CENTER for PRODUCE SAFETY

CPS 2020 RFP
FINAL PROJECT REPORT

Project Title
Field evaluation of microfluidic paper-based analytical devices for microbial source tracking

Project Period
January 1, 2021 – December 31, 2022 (extended to January 31, 2023)

Principal Investigator
Mohit Verma, PhD
Purdue University
Agricultural and Biological Engineering
ABE 3016, 225 S University St.
West Lafayette, IN 47907-2093
T: 765-496-3687
E: msverma@purdue.edu

Co-Principal Investigator
Aaron Ault, PhD
Purdue University
Electrical and Computer Engineering
West Lafayette, IN 47907-2035
T: 765-494-3530
E: aultac@purdue.edu

Objectives
1. *Establish background levels of fecal and pathogenic contamination in the field to determine the limits of detection that are needed for a field-based assay.*
2. *Design and test a portable microfluidic paper-based analytical device (μPAD) that can detect contamination and provide results within an hour in the field.*

Funding for this project was provided partly through the CPS Campaign for Research.

FINAL REPORT

Abstract

Fecal contamination in fresh produce poses a significant health risk due to the presence of pathogens that can cause foodborne illnesses. Existing methods for evaluating the risk of fecal contamination have certain limitations, such as they are costly, time-consuming, and require laboratory infrastructure. To overcome these limitations, our research team focused on using *Bacteroidales*—an obligate anaerobe that constitutes a large portion of fecal bacteria and has high host specificity and low abundance in non-fecal sources—as an indicator of fecal contamination. In this project, we developed a novel risk assessment tool using loop-mediated isothermal amplification (LAMP), a molecular technology that enables on-site evaluation of fecal contamination risk within an hour. The primers for LAMP were designed based on the conserved region of 16S ribosomal RNA (16S rRNA) gene. We conducted baseline studies to determine the background concentrations of *Bacteroidales* in fresh produce fields through the growing and harvesting phases of production. The team quantified *Bacteroidales* concentrations in both high-risk fields (adjacent to animal feeding operations) and low-risk fields (commercial fresh produce farms). The results showed a 10^4-fold difference in concentration between the two environments.

To validate our assay, we tested it in a variety of conditions: a controlled lab setting, around animal feeding operations, and in fresh produce fields during the growing/harvesting operations. DECODE (Device for Easy Cross-species tracking Of DNA in the Environment), droplet dispensers, and other devices were developed to make the testing more accessible to minimally trained users. Field trials were conducted during the growing/harvesting seasons using microfluidic paper-based analytical devices (μPADs) and DECODE. Ultimately, the field trial results were validated in the lab, which showed good agreement. Our fully-integrated LAMP testing platform with features of fluid delivery, heating, paper-based LAMP assay, and imaging enabled on-site identification of fecal contamination within 60 min of sampling. The assay only needs a water bath and has minimal sample processing; DNA extraction and purification are not required. The ability to implement the test in low-resource settings could promote widespread adoption. We anticipate that due to the simple nature of the assay, it can be coupled with the current food safety approaches for fresh produce and help reduce outbreaks of foodborne illness or food contamination incidents.

Background

Foodborne outbreaks caused by fecal contamination of fresh produce represent a serious concern to public health and the economy (Carstens et al., 2019). To address this issue, the California and Arizona Leafy Greens Marketing agreements have updated their metrics to take into account potential sources of contamination, such as atmospheric particles/aerosols and irrigation water quality. The U.S. Food and Drug Administration (FDA) has also responded by implementing the FDA Food Safety Modernization Act (FSMA) with new requirements for growers to enhance food safety.

To assess the level of fecal contamination, it is common practice to quantify the abundance of one or more fecal indicator bacteria (FIB) (Brauwere et al., 2014). FIBs, such as *Escherichia coli*, *Enterococcus faecalis*, and *Bacteroidales*, have been selected as indicators of fecal contamination (Allende et al., 2018; Denis et al., 2016; Tambalo et al., 2012). *Bacteroidales* are particularly useful because they are restricted to warm-blooded animals and are a key component of gut microflora (Bernhard & Field, 2000a). Furthermore, as obligate anaerobes, *Bacteroidales* are unable to proliferate in standard atmospheric conditions, making it

