Effectiveness of Triple Washing or Organic Sanitizer Treatment in Reducing *E.coli* Levels in Leafy Green Wash Water and its Relationship to Incoming *E. coli* load

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Study Objectives:

1. To determine the effectiveness of two procedures in reducing *E. coli* levels in leafy greens wash water to include:

- triple washing without sanitizer, and
- single washing with an organic (OMRI-approved) sanitizer.

2. To assess the level of *E. coli* contamination at which these treatments are no longer effective.

Background:

Vermont vegetable growers are interested in practical techniques for reducing food safety risks. *E. coli*, a species of bacteria containing many strains found in the mammalian gastrointestinal tract, may be present in farm soils as a result of animal manure applications, irrigation with surface water, and/or wildlife activity. Currently, little is known about the level of *E. coli* and other bacteria in vegetable wash water or the effectiveness of triple washing in reducing those levels. Reducing levels of bacteria in wash water is one way to reduce food safety risks, specifically the risk of cross-contamination whereby one contaminated item leads to the spread of bacteria to other items being washed in the same water.

A preliminary set of on-farm wash water samples taken in 2011 found that triple washing or treatment with SaniDate 5.0, a sanitizer allowed for use on organic farms, consistently and dramatically reduced *E. coli* levels compared to a single wash of lettuce. This study reflects the additional data collected in 2012 in order to develop a basis for making recommendations to growers.

A range of washing systems exist on small diversified vegetable farms that characterize Vermont vegetable production; the effectiveness of such wash systems is not as well studied as large processing systems using sophisticated methods of wash water disinfection. On all farms, special food safety consideration should be given to delicate crops typically eaten raw, such as leafy greens and lettuce. For these crops many Vermont vegetable farms either: 1) use a rinse system with one to three rinses, 2) add disinfectant to the wash water, or 3) use a combination of these two methods.

The main benefit of using a disinfectant in water is to reduce the population of organisms that can lead to cross contamination within the wash vessel. Similarly, the presence of disinfectant in wash water can protect produce quality if there is a temperature-generated pressure differential that forces wash water into crop tissues. Secondary effects may include the reduction of microbes on the surface of the produce. The use of disinfectants requires additional expense, monitoring, and, in the case of organic growers using chlorine, a method of diluting wash water prior to discharge in order to comply with organic certification standards. Sanitizers also raise human and environmental health concerns if improperly used or disposed.

The marketing benefit of triple washing, particularly for crops like leafy greens grown near the soil surface, is a cleaner crop with less grit. Triple washing may reduce or eliminate the need for the use of a disinfectant; however, there is a lack of studies on the effectiveness of triple washing without sanitizer in pathogen reduction. Triple washing requires more infrastructure (three vessels compared with one dump tank) and larger quantities of water.

An indicator of fecal contamination, the presence of *E. coli* may not pose a direct threat to public health but it does suggest the potential for contamination with human pathogens of public health concern. In this study, we tested for generic *E. coli* levels in wash water from three farms using both a triple rinse system without sanitizer and a single rinse system with sanitizer. Compared with the first wash, we assessed the effectiveness of subsequent washes and the addition of sanitizer to the first and second wash in reducing *E. coli* levels in wash water.

We also separately (off-farm) intentionally contaminated water of a first triple wash with mammalian livestock manure in an attempt to determine the levels of *E. coli* at which the effectiveness of triple washing or adding sanitizer would be overwhelmed. The detection of *E. coli* in the final triple wash water (without sanitizer) and/or in the first triple wash water after the addition of sanitizer suggests a threshold level of *E. coli* in the first wash water at which these systems are no longer effective.

Methods:

<u>Farm Site Selection</u>

Three certified organic Vermont vegetable farms representing different scales of production and geographic location were selected for the study. All farms used composted manure on their soils within the last year to improve soil fertility, and all farms had an un-chlorinated but potable water supply for washing vegetables.

Participating farms used different washing methods, as dictated by their infrastructure and markets. Samples were collected approximately weekly from June – September, 2012 (sampling time frame varied by farm) by UVM Extension personnel.

Sanitizer

SaniDate® 5.0, BiosafeSystems, LLC, is a sanitizer/ disinfectant containing the active ingredients hydrogen peroxide (23%) and peroxy acetic acid (5.3%) which is OMRI-labeled and thus acceptable for use on organic farms. SaniDate® 5.0 is labeled for post-harvest use of fruit and vegetable processing water at a rate of 0.5oz (15 ml) / 10 gallons (38 L) water, a 1:1000 dilution.

