovery, quantification and detection of applied bacteria. Results from this experimental effort were already submitted in the previous report to the California Leafy Green Research Board. In the second related research activity, collected samples were analyzed using enrichment without the incorporation of a selective agent, thus we could evaluate recovery and detection using

commercial enrichment media and kits for molecular detection. For each sample, we performed enrichment for up to 18 hours at 37 and 42^{o}C for *S. enterica* and *E. coli* O157:H7, respectively. The ratio of leaf sample mass to enrichment broth is indicated in each section (see below). After enrichment, *the same sample was subjected to molecular detection and culture confirmation for each method utilized*. For culture confirmation, recover the inoculated microorganism from the primary enrichment was greatly facilitated by amending rifampicin to the selective media, including Chromogenic agar O157 and XLT-4 for recovery of E. coli O157:H7 and *Salmonella,* respectively.

During previous trials it was noticed that field-collected samples analyzed only through molecular detection resulted in predominantly negative detection outcomes, including the internal positive controls (IPC). The IPC serves as both a positive culturable enrichment control and an internal PCR amplification control to identify the presence of interfering inhibitors to the PCR reactions. From these prior results we suspected that inhibition of the PCR reaction associated with the plant material may have been responsible. However, this same outcome was observed with samples collected during summer 2011 which were all subjected to a secondary enrichment consisting of a 4 hour incubation in 42^{o}C preconditioned media (Brain Heart Infusion; BHI) prior to processing for PCR detection. For samples collected during fall of 2011, with previous knowledge of potential inhibition of PCR reaction, 2 mL of enrichment were collected and centrifuged at 14,000 rpm for 2 min, the supernatant discarded and the resultant pellet was washed twice with Butterfield's Phosphate Buffer. The final cleaned sample was utilized for downstream applications, including colony confirmation and molecular detection. All results obtained through molecular detection kits were considered negative only when the IPC was determined to be acceptable.

Results:

Survival and detection of *E. coli* O157:H7 and *Salmonella enterica* on leafy greens during field production.

A. Methods associated with standard mass: enrichment broth protocol.

Samples of three lettuce types (for convenience referred to as cultivars), Green Oak, Green Romaine and Red Oak were utilized in this experiment. Subset samples of 25 g of the plant material was enriched with Brain Heart Infusion media (BHI) or Buffered peptone water (BPW) for enrichment of *Salmonella* and with Proprietary media A or Proprietary media B for enrichment of *E. coli* O157:H7. For both target pathogens a ratio of 1:10 (plant mass to enrichment broth) was used as is standard for AOAC certified test methods and commonly used in FDA BAM protocols. Prior to introduction of the leaf mass, all enrichment media was pre-conditioned to 37 or 42^{o}C for *Salmonella* and *E. coli* O157:H7, respectively. After addition to

enrichment broth, leaf material were thoroughly massaged manually and compressed into the broth volume at the base of the sample bag to ensure the tissue was fully submerged in the broth during incubation. Samples of the enrichment culture were collected after 4, 8, 12 and 18 h of enrichment, except for the Media A, which was requested by the supplier to be also tested after 6 h. Results obtained after 18 hours and by colony confirmation were considered as definitive to calculate the total number of positive samples. All samples were subjected to molecular testing for pathogen detection using a probe-based detection system, immunomagnetic-separation concentration, and a fluorescent dye-based PCR detection system. As these studies utilized non-certified protocols and to accommodate the requests of the cooperating commercial kit suppliers, all results are coded as Kits A, B, C and Z.

Results A.