less likely that they would be overrepresented in assessments of fecal contamination. Laboratory methods used to detect FIB include culture-based techniques (Hoadley & Cheng, 1974) and DNA-based approaches (Gómez-Doñate et al., 2016; Kildare et al., 2007). Culture-dependent techniques have limitations in detecting the Viable but Non-Culturable (VBNC) state (Zhao et al., 2017) and require overnight incubation, which can delay results and prevent prompt implementation of contamination control measures. On the other hand, DNA-based methods, such as the polymerase chain reaction (PCR), are rapid but require a laboratory, specialized personnel, and expensive equipment, making in-field assessments difficult. LAMP-based methods offer a simple and effective alternative for on-field pathogen detection (Davidson et al., 2021; Mohan et al., 2021; Notomi et al., 2000; Pascual-Garrigos et al., 2021; Wang et al., 2021, 2023). The method only requires a single temperature of 65 °C and is resistant to common PCR inhibitors (Francois et al., 2011; Thio et al., 2021).

In this project, the team established the baseline measurement of *Bacteroidales* in fields with low and high fecal contamination risks to support the use of *Bacteroidales* in the fresh produce industry for assessing the risk of both general fecal contamination and microbial source tracking. In addition, we developed a fully integrated LAMP testing platform which included components such as heating, imaging, fluid delivery, and paper-based LAMP assay, and deployed it on a commercial lettuce farm. The unit, operating in the back of a car, was powered by a portable power station. Our platform enabled *in-situ* identification of fecal contamination within 60 min of sampling. We are the first to implement a potable paper-based LAMP testing platform in fresh produce farms. It serves as an enabler for establishing future nucleic acid amplification tests (NAATs) as part of standard growing and harvesting practices during fresh produce production.

Research Methods and Results

Objective 1: Establish background levels of fecal and pathogenic contamination in the field to determine the limits of detection that are needed for a field-based assay.

Bacteroidales provide an indication of fecal contamination. Certain features of *Bacteroidales* make them superior to other FIB. These features include high prevalence in feces (constituting 30%–40% of total fecal bacteria, 10^9 to 10^{11} colony forming units (CFU)/g), obligate anaerobicity (preventing their growth and multiplication in the ambient environment), low natural abundance from non-fecal sources, and high host-specificity (various sequences of the 16S rRNA gene have been designed to detect fecal pollution from specific hosts) (Mascorro et al., 2018; Ordaz et al., 2019). As a result, *Bacteroidales* serve as a valuable—and potentially quantitative—marker to assess the risk of contamination in each farm. However, the levels of *Bacteroidales* naturally present in the environment of various fresh produce operations, as well as the concentration near animal feeding operations, were not previously known. Quantitative PCR (qPCR) is the gold standard test for nucleic acid-based assays. We used qPCR to establish background levels of fecal contamination in both fresh produce operations and fields near animal feeding operations.

<u>Improving sampling method for establishing background levels of fecal contamination</u>

In the field studies, the team evaluated three different sampling strategies for collecting bacterial samples. Methods included: 1) swabbing the leaves and resuspending the swab in water, 2) washing the leaves and using the wash water as a microbiological sample, and 3) using collection flags (clean plastic sheets) to capture bacterial samples and swabbing the flags. The collection flags were assembled (**Figure 1**) using bamboo skewers (29.8 cm), transparent film (Apollo Plain Paper Copier Transparency Film), a stapler, and a paper-cutter. The

transparent film was pre-cut into 7.62 × 21.59 cm (3 × 8.5 inch) strips. Four pieces of the film were stapled together at the edge to form a loop. A bamboo skewer was inserted through the loop to make a collection flag. The methods were compared with each other to see which approach is more reliable in the field experiment.

Establish background levels of *Bacteroidales* near animal feeding operations

Ten lettuce plants and ten collection flags (per spot) were placed at three different distances (distance varies circumstantially due to the availability of space around animal units) away from each animal operation facility, with three replicates in each row (**Figure 2**). Both the plants and collection flags were encoded with a unique identifier and the location associated with the plant/flag's identifier was recorded. A group of ten lettuce plants and ten collection flags were placed in the greenhouse, which served as the negative control. After 7 days, all lettuce and collection flags were collected. Following U.S. Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM) for isolating specific pathogens from fresh vegetable samples, 25 g of lettuce (approximately four leaves) or four pieces of transparency film were swabbed using a wet polyester-tipped swab (263000, BD BBL, USA). Each swab was resuspended in 200 µL of molecular biology grade water. The resuspension was directly used for qPCR without performing DNA extraction. The qPCR primers information is shown in **Table 1**.