Washing and Water Sample Collection

Farm 1 – Heads of lettuce, leafy greens and other vegetables were washed by gentle agitation for 5-10 seconds per bay in a triple bay stainless steel sink with each bay containing 10 gallons of water without sanitizer. Wash water samples were collected from each bin. A full dose of SaniDate (15 ml) was then added to the first bin and a half-rate of SaniDate (7.5 ml) was added to the second bin (the addition of half-rate SaniDate to the second bin was limited to samples taken towards the end of the study period). Wash water samples were then collected promptly after gentle mixing from the first and second bin following the addition of SaniDate. At the end of the washing and sampling, all three bays were emptied, thoroughly rinsed with potable water and refilled for the next batch of produce.

Farm 2 – Leafy green salad mixes were triple washed in 150 gallon tubs containing approximately 135 gallons of potable water without sanitizer. Typically one batch contained two to four harvest bins of salad greens, each weighing approximately 30 lbs. After each batch the water from the first wash was drained and refilled with fresh water. For the next batch of greens, the wash tub that had been used for the second wash was used for the first wash, the wash tub that had been used for the third wash was used for the second wash, and the newly filled tub (previously the first wash but now with fresh water) became the third and final wash. The goal of the tub rotation is to conserve water (only one tub is changed out per batch instead of three) without compromising water quality, particularly in the final wash. Wash water samples were collected at the end of a batch from each tub. Ten gallons was removed from tub one into a 12-gallon tote. To the sample ten gallons, a half rate of SaniDate (7.5ml) was added, stirred, and a water sample was collected. An additional 7.5ml SaniDate was then added to achieve a full dose, stirred, and a wash water sample was again collected.

Photo 1 - Triple wash system for salad greens at Farm 2. Gray harvest containers (right in photo) contain salad greens from the field. Three stock tanks are used to triple wash greens. Greens are moved from tank to tank inside of a net with pulley. Greens are then moved to a sloping board to drain and spun dry in the spin cycle of a washing machine. Greens are bagged and sold directly to restaurants and institutions.

Farm 3 – Mixed produce was triple washed in 100 gallon tubs containing approximately 85 gallons of water without sanitizer. Produce varied weekly depending on the harvest and wash water was not changed in between produce types. After washing, wash water samples were collected from each bin. A full dose of SaniDate (120 ml) was then added to the first bin and a half-rate of SaniDate (60 ml) was added to the second bin. Wash water samples were again collected from the first and second bin following the addition of SaniDate.

Intentional Contamination

In order obtain wash water samples with sufficiently high *E. coli* levels needed to overwhelm triple washing and the addition of sanitizer, initial wash water was intentionally contaminated with llama manure at a third site as follows:

Phase 1: Goat and llama manure in natural (pellet) and slurry form were mixed into 10 gallons of water to determine baseline levels in what would be equivalent to the first wash. After 5 minutes, water samples were taken. **Phase 2:** Using the manure load from Phase 1 as a guide, manure was added to the first wash of a triple wash system set up to wash 8 lbs of leafy green (kale or Swiss chard) in 10 gallons of water. Samples were collected from:

- each of the triple wash totes without sanitizer (n=3)
- first wash with a half rate of sanitizer
- first wash with a full rate of sanitizer
- second wash with a half rate of sanitizer

Laboratory Analysis

Water samples were tested for *E. coli* (MPN CFU /100ml) by the Vermont Department of Health Laboratory using an Enzyme Substrate Test (EST)-Quantitation from IDEXX. When counts were expected to be high (i.e., the first wash), a portion of the sample was set aside to use for dilution (1:100), if necessary. Results are reported as Most Probable Number (MPN) / 100ml.

Data Analysis

Data were entered and analyzed using Microsoft Excel 2010. The range of *E. coli* levels in the initial untreated wash water from the three farms were used to reflect the natural range of incoming levels of *E. coli* on produce. Reduction of *E. coli* levels with each additional wash and following the addition of SaniDate® 5.0 in half and full concentrations to the first wash and/or half concentrations to the second wash were calculated both as log10 and percent reductions. An artificial value of 0.5 MPN / 100ml was inserted for zero values for the purposes of calculating log10 reductions. Percent reduction means were compared using a two-tailed paired t-test when comparing washes and treatments from the same sample sets (e.g., double vs. triple washing). Percent reduction means were compared using a two-tailed Student's t-test when comparing different wash types (e.g, greens vs. non-greens washes) or different sites. Groups were tested for equal variances using the f-test to determine the type of Student's t-test used.