Survival and detection of both attenuated pathogens was determined to be highly dose dependent and impacted by the duration of post-inoculation environmental exposure; greater frequency of detection by colony confirmation was achieved on plant material inoculated with log 5 CFU/mL than by log 3 CFU/g and a corresponding greater number of samples were found to be positive 3 days post inoculation as compared to 10 days (*Tables 1A-B*). At both inoculum densities under the ambient conditions, quantification without subsequent processing (e.g. concentration or capture filtration) was below the limit of detection. Therefore it was necessary that all samples be subjected to pre-detection enrichment. These results are in agreement with previous survival and persistence experiments on the same cultivars and field location in which detection was performed using rifampicin as a selective agent, as well with diverse experiments performed by the Suslow Lab (See Background Literature). Contrasting the outcomes of the two attenuated pathogens, the attenuated *Salmonella* exhibited a better environmental fitness than the attenuated *E. coli* O157:H7. *Salmonella* survival was more prevalent for both inoculation doses and across the duration of the experiment. This observation was particularly apparent at the high inoculum dose; the frequency of positive detections remained mostly unchanged between 3 and 10 days after inoculation (*Table 1A*). In contrast, *E. coli* O157:H7, at the high inoculum dose, experienced a steep drop in the rate of positive samples from 80% to about 22% (*Table 1B*). Samples treated with low inoculation dose were determined to result in a rapid die-off after just three days from the time of inoculation.

Table 1. Survival of (A) *Salmonella enterica* and (B) *E. coli* O157:H7 during a field study; recovery after 18 h of enrichment.

(A)	Low inoculum dose[a]		High inoculum dose[a]	
Days post inoculation	**3**	**10**	**3**	**10**
Lettuce cultivar	**Total number of positive detection[b]**			
Green Oak	12	2	14	17
Green Romaine	10	5	18	12
Red Oak	8	1	17	14
Total number of positive samples	*30*	*8*	*49*	*43*

(B)	Low inoculum dose[a]		High inoculum dose[a]	
Days post inoculation	**3**	**10**	**3**	**10**
Lettuce cultivar	**Total number of positive detection[b]**			
Green Oak	1	0	16	3
Green Romaine	3	2	15	5
Red Oak	0	0	13	4
Total number of positive samples	4	2	44	12

(a) Initial inoculation dose at the beginning of the field trial were log 3 and log 5 CFU/mL that represent low and high inoculum doses respectively.

(b) Positive samples correspond to samples where the attenuated pathogens were detected and confirmation through culture with rifampicin selective media after 18 h of enrichment was achieved. (n=18 samples per sampling point)

Effect of time and enrichment composition in the detection of E. coli O157:H7 and *Salmonella.*

Comparison among media showed little effect in the detection of both attenuated pathogens, surviving following open-field inoculation, after 18 h of enrichment (*Table 2*). For the case of *Salmonella*, 68 and 53% of total samples were observed with BHI and BPW, respectively, while 25 and 31% of the total collected samples were positive with samples enriched for *E. coli* O157 with Media A and B, respectively. Upon analyzing the results according to the duration of enrichment, it was noticed that for leafy greens inoculated with *Salmonella*, 12 hours of enrichment were insufficient to ensure detection in all samples by colony confirmation that were known positive by inoculation; only slightly over 50% of the samples were positive. In contrast, for *E. coli* O157:H7, after 6 h of enrichment in Media A positive colony confirmation was observed in 96% of the samples, while with Media B between 12 and 18 hours of incubation were necessary to achieve 100% positives. For both *Salmonella*

and *E. coli* O157:H7, Media A and B seem to have a more selective effect that allows earlier detection of the pathogen as compared to BHI or BPW (*Table 2*).

Table 2. Influence of enrichment media and incubation time in the detection of *Salmonella enterica* and *E. coli* O157:H7 from field inoculated lettuce.

	Salmonella		***E. coli* O157:H7**	
	Brain heart infusion	**Buffered peptone water**	**Media A**	**Media B**
Time of enrichment (h)	% of positive detection by colony confirmation[a]			
4	42	38	67	82
6	nd	nd	96	nd
8	58	51	100	82
12	58	52	100	88
18	100	100	100	100
Ratio of positive samples/total samples analyzed[b]	67/108	58/108	28/108	34/108

(a) The percentage was based in the total number of positives that were culture confirmed after 18 hours of enrichment. Positive samples correspond to samples where the attenuated pathogens were detected and confirmation through culture with rifampicin selective media after 18 h of enrichment was achieved. Results for all inoculation levels and lettuce cultivars were pooled. (nd) not determined for this time-point