To construct a fecal contamination risk evaluation map, we converted the cycle threshold (Ct) value of each qPCR reaction to $\log_{10}$ (copies/cm^2) via a linear fit to log-transformed concentrations (**Figure 3**). The heatmap demonstrates there are more than 10^3 copies of *Bacteroidales* cells per cm^2 around animal operations (**Figure 3**). We also demonstrated that collection flag samples have higher consistency than lettuce swab samples. Some of the swab samples from lettuce placed next to animal units did not amplify, and the amplification curves had high variability in the time-to-amplification (**Figure 2**). This could be due to the rough foliage topography, which makes consistent swabbing challenging. Thus, we decided to use exclude method 1 and use methods 2 and 3 for establishing background levels of fecal contamination in the fresh produce fields.

Establish background levels of *Bacteroidales* in fresh produce fields during the growing seasons

Samples were collected between May 2021 and August 2021 over two growing seasons in two commercial romaine lettuce fields in Salinas, California. The fields were labeled with row and column numbers, with the distance between each row and column to be 6 meters. Samples were collected at the intersection of each row and column (approximately 100 sampling sites per acre of field). Two types of samples were collected at each sampling site: 1) 25 g of romaine lettuce leaf sample (approximately four leaves), and 2) collection flag sample. The sample size for the romaine lettuce leaf sample was determined following FDA BAM for isolating specific pathogens from fresh vegetable samples (FDA, 2021). The fabrication and deploying method for the collection flags are described in the previous section.

During the first growing season (May 2021), we collected 336 romaine lettuce leaf samples and 336 collection flag samples over a field size of 3.3 acres (16 rows, 21 columns) (**Figure 4**). In the second growing season (August 2021), we collected 480 romaine lettuce leaf samples and 480 collection flag samples over a field size of 4.8 acres (8 rows, 60 columns) (**Figure 5**). We collected the samples while wearing a Tyvek suit, gloves, and a mask to avoid contaminating the samples. Before collecting each sample, we used 70% ethanol to sanitize gloves and sleeves to avoid cross-contamination. Each sample was placed in an individual, pre-labeled Ziploc resealable storage bag (B07NQVYCG3, Amazon, USA). The collected samples were kept on ice and shipped back to West Lafayette in a cooler box with ice packs via FedEx Priority Overnight. Following sample collection, the remaining lettuce in the experimental fields was destroyed in the field.

The romaine lettuce leaf samples were processed by a washing and filtering method modified from the FDA BAM for isolating specific pathogens from fresh vegetable samples (FDA, 2021). Briefly, 225 mL of ultrapure water (PURELAB flex, ELGA, USA) was added to the sealed bag with 25 g of lettuce leaf sample. The bag was hand-shaken for 1 min to elute any bacteria into the solution. The wash solution was then filtered using a 90 mm, 0.22 µm, cellulose acetate (CA) membrane (FBM090CA022, Filter-Bio, China). The filtered membrane was immersed into 1 mL of nuclease-free water inside a 2 mL centrifuge tube and the tube was vortexed at maximum speed for 1 min. Finally, the tube was centrifuged at 10,000 rpm for 1 min to recover the resuspension. The membrane was removed from the tube after centrifugation. Each collection flag was swabbed using a wet polyester-tipped swab (263000, BD BBL, USA) and was resuspended in 200 µL of nuclease-free water. All samples were kept at -20 °C until the experiment.