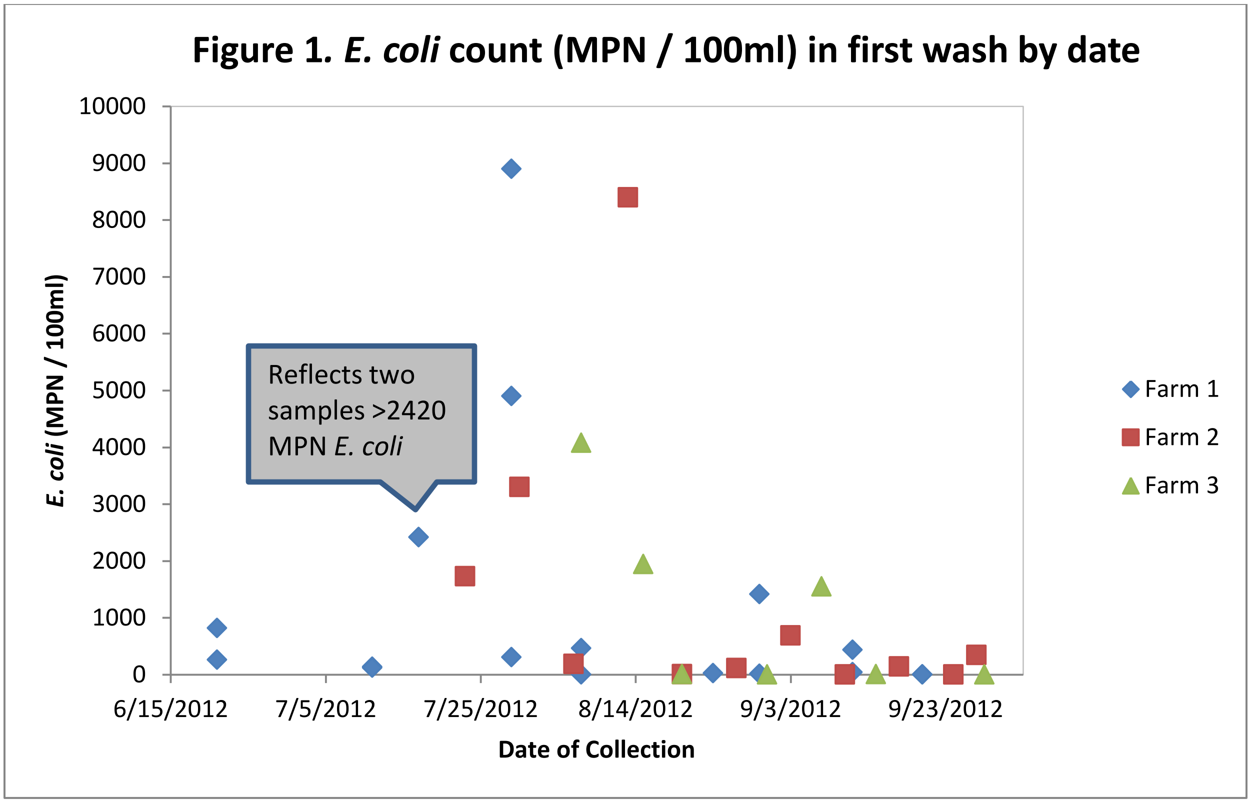
Results:

Farm Sites

A total of 36 sets of wash water samples were collected: eighteen from Farm 1, eleven from Farm 2, and seven from Farm 3. Two of the first washes were not diluted and had results reported as >2420 MPN / 100ml E. coli, indicating E. coli levels that exceeded the upper limit of detection of the test without dilution.

E. coli levels in the initial wash varied widely from 0-8900 MPN / 100ml. High levels were observed at all farms and for a variety of produce types in late July to early August. *E. coli* levels were consistently lower in late August and early September (see Figure 1). Although we did not systematically collect data on weather conditions and the appearance of the wash water, levels of *E. coli* did not appear to be affected by these variables.

Figure 1.



Of the 36 sample sets, 13 were excluded from reduction analysis due to E. coli counts <50 MPN / 100ml in the first wash. Including these set in the analysis would have increased the rates of reduction since subsequent washes typically yielded 0 counts (resulting in low actual but high percent (typically 100%) reduction). Two additional sets were excluded from analysis because the initial reading was reported as >2420 MPN, the limit of detection of the test without dilution.