Molecular detection of both attenuated pathogens was also assessed during the time intervals of enrichment. Samples subjected to this analysis were those treated with the higher inoculum level (log 5 CFU/mL) and collected 10 days post-inoculation. The purpose was to determine the ability of the enrichment procedures to detect both pathogens once they have been exposed to and survived open-field environmental stresses. Comparing results for colony confirmation (*Table 2*) with those using molecular detection (*Table 3*), the percentage of positive samples was less by molecular detection than by culture confirmation after 12 hours for both attenuated pathogens and with both molecular detection systems tested. The number of positive samples ranged between 40-60% compared to 50-80% by culture confirmation. The outcomes relative to the number of replicates and potential for random variability are not dramatic and are not intended to support a suggestion that a culture-based detection approach would be a more practical or sensitive method. It is important to reiterate that results were generated with the use of rifampicin as a selective agent so we can ensure and facilitate the recovery of the applied strain if it was present in the sample. However this data does provide compelling evidence that even in pre-conditioned media, if the target bacterial pathogen has been exposed and has adapted to environmental stress, a period of enrichment greater than 12 hours might be necessary to minimize the potential for false negative outcomes during the application of molecular detection platforms.

Table 3. Detection of *Salmonella enterica* and *E. coli* O157:H7 through though molecular platforms after 10 days post-inoculation exposure on lettuces in the field.

	Salmonella		***E. coli* O157:H7**	
	Kit A	**Kit B**	**Kit A**	**Kit B**
Time of enrichment (h)	**% of positive samples[a]**			
4	35	20	33	8
8	63	32	50	33
12	55	38	58	42
Total number of positive samples/total samples[b]	43/54		12/54	

(a) The percentage was based in the total number of positives that were culture confirmed after 18 hours of enrichment from samples collected after 10 days of inoculation with high dose of inoculum (log 5 CFU/mL)

(b) Positive samples correspond to samples where the attenuated pathogens were detected and confirmation through culture with rifampicin selective media after 18 h of enrichment was achieved. Results for all lettuce cultivars were pooled.

Effect of sample mass and compositing in the molecular detection of E. coli O157:H7 *and* Salmonella enterica.

In an effort to minimize the number of samples during product testing, it has been suggested (and in some commercial applications put into practice without adequate validation) that by increasing the amount of leaf mass collected in each lot-acceptance test unit, the sensitivity during the detection would be enhanced while minimizing the base cost per test employed for routine screening programs. One of the primary practical obstacles often cited for this approach is that the ratio of plant material to enrichment broth needs to be reduced in order to have a test sample unit that can be practically incubated and handled as incubator space may be a limiting factor. For instance, working with samples that are 375 g (fifteen times more the usual sample size of 25 g), it would be necessary to add close to 4 L of media to maintain a standard 1:10 ratio. In this study we evaluated the use of a 1:4 ratio, based on prior reports for ground beef and our own internal studies, in samples containing 375 and 125 g, which was determined to be sufficient to immerse the entire amount of the plant mass. Additionally, a control sample containing 25 g and a 1:10 ratio of plant to enrichment media was also evaluated.

The experiment was done initially with plant material that was collected from the field utilizing non-treated blocks, thus the collected leaf material had not been inoculated with the attenuated pathogens. Samples containing 375, 125 or 25 g were inoculated with approximately 10 CFU/replicate test-unit of the attenuated strains of *E. coli* O157:H7 and *Salmonella* and stored for 8 hours at 5 ^{o}C. Enrichment broth, pre-conditioned to the final incubation temperature, was

added to the samples and aliquots of the enrichment cultures were collected after 8 and 18 hours of incubation. Samples were analyzed by colony confirmation using rifampicin as a selective agent as previously mentioned, and *the same sample* was also utilized for detection of the attenuated pathogens using molecular detection techniques.