Based on *Bacteroidales* qPCR assays on a total of 1632 samples (lettuce leaves and collection flags, over a total of two trips), the team built heatmap contours of distribution of fecal contamination in the fields (**Figures 4** and **5**). In both cases, the levels of *Bacteroidales* were extremely low as expected for background measurements in clean low-risk fields (i.e., fields away from animal operations in areas normally used for commercial production). This suggests that extremely low background levels of *Bacteroidales* are present in the soil naturally. The team also conducted microbial source tracking on samples that returned a concentration higher than 1 copy/reaction (17 samples) for universal *Bacteroidales.* Since the host-specific populations represent a small group within the general *Bacteroidales* population (Bernhard & Field, 2000b; Lamendella et al., 2007), a digital PCR (dPCR) method was adopted for the microbial source tracking experiment to achieve a higher level of sensitivity (Milbury et al., 2014) by amplifying only *Bacteroidales* derived from a single host species. Due to the inherent nature of dPCR, the assay has a high tolerance to biological inhibitors and has better performance on trace detection for a minority target (Milbury et al., 2014; Perkins et al., 2017).

The dPCR reactions were performed in a total volume of 12 µL, containing 3 µL of 4X Probe PCR Master Mix (250102, Qiagen, USA) (final concentration 1X), 1.2 µL of 10X primer-probe mix (final concentration 1X, 0.8 µM forward primer, 0.8 µM reverse primer, 0.4 µM FAM probe), 2.8 µL of nuclease-free water, and 5 µL of the template or 5 µL of nuclease-free water for no template control (NTC). 10X primer-probe mix was one of the host-specific qPCR primer-probe set in **Table 1** (cattle-specific *Bacteroidales*, swine-specific *Bacteroidales*, human-specific *Bacteroidales*, and poultry-specific *Bacteroidales*). The dPCR reactions were performed in an 8.5K 96-well Nanoplate (250021, Qiagen, USA) on a 5-plex QIAcuity One digital PCR instrument (911021, Qiagen, USA). The thermal cycling conditions were implemented using the following program: initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s, and 60 °C for 30 s.

Following plate preparation, the system partitioned each sample into approximately 8500 partitions, with approximately 8300 valid counts. Each partition was individually sealed following 40 cycles of thermocycling. The plate was then imaged to count the number of positive/fluorescent partitions for each sample. The fluorescent threshold was determined to be 20 relative fluorescence units (RFU) based on the NTC. 16 partitions were counted as positive, including 2 positive partitions for cattle-specific *Bacteroidales*, 3 positive partitions for swine-specific *Bacteroidales*, 2 positive partitions for human-specific *Bacteroidales*, and 9 positive partitions for poultry-specific *Bacteroidales* (**Figure 6**). We were unable to make definitive statements about microbial source tracking due to the low copy number of host-specific *Bacteroidales*.

Establish background levels of *Bacteroidales* in fresh produce fields during harvesting seasons

Samples were collected in July 2022. The Purdue team traveled to Salinas, CA, and collected 246 swab samples from fresh-pack lettuce harvesters (**Figure 7**), and 210 swab samples from processed lettuce harvesters (**Figure 8**). The collected samples were shipped back to Purdue to perform molecular tests. For the fresh-pack harvester samples, we swabbed a 10*10 cm^2 area of the packing table before and after harvesting (Figure 7). For the processed lettuce harvesters we swabbed the conveyer belt, conveyer belt wall, curtain, tunnel, elevator, and funnel surfaces. The sampling sites were selected on the basis of how lettuce comes in contact with the harvester surfaces: from the ground to the collection box (Figure 8). For both types of harvesters, we swabbed the surfaces using a BD CultureSwab™ Sterile Double Swab (220135, BD, USA) and resuspended the swab in 500 µL of nuclease-free water. The team conducted qPCR assays to detect *Bacteroidales* as markers of fecal contamination on all 456 samples (**Figure 9**).

For fresh-pack lettuce harvester samples, the qPCR results showed a significant difference in *Bacteriodales* levels between preharvest (mean = 10^0.11 copies/cm^2, SD = 10^0.28 copies/cm^2) and postharvest (mean = 10^0.04 copies/cm^2, SD = 10^0.14 copies/cm^2; $p < 0.05$). The concentration level of *Bacteriodales* decreased after harvest. For processed lettuce harvester samples, the qPCR results did not show a significant difference in *Bacteriodales* levels between preharvest (mean = 10^0.05 copies/cm^2, SD = 10^0.23 copies/cm^2) and postharvest (mean = 10^0.12 copies/cm^2, SD = 10^0.26 copies/cm^2; $p = 0.06$). We observed a trend of decreasing *Bacteriodales* levels among the harvester sites from the conveyer belt to the funnel, which corresponded to the travel distance of lettuce from the ground and through the harvester. However, the data was not statistically significant (one-factor analysis of variance (ANOVA), $p = 0.06$).