An accurate result for the first wash is necessary for accurate percent and log10 reduction calculations. Thus, twenty-one sample sets were used for on-farm reduction analysis. The mean reduction of *E. coli* in the single wash with a full dose of sanitizer was 99.8%, which outperformed all other washes and treatment ($p<.01$). Triple washing produced significantly greater reductions than double washing ($p<.01$) or single washing with a half dose of sanitizer (Farm 2 only, $p=.03$).

On average, the second wash reduced *E. coli* levels by 87.9 %. The second wash reduction was higher (90.9%) for greens only than for non-greens (78.5%) but there was no significant difference between greens and non-greens produce types for any of washes or treatments due, at least in part, to the small sample size (n=5) of non-greens produce. Double washing with a half dose of sanitizer appeared to perform well (98.3% reduction), although this sample size (n=5) limited statistical comparisons with other treatments. A single wash with a half dose of sanitizer did not perform as well as a single wash with the full dose, 90.8% vs. 99.8% reduction, respectively ($p=.01$), suggesting that the full dose recommendation is necessary for maximum effectiveness in the case of a high level of contamination. Tables 1 and 2 display percent and log10 reductions, respectively.

	Table 1. Mean and (range) Percent Reduction of *E. coli* by Wash and/or Treatment				
Farm / Produce Type	**Double Wash**	**Triple Wash**	**Double Wash + Half-rate SaniDate***	**Single Wash + Half-rate SaniDate****	**Single Wash + Full-rate SaniDate**
Farm 1 n=10*	88.54 (51.7 - 98.2	97.9 (94.9 - 100)	98.7 (96.3 - 99.9)	--	99.9 (99.2 - 100)
Farm 1 Greens Only n=7	93.8 (80.2 - 98.7)	98.1 (94.9 - 100)	98.7 (96.3 - 99.9)	--	100 (99.9 - 100)
Farm 1 Non-Greens n=3	76.2 (51.7 - 99.2)	97.2 (96.4 - 98.1)	--	--	99.7 (99.2 - 100)
Farm 2 n=8	88.0 (73.9 - 98.8)	97.0 (89.6 - 99.6)	--	90.8 (79.9 - 99.8)	100 (100 - 100)
Farm 3 n=3	85.9 (74.3 - 94.1)	93.4 (88.0 - 97.0)	97.7 (96.9 - 99.4)	--	99.0 (96.9 - 100)
All Farms All Produce Types n=21	87.9 (51.7 - 98.8)	96.9 (88.0 - 100)	98.3 (96.3 - 99.9)	90.8 (79.9 - 99.8)	99.8 (96.9 – 100)
All Farms Greens Only n=16	90.9 (73.9 - 98.8)	97.5 (94.9 - 100)	98.7 (96.3 - 99.9)	90.8 (79.9 - 99.8)	99.8 (96.9-100)
All Farms Non-greens N=5	78.5 (51.7 – 99.3)	95.0 (88.0 – 98.1)	97.7 (96.0 – 99.4)	--	99.8 (99.2 – 100)

*n=3 for Farm 1, Farm 1 Greens Only, and All Farms Greens Only. n=2 for Farm 3. n=5 for All Farms All Produce Types.

**n=8 for Farm 2, All farms All Produce Types, and All Farms Greens Only.

	Figure 2. Mean and (range) Log10 Reduction *E. coli* by Wash and/or Treatment				
Farm / Produce Type	**Double Wash**	**Triple Wash**	**Double Wash + Half-rate SaniDate***	**Single Wash + Half-rate SaniDate****	**Single Wash + Full-rate SaniDate**
Farm 1 n=10*	1.28 (0.32 -2.14)	1.93 (1.30 - 3.38)	2.42 (1.43 - 3.15)	--	3.82 (2.12 - 5.25)
Farm 1 Greens Only n=7	1.38 (0.70 - 1.89)	2.09 (1.30 - 3.38)	2.42 (1.43 - 3.15)	--	3.92 (2.91 - 4.99)
Farm 1 Non-greens n=3	1.03 (0.32 - 2.14)	1.57 (1.44 - 1,72)	--	--	3.60 (2.12 - 5.25)
Farm 2 n=8	1.09 (0.58 -1.93)	1.69 (0.98 -2.37)	--	1.35 (0.70 - 2.64)	3.12 (2.32 - 4.23)
Farm 3 n=3	0.93 (0.59, 1.23)	1.26 (0.92 - 1.53)	1.82 (1.40 - 2.24)	--	2.87 (1.51 - 3.61)
All farms All Produce Types N=21	1.16 (0.32 - 2.14)	1.74 (0.92 - 3,38)	2.18 (1.40 - 3.15)	1.35 (0.70 - 2.64)	3.42 (1.51 - 5.25)
All farms Greens Only n=16	1.23 (0.58 - 1.93)	1.85 (0.98 - 3,38)	2.42 (1.43 - 3.15)	1.35 (0.70 - 2.64)	3.37 (1.51- 4.99)
All farms Non-greens N=5	0.93 (0.31 – 2.14)	1.39 (0.92 – 1.72)	1.82 (1.40 – 2.24)	--	3.58 (2.12 – 5.25)