After 8 hour of enrichment, all 375 and 125 samples were negative, while all 25 g samples were positive by colony confirmation and molecular testing (data not shown). After 18 hours of enrichment all 'spiked' samples were positive by colony confirmation irrespective of the amount of included plant mass. In contrast, 0 to 50% of the samples was negative by molecular testing in samples containing 375 g of plant material, and about 50-100% of the samples was positive for those containing 125 g (*Table 4*). Using a low challenge dose against indigenous microbiota, this information suggests that a substantial increment in the amount of plant material employed for a lot-qualifying or investigative detection system could be a factor that can contribute to the frequency of false negative outcomes. These observations may be the result of a reduced threshold number of the targeted pathogen cells after enrichment, likely resulting from competition with background bacteria. Therefore, following manufacturer instructions and validated or process-approved protocols for use, adhering to both the amount of sample and the corresponding ratio with the enrichment broth, is essential.

Table 4. Effect of sample size in the molecular detection of (A) *E. coli* O157:H7 and (B) *Salmonella* in leafy greens lab-inoculated prior to enrichment.

	(A)	**Colony confirmation**			**Kit B**			**Kit A**		
		Total number of positives/total number of samples[a]								
	Sample size (g)	**375**	**125**	**25**	**375**	**125**	**25**	**375**	**125**	**25**
Media	Cultivar									
	Green Oak	1/1	3/3	3/3	0/1	2/3	3/3	1/1	3/3	3/3
B	Lolla Rosa	1/1	3/3	3/3	0/1	1/3	3/3	1/1	3/3	3/3
	Total	***2/2***	***6/6***	***6/6***	***0/2***	***3/6***	***6/6***	***2/2***	***6/6***	***6/6***
	Green Oak	1/1	3/3	3/3	0/1	3/3	2/3	1/1	3/3	2/3
BHI	Lolla Rosa	1/1	3/3	3/3	0/1	0/3	3/3	0/1	3/3	3/3
	Total	***2/2***	***6/6***	***6/6***	***0/2***	***3/6***	***5/6***	***1/2***	***6/6***	***5/6***

	(B)	**Colony confirmation**			**Kit B**			**Kit A**		
		Total number of positives/total number of samples[a]								
	Sample size (g)	**375**	**125**	**25**	**375**	**125**	**25**	**375**	**125**	**25**
Media	Cultivar									
	Green Oak	1/1	3/3	3/3	0/1	1/3	3/3	0/1	0/3	3/3
B	Lolla Rosa	1/1	3/3	3/3	0/1	3/3	2/3	0/1	2/3	3/3
	Total	***2/2***	***6/6***	***6/6***	***0/2***	***4/6***	***5/6***	***0/2***	***2/6***	***6/6***
	Green Oak	1/1	3/3	3/3	0/1	3/3	3/3	1/1	3/3	3/3
BHI	Lolla Rosa	1/1	3/3	3/3	1/1	3/3	3/3	0/1	3/3	3/3
	Total	***2/2***	***6/6***	***6/6***	***0/2***	***3/6***	***5/6***	***1/2***	***6/6***	***5/6***

(a) Results were obtained after 18 hours of enrichment. Sampling was done after 8 hours, in which samples containing 25 g of leaf material were positives by all detection methods. The ratio of leaf material to enrichment broth was 1:4 for 375 and 125 grams and 1:10 for 25 g samples.

A second trial was performed with 375 and 125 g of plant material that had been previously inoculated in the field with log 3 CFU/mL and collected 48 h post-inoculation. For these studies, in addition to probe based Kits A and B , two other commercial kits (Kit Y and Kit Z) for detection of *E. coli* O157:H7 and *Salmonella* detection kits were utilized; in all cases leaf mass samples were enriched using a 1:4 ratio. Detection of the attenuated pathogens using Kit C which involves immune-capture prior to molecular detection through PCR and probe based kit Z *E. coli* O157:H7 and *Salmonella* detection kits resulted in no detection for both sample mass

sizes, however the IPC was unacceptable indicating inhibition of reactions even when the samples were further processed and cleaned.

In field assessments with *E. coli* O157:H7, recovery by culture confirmation was observed in 70-100% of the collected samples, however the use of molecular detection methods with 375 g of samples resulted in 0 to 30% positive detection and 0-40% for 125 g samples *(Table 5A)*. In each case the result was a lower rate of detection as compared to leaf material taken from the field and inoculated in the laboratory using standard protocols *(Table 4)*. These results underscore the importance of the physiological status of the pathogen prior to the detection-recovery interval.