Objective 2: Design and test a portable µPAD that can detect contamination and provide results within an hour in the field.

Design and fabrication of the Device for Easy Cross-species tracking Of DNA in the Environment (DECODE)

We designed and fabricated a water-bath heater for running LAMP assays on the farm (**Figure 10**). All designs were prepared using Fusion 360 software (Autodesk, CA). The heating unit consists of a 3D printed cavity, made from Rigid 4000 V1 resin (RS-F2-RGWH-01, Formlabs, USA), with a 3 mm transparent acrylic sheet (B099J2XVRW, Amazon, USA) attached at its bottom. All 3D printings were performed using a Form 3B stereolithography 3D printer (Formlabs, USA). The transparent window under the heating cavity allowed for coupling the heating unit with an imaging unit to track the color changes in the reaction pads. The heater used a 200 W, 110 V hot rod Dernord heating element (B08LKFDGJD, Amazon, USA) to heat the water as well as a 12 V submersible mini water pump (B08RWP6GJF, Amazon, USA) to improve the temperature uniformity. The water temperature was monitored using a waterproof digital temperature sensor (DS18B20, Gikfun, USA), controlled by a PID control algorithm, run on a Raspberry Pi 4B (B07TD42S27, Amazon, USA) minicomputer. The heater provided fast operation, reaching 65°C in under 20 min. The camera in the imaging unit captured a shot of the paper pads every minute. In conclusion, the device provided us with a portable means to successfully carry out paper LAMP assays in the field.

Fabrication of micro-droplet dispensers

To measure and create precise volumes of the microbial samples in the field, we designed a micro-droplet dispenser with a capacity of 27 µL of droplet volume. The dispenser consists of a liquid holder, a plunger, and two O-rings (Airy-Acc-Oring-2.5×6mm, Helipal, USA).

The liquid holders and plungers were 3D printed using High Temp V2 resin (RS-F2-HTAM-02, Formlabs, USA). After printing, the parts were washed with isopropyl alcohol (IPA) for 30 min and cured under UV light at 55 °C for 30 min. The tip of the liquid holders was wrapped in parafilm (S37440, Fisher Scientific, USA) wrapping film prior to surface treatment. The liquid holders were surface-treated with air plasma for 2 min at 0.2 Torr using a plasma generator (PE-25, Plasma Etch, Inc., USA) and polyethylene glycol (PEG) 400 (P167-1, Fisher Scientific, USA) for 24 h. Following surface treatment, all components were washed with IPA, then with ultrapure water (ELGA, PURELAB flex, USA), dried with an air gun, and stored in separate reclosable polypropylene bags (S-17954, Uline, USA) for future use. The dispensers were used to carry out paper LAMP assays in the field, eliminating the need for a pipette that would require additional training to operate.

Design new primers for loop-mediated isothermal amplification (LAMP)

The portable nature of LAMP, which only requires a heat source, makes it well-suited for use in a field-based assay. In an effort to assess the risk of fecal contamination, we have designed a novel LAMP primer set for *Bacteroidales* (**Table 2**). This set is based on a conservative region of the *Bacteroidales* 16S ribosomal RNA gene, which was determined through the alignment of the first 1000 hits of the gene obtained from NCBI BLAST using the NCBI Multiple Sequence Alignment Viewer (MSA). The LAMP primers were designed using PrimerExplorer V5 (http://primerexplorer.jp/lampv5e/index.html) with default parameters.

Fabrication and preparation of microfluidic paper-based analytical devices (μPADs)

Paper-based devices were used for running LAMP assays in the field (Wang et al., 2021). Each device consisted of two paper pads of 5 mm × 6 mm chromatography paper (Grade 222, Ahlstrom-Munksjo, USA) attached on a double-sided adhesive (90178, Adhesives Research, USA). The pads were separated by 2.5 mm × 6 mm, 20-mm polystyrene spacers (40047020, Tekra, USA). After fabrication, the paper-based devices were stored in reclosable polypropylene bags (S-17954, Uline, USA) for future use.