*n=3 for Farm 1, Farm 1 Greens Only, and All farms Greens Only. n=2 for Farm 3. n=5 for All farms All Produce Types.

**n=8 for Farm 2, All farms All Produce Types, and All Farms Greens Only.

Intentional Contamination

The pellet form of goat and llama manure added to 10 gallons of water yielded low E. coli results. Greater E. coli counts were achieved with manure slurry (Table 3), so llama slurry was selected to test the upper range of treatment effectiveness.

Table 3. E coli in MPN / 100ml by manure type, quantity, and preparation			
Species	**Number of pellets**	**Preparation**	**E. coli (MPN / 100 ml)**
Llama	5	Pellet	77
Goat	20	Pellet	15
Goat	20	Slurry	193
Llama	15	Slurry	>2420*

Four triple washes were conducted with 15 llama manure pellets in slurry preparation. Initial wash readings varied widely, from 840 - 173,290 MPN / 100ml *E coli.* Reduction rates were consistently high for all samples. Average reduction rates were significantly higher for double and triple washes and half-rate SaniDate® 5.0 in the first wash than similar reduction rates for farms ($p<.01$). See table 4.

Table 4. Mean and (range) Percent and Log10 Reduction E. coli Intentional Contamination n=4					
Reductions	**Double Wash**	**Triple Wash**	**Double Wash + Half-rate SaniDate***	**Single Wash + Half-rate SaniDate**	**Single Wash + Full-rate SaniDate**
% Reduction	97.8 (96.7- 98.6	99.9 (99.8 - 100)	100 (100 - 100)	99.5 (98.1 - 100)	100 (100 - 100)
Log10 Reduction	1.69 (1.48 - 1.85)	3.22 (2.81 - 3.65)	4.38 (3.23 - 5.54)	3.39 (1.71 - 4.57)	4.38 (3.22 - 5.54)

Photo 2 – Intentional Contamination Samples Collection bottles containing water samples from the first, second and third washes (left to right). The first sample is tan, but the second and third samples are clear. Samples showed this trend throughout the study; although data was not collected there was no apparent correlation of turbidity with the level of *E. coli* in wash water.

Discussion:

Incoming *E. coli* levels varied widely (0-8900 MPN / 100ml) throughout the study period. High levels were not necessarily associated with turbid water as first washes were often turbid even when levels of *E. coli* were very low. High levels of *E. coli* were primarily observed early in the study period on all farms. This study did not assess potential causes for high *E. coli* levels, but did demonstrate that very high levels can occur in the absence of obvious cues suggesting contamination (e.g., extreme weather events, dirty water after washing).

Additional produce washes beyond the first wash greatly reduced the amount of *E. coli* in wash water. On average, *E. coli* levels are reduced by 88% with double washing and 97% with triple washing, demonstrating that large reductions are possible without the addition of sanitizer.

The addition of an OMRI-approved sanitizer, SaniDate® 5.0, at concentrations recommended by the manufacturer most successfully and consistently reduced *E. coli* counts (99.8%). In 17 of 21 samples, reduction was 100% (meaning no *E. coli* was detected in wash water samples). The addition of SaniDate® 5.0 to the first wash at half the recommended concentration did not reduce *E coli* levels to the same extent and was comparable to double washing, particularly for greens. Although our sample size is small, the addition of a half rate of sanitizer to the second wash produced results similar to triple rinsing and the use of a full dose of sanitizer in the first wash.