Table 5A. Effect of sample mass on the molecular detection of *E. coli* O157:H7 in field inoculated leafy greens.

	(A)	**Colony confirmation**		**Kit B**		**Kit A**	
		Total number of positives/total number of samples[a]					
	Sample size (g)	**375**	**125**	**375**	**125**	**375**	**125**
Media	**Lettuce cultivar**						
B	Green Oak	3/3	6/6	0/3	0/6	0/3	1/6
	Green leaf	1/3	0/6	0/3	0/6	0/3	0/6
	Red Oak	0/2	2/6	0/2	0/6	0/3	0/6
	Lolla Rosa	3/3	1/6	0/3	0/6	2/3	0/6
	Total	***7/11***	***9/24***	***0/11***	***0/24***	***2/12***	***1/24***
		Colony confirmation		**Kit C**		**Kit A**	
	Sample size (g)	**375**	**125**	**375**	**125**	**375**	**125**
BHI	Green Oak	3/3	6/6	0/3	2/6	2/3	2/6
	Green leaf	3/3	4/4	0/2	2/4	0/3	1/4
	Red Oak	0/0	0/0	0/0	0/0	0/0	0/0
	Lolla Rosa	3/3	6/6	0/3	6/6	1/3	4/6
	Total	***9/9***	***16/16***	***0/8***	***4/16***	***3/9***	***7/16***

In the case of samples inoculated with *Salmonella* there is a greater percentage of recovery of positive samples compared to *E. coli* O157:H7 which corresponds to the findings reported in Table 1. Similar to *E. coli* O157:H7 outcomes, those containing 375 g had a lower detection rate than with 125 g *(Table 5B)*. Different from *E. coli* O157:H7 detection, the Kit A assay provided a greater number of positive observations than Kit B or Kit C system when they are compared with the results obtained by culture confirmation (*Table 5B*). It is likely that despite a presumptively comparable population of background bacteria from field grown plants

competing in the enrichment, the imposition of pre-sampling exposure and adaptation of the target pathogens to environmental stress factors limited the PCR-based detection.

Taken collectively, these experimental outcomes point to the need for validation study design that incorporates the plausible physiological status and lag-growth phases of target bacteria and sample mass size in combination with the enrichment culture volume. Ignoring any of these variables, within the understandable objective of reducing the cost of testing and increasing the statistical probability of detecting product defects, may inadvertently increase the risk of false negative outcomes.

Table 5B. Effect of sample mass on the molecular detection of *Salmonella* in field inoculated leafy greens.

	(B)	**Colony confirmation**		**Kit B**		**Kit A**	
		Total number of positives/total number of samples[a]					
Media	**Sample size (g)**	**375**	**125**	**375**	**125**	**375**	**125**
	Lettuce cultivar						
B	Green Oak	3/3	6/6	2/3	5/6	3/3	4/6
	Green leaf	2/3	2/6	0/3	0/6	0/3	1/6
	Red Oak	2/3	5/5	1/3	0/6	0/3	0/5
	Lolla Rosa	3/3	4/6	1/3	1/6	1/3	1/6
	Total	***10/12***	***17/24***	***4/12***	***6/24***	***4/12***	***6/24***
		Colony confirmation		**Kit C**		**Kit A**	
	Sample size (g)	**375**	**125**	**375**	**125**	**375**	**125**
BHI	Green Oak	3/3	4/4	0/3	0/4	3/3	4/4
	Green leaf	3/3	6/6	0/3	1/6	3/3	4/4
	Red Oak	1/1	5/5	0/1	0/2	1/1	5/5
	Lolla Rosa	3/3	6/6	1/3	1/6	3/3	6/6
	Total	***10/10***	***21/21***	***1/10***	***2/18***	***10/10***	***19/19***

(a) Results were obtained after 18 hours of enrichment; however sampling was done after 8 hours but detection was negative for most samples. All internal positive controls worked adequately for molecular detection methods. Kit Z, which is a probe based molecular detection system for both pathogens was also tested, however all outcomes were negative, potentially a result of inhibition as all internal controls were negative.