To prepare 1000 μL 2x LAMP mix, the solution consisted of 100 μL KCl (1000 mM; P9541, Sigma-Aldrich, USA), 160 μL MgSO4 (100 mM; M2773Sigma-Aldrich,), 280 μL deoxynucleotide triphosphate (dNTP) (10 mM; FERR0182, Fisher Scientific, USA), 2.8 μL deoxyuridine triphosphate (dUTP) (100 mM; FERR0133, Fisher Scientific, USA), 0.4 μL Antarctic Thermolabile UDG (1 U/μL; M0372S, New England Biolabs, USA), 5.4 μL Bst 2.0 DNA Polymerase (120 U/μL; M0537M, New England Biolabs, USA), 20 μL phenol red solution (25 mM; P3532, Sigma-Aldrich, USA), 100 μL tween 20 (20%; P9416, Sigma-Aldrich, USA), and 331.4 μL nuclease-free water (43-879-36, Fisher Scientific, USA). After mixing, using a micro-pH electrode (11-747-328, Fisher Scientific, USA), the pH was adjusted to 7.8-7.9 using KOH (0.1 M or 1 M), to get a red solution.

To prepare 200 μL of LAMP master mix, we mixed 125 μL of the 2x mix, 25 μL of 10x primer mix (16 μM FIP/BIP, 2 μM F3/B3, 4 μM LF/LB) (final concentration 1.6 μM FIP/BIP, 0.2 μM F3/B3, 0.4 μM LF/LB) (Table 1), 0.67 μL Bst 2.0 DNA Polymerase, and 1 μL of betaine (5 M; B0300-5VL, Sigma-Aldrich, USA), 3.13 μL bovine serum albumin (BSA) (40 mg/mL; A2153, Sigma-Aldrich, USA), 36.0 μL trehalose (50% (w/v); 182550250, Thermo Scientific Chemicals, USA), and 9.2 μL nuclease-free water. The pH of the master mix was adjusted to about 7.8-7.9 using KOH (0.1 M), to get a red solution. A 30-μL aliquot of the final master mix was added to each paper pad and left inside a PCR workstation (MY-PCR32, Mystaire, USA) to dry for 2 h. For each paper device, one paper pad included a master mix with the LAMP primer mix and the other one without the primer mix. When no primer mix was used, the same volume of nuclease-free water was added to the mix instead. The dried paper devices were packed in separate reclosable polypropylene bags (S-17954, Uline, USA) and stored at -18 °C until usage. Paper

devices used for the field test were shipped to the field (Salinas, CA) and kept in a household refrigerator (-18 °C) until usage.

Limit of detection (LoD) of LAMP assays using µPADs

LoD experiments were performed on paper devices to evaluate the assay's sensitivity. We used five levels of *B. fragilis* DNA (ATCC® 25285™) concentration (50,000, 5,000, 500, 100, and 50 copies per reaction) as well as NTC. All reactions were done in triplicates. 27 µL of the template (*B. fragilis* DNA or nuclease-free water for NTC) was added to both paper pads (control pad and reaction pad). The paper strips were separately sealed inside reclosable polypropylene bags (S-17954, Uline, USA) and heated at 65 °C for 60 min in a water bath (ANTC01, Anova, USA). Time-lapse video of the paper strips was taken from 0 to 60 min using a HERO8 Black digital camera (SPJB1, GoPro, USA). We also scanned the paper strips at 0 min and 60 min using a flatbed scanner (B11B223201, Epson, USA). dPCR quantified genomic DNA was used to test the limit of detection of the paper LAMP assay. The criterion for LoD determination is the concentration of DNA that show consistent amplification in all three replicates; in this case, we observed an LoD of 500 copies/reaction (**Figure 11**).

Conduct field trials during the growing seasons using µPADs and DECODE

The surveyed field was labeled with row and column numbers, with the distance between each row and column to be 6 meters. Samples were collected at the intersection of each row and column (approximately 100 sampling sites per acre of field). To collect airborne microbiological samples from the field, 96 collection flags were put at each sampling location 7 days before sample collection. Each collection flag was encoded with a unique identifier and the location associated with the flag's identifier was recorded. After 7 days, all collection flags were collected, and each flag was placed in an individual, pre-labeled Ziploc resealable storage bag (B07NQVYCG3, Amazon, USA). Each collection flag was swabbed