The intentional introduction of manure resulted in highly variable *E. coli* counts. It is possible that the manure (and by extension the *E. coli*) was not evenly distributed through the wash water. If manure aggregates were present, sampling could under or over represent the average number of *E. coli* in the wash water. Large reductions were seen in these washes, likely because the *E. coli* was introduced into the water rather than via produce. The five minutes of wash time may not have been sufficient *for E. coli* to adhere to plant tissues. These samples may better represent reduction achieved when clean produced are exposed to bacteria through cross contamination. Because these reductions are not consistent with on-farm reductions, we do not feel that we can assess the level of *E. coli* in which the practices of triple washing or the addition of SaniDate® 5.0 are overwhelmed using these data.

Although limited, on-farm data provides the best information on the limits of the system. For all incoming *E. coli* levels, there was $\geq$97% reduction with a full concentration of SaniDate® 5.0 in the first wash. Triple washing was able to reduce *E. coli* counts to <100 counts in 19 of 21 samples. The initial (first wash) counts associated with the triple wash counts $\geq$100 were high (8900 MPN / 100ml and 4080 MPN / 100ml).

Study Limitations:

This study has several limitations. First, we used generic *E. coli* levels in wash water as a proxy for *E. coli* on produce. It is possible that the levels of bacteria clinging to or absorbed by the produce are not reduced to the same level as bacteria present in wash water. The levels in wash water reflect the bacteria that were dislodged from the produce, not the levels that continue to adhere to the produce which will be ingested by consumers. It is the presence of pathogens **on** food, not in the wash water, which is the direct public health concern.

Second, our study uses generic E. coli as a proxy for pathogenic bacteria such as *Salmonella*, *Campylocater* and pathogenic *E. coli* that could also be in vegetable wash water. The use of ubiquitous generic *E. coli* was a necessary first step for on-farm testing, as pathogens are much less common and it is unacceptable to introduce them into the on-farm setting for the purposes of research. It is possible that the same level of reduction is not achieved by triple washing and/or the addition of sanitizer for pathogenic bacteria, viruses or parasites as we observed with generic *E. coli*. However, the availability of a low-cost lab test for generic *E. coli* in water makes it a practical indicator for farms to use in managing food safety risks that may be associated with vegetable wash water.

Although the presence of *E. coli* in wash water indicates a potential pathway for contamination of food with human pathogens, there are no standards or guidelines for levels of *E. coli* in wash water, nor have studies been conducted that estimate the risk of foodborne illness associated with wash water *E. coli* levels. Therefore, we are unable to calculate the risk reduction of foodborne illness attributable to triple washing and the use of disinfectant in wash water.

Finally, intentional contamination of wash water with mammalian livestock manure may not reflect natural contamination events within farm ecosystems. The goal of intentionally contaminating the water was to estimate the *E. coli* levels in initial wash water at which a triple wash system and the addition of sanitizer are overwhelmed. The high reductions levels suggest that this system was dissimilar to natural on-farm systems.

Summary:

This study suggests that both triple washing produce and the addition of SaniDate® 5.0 at the manufacturer's recommended concentration to the first wash are highly effective in reducing *E. coli* levels in wash water. These practices are affordable and require a small amount of additional management on the part of farmers, and should be considered for adoption. Addition of a half-rate of SaniDate® 5.0 to the second wash also appears effective, although data are limited. Double washing and a half dose of sanitizer in the first wash also reduce *E. coli* levels, but to a lesser extent than other methods. Given the limited savings of time and money for farmers relative to triple rinsing or full dose of sanitizer, these less-effective practices are not recommended, though they are clearly preferable to single washing and no sanitizer use.

The introduction of manure as a natural form of *E. coli* directly into wash water does not appear to be comparable to field contamination of produce, but may be a reasonable proxy for cross contamination events.

Further investigation is needed to assess on-farm reduction levels of *E. coli* in produce. We suggest a similar on-farm study design, collecting and sampling for *E. coli* on produce prior to and following each wash and/or addition of sanitizer.

Beneficiaries:

This study is applicable to most of the approximately 500 produce growers in Vermont, specifically those growing greens. The results are generalizable to produce growers outside of Vermont who have small to mid-size operations. This is a first step in accumulating scientifically-backed recommendations for washing produce in small and mid-sized operations with the ultimate goal of preventing foodborne illness associated with eating fresh produce.

Lessons Learned:

The on-farm study methodology provided consistent results with a dose response, and appears to be a promising methodology for assessing the effectiveness of triple washing and sanitizer.

The introduction of fecal material into the first wash water does not appear to approximate contaminated produce, but rather may be more similar to a cross contamination event (which we did not assess in this study).

Future studies should test produce rather than wash water for E. coli and/or pathogen levels after various washes and/or sanitizer treatments.

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