The comparison of different ratios of plant:broth material was also assessed. For detection of *Salmonella* the effect of sample mass and the ratio of plant to broth material utilized did not have any effect on the detection and overall, detection was achieved in more than 96% of the inoculated samples containing 10 CFU before enrichment (*Table* 6A), additionally quantification of the population of *Salmonella* in the enrichment showed that regarding the

conditions of enrichment employed as well as the composition of the broth, it is always possible to achieve a population larger than log 4 CFU/mL (*Table 7A*), which is estimated to be sufficient to be detected using available molecular detection methods. In addition, this also suggests that *Salmonella* on leafy greens could be easily enriched.

In contrast, detection of *E. coli* O157L:H7 was highly dependent of the type of media utilized, the sample mass as well as the enrichment broth employed for its recovery and detection (*Table 6B*), which was also supported by the final population achieved in the final enrichment (*Table 7B*). Overall, better detection was achieved when a lower amount of sample mass is utilized in combination with a high plant:broth ratio. This strongly suggests that the presence of background bacteria present on the leaf surfaces is highly competitive with *E. coli* O157:H7 (variability in fitness and competitiveness is known among different pathogenic isolates) during the enrichment process. Due to weather conditions the experiment could not be repeated using leaves inoculated on the field. Thus, results need to be cautiously considered as it is possible that environmental stress could add elements that favor the frequency of false negatives.

Table 6. Effect of sample mass and ratio plant material to enrichment broth in the detection of (A) *S. enterica* and (B) *E. coli* O157:H7

	Sample mass (plant:broth ratio)									
	375 (1:4)		**125 (1:4)**		**125 (1:10)**		**25 (1:4)**		**25 (1:10)**	
(A)	**Kit A**	**KitB**	**Kit A**	**KitB**	**Kit A**	**KitB**	**Kit A**	**KitB**	**Kit A**	**KitB**
BPW	3/3	3/3	3/3	3/3	3/3	3/3	5/5	5/5	4/5	4/5
Media B	3/3	3/3	3/3	3/3	3/3	3/3	5/5	5/5	5/5	4/5
Media A	3/3	3/3	2/3	3/3	3/3	3/3	4/5	5/5	5/5	5/5
BHI	3/3	3/3	2/3	3/3	3/3	3/3	5/5	4/5	4/5	5/5
Total detected	100%		92%		100%		96%		92%	

	375 (1:4)		**125 (1:4)**		**125 (1:10)**		**25 (1:4)**		**25 (1:10)**	
(B)	**Kit A**	**KitB**	**Kit A**	**KitB**	**Kit A**	**KitB**	**Kit A**	**KitB**	**Kit A**	**KitB**
TSB	2/3	3/3	3/3	0/3	3/3	0/3	5/5	5/5	5/5	5/5
Media B	1/3	1/3	0/3	0/3	3/3	0/3	5/5	5/5	5/5	5/5
Media A	2/3	0/3	3/3	3/3	3/3	2/3	5/5	4/5	4/5	4/5
Media C	3/3	1/3	3/3	2/3	3/3	3/3	5/5	5/5	5/5	5/5
Total detected	54%		58%		71%		98%		96%	

Results represent total number of positives/total samples tested. Initial inoculation dose was 10 CFU for *E. coli* O157:H7 and samples were spiked in the laboratory, while for *Salmonella* samples were inoculated in the field and they were below the limit of detection by direct plating (log 1.43 CFU/g) at the time of collection.

Results were obtained after 18 hours of enrichment; however sampling was done after 8 hours but detection was negative for most samples. All internal positive controls worked adequately for molecular detection methods.

Table 7. Effect of sample mass in the final population of (A) *S. enterica* and (B) *E. coli* O157:H7 in the enrichment after 18 hour of incubation.

(A)	Sample mass	375 g			125 g			25 g		
	Ratio	1 : 4	1 : 10		1 : 4	1 : 10		1 : 4		
Broth	BHI	4.52 ± 1.34^{a,1}	5.26 ± 1.24^{a,1}		3.78 ± 0.44^{a,1}	5.04 ± 0.50^{b,1}		4.00 ± 0.71^{b,1}		
	BPW	5.46 ± 0.39^{a,1}	5.20 ± 0.61^{a,1}		5.41 ± 1.33^{a,1}	5.49 ± 0.53^{a,b,1}		5.60 ± 0.73^{a,1}		
	Media B	4.35 ± 1.00^{a,1}	4.74 ± 0.31^{a,1}		4.67 ± 0.03^{a,1}	4.93 ± 0.30^{b,1}		4.44 ± 0.67^{a,b,1}		
	Media A	4.13 ± 1.03^{a,1}	5.25 ± 0.98^{a,1,2}		4.49 ± 1.15^{a,1,2}	6.43 ± 0.77^{a,1}		5.22 ± 1.12^{a,b,1,2}		

(B)	Sample mass	375 g			125 g			25 g		
	Ratio	1 : 4	1 : 10		1 : 4	1 : 10		1 : 4		
Broth	Media C	3.36 ± 0.32^{a,3}	5.05 ± 0.63^{a,1,2}		3.06 ± 2.00^{a,2,3}	5.79 ± 0.18^{a,1}		5.63 ± 0.5^{a,1}		
	Media B	1.83 ± 0.62^{a,2}	1.69 ± 0.65^{c,2}		2.13 ± 0.09^{b,2}	5.25 ± 0.16^{a,b,1}		4.70 ± 0.34^{a,b,1}		
	TSB	2.95 ± 1.17^{a,2}	2.92 ± 0.31^{b,c,2}		2.24 ± 0.46^{b,2}	5.12 ± 0.77^{a,b,1}		4.97 ± 0.46^{a,b,1}		
	Media A	2.14 ± 0.49^{a,2}	3.91 ± 0.59^{a,b,1}		4.29 ± 0.60^{a,1}	4.58 ± 0.30^{b,1}		4.40 ± 0.70^{b,1}		

Results represent the mean ± standard deviation in CFU/mL of enrichment (n=5). Samples were collected after 18 h and were plated on TSA supplemented with 80 μg/mL of rifampicin. Different letter within the same column denote significant difference among broth tested ($p<0.05$). Different number within each row, denote significant difference among different sample mass and plant:broth ratio combination ($p<0.05$).

BHI (Brain heart infusion), TSB (Tryptic soy broth)

A statistically valid alternative strategy would be to perform the enrichment of various standard, validated sample mass units (25 g) and then composite the final enrichments into a single sample for pathogen detection processing. To test this approach, we collected aliquots of an enrichment end-point sample that was positive by culture confirmation and molecular detection and mixed this with enrichment cultures that originated from non-inoculated plant material. A total of 1 mL of each sample enrichment was mixed in a Falcon tube, homogenized, and the resultant mixture was utilized for molecular detection. Thus, an enrichment culture from one positive sample was mixed with 3, 5 or 10 known negative samples. Detection of both *E. coli* O157:H7 and *Salmonella* was positive for all composites (*Table 7*). Therefore, preparation of a composite could minimize the cost of product testing without compromising the final result, assuming that the most significant cost is associated with the PCR kit and subsequent detection processing.

Table 7. Effect of sample pooling in the detection of *E. coli* O157:H7 and *Salmonella* on Romaine lettuce using molecular detection methods

	Kit A		**Kit B**	
Mixing ratios[a]	*E. coli* O157:H7	*Salmonella*	*E. coli* O157:H7	*Salmonella*
	Ratio of positive samples/total analyzed composites[b]			
10:1	3/3	3/3	3/3	3/3
5:1	3/3	3/3	3/3	3/3
3:1	3/3	3/3	3/3	3/3

(a) Total number of negative samples:total number of positive samples. The total volume used to create the composite was of 1 mL from each sample.

(b) Enrichment was performed in BPW and MP media for enrichment of *Salmonella* and *E. coli* O157:H7 respectively for 18 hours with a 1:10 ratio of plant material and enrichment broth. Initial inoculation dose was of 10 CFU.

***Effect of non-target Enterobacteriaceae* in the detection of pathogenic bacteria during enrichment.**

During the sample enrichment, especially non-selective conditions, it is expected that the population of non-target target microorganisms will increase. This can be avoided or minimized by utilizing various selective agents (antibiotics, salts, acids etc.), combined with specific incubation temperatures and other selective condition strategies. Despite these semi-selective or highly selective conditions, closely related microorganisms, part of the natural microbiota of the sample, will also be enriched, competing for resources with the target microbe and potentially interfering with detection. In this study, we enriched Romaine lettuce samples that were previously inoculated with 10 CFU of either *E. coli* O157:H7 or *Salmonella.* In addition, samples

were inoculated with 10^5 CFU of commensal *E. coli*, *Citrobacter freundii* or *Enterobacter aerogenes* which are taxonomically related and routinely encountered on most non-cultivated and commercially cultivated plants, including lettuce and leafy greens. Samples were enriched for up to 18 hours and molecular testing was performed, as described above.

Samples that were inoculated individually with each of the challenge strains but that were not inoculated with the pathogens, as well as non-treated material, resulted in a negative outcome after enrichment (data not shown). Samples that contained the three enterobacteria in addition to either *E. coli* O157:H7 and *Salmonella*, were negative in detection for most samples *(Table 8).* Similarly, samples containing commensal *E. coli* as a single challenge strain were uniformly negative in detection using the Kit B for *E. coli* O157:H7, however *Salmonella* detection was positive in every replicate enrichment. Detection of both pathogens in the presence of co-inoculated *C. freundii* or *E. aerogenes* was positive in all samples *(Table 8).* Results suggest that some commensal *E. coli* could interfere with the detection of pathogenic strains following enrichment.

Table 8. Competition assays, effect of enteric bacteria in the detection of (A) *E. coli* O157:H7 and (B) *Salmonella enterica* inoculated on romaine lettuce during enrichment.

(A) Microorganisms applied to the enrichment[a]				**Detection method**	
				Kit B	**Kit A**
Generic E. coli	*C. freundii*	*E. aerogenes*	*E. coli* O157:H7	Positive samples/total samples analyzed	
A	A	A	A	0/3	0/3
P	P	P	A	0/3	0/3
P	P	P	P	0/3	2/3
P	A	A	P	0/3	3/3
A	P	A	P	3/3	3/3
A	A	P	P	3/3	3/3
A	A	A	P	3/3	3/3

(B) Microorganisms applied to the enrichment[a]				**Detection method**	
				Kit B	**Kit A**
Generic E. coli	*C. freundii*	*E.aerogenes*	*Salmonella*	Positive samples/total samples analyzed[b]	
A	A	A	A	0/3	0/3
P	P	P	A	0/3	0/3
P	P	P	P	1/3	2/3
P	A	A	P	3/3	3/3
A	P	A	P	3/3	3/3
A	A	P	P	3/3	3/3
A	A	A	P	3/3	3/3

(a) A total of 10^5 CFU of generic *E. coli*¸ *C. freundii* and *E. aerogenes* and 10 CFU of *E. coli* O157:H7 or *Salmonella* were added to the enrichment. (A)=absent (P)=present

(b) Enrichment was performed in BPW and media B media for enrichment of *Salmonella* and *E. coli* O157:H7 respectively for 18 hours with a 1:10 ratio of plant material and enrichment broth.

Conclusions:

Molecular detection of pathogenic bacteria in produce can be impacted by several factors including the duration of incubation and composition of enrichment media, sample mass and composition, background microbiota and the physiological status of the target microbe. Although this study primarily utilized a Kits A and B pathogen detection platforms, the study was not intended to specifically compare these methods or to study their efficiency outside of the validated and approved test method intended for current commercial application by qualified laboratories. The results of this study did fulfill the purpose of highlighting the importance of validation of molecular detection platforms that include environmental conditions specific and applicable for leafy greens. Similar studies should be performed for any commercial kits that are intended for pathogen detection across a diversity of fresh produce.

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Background Literature from CLGRB funded research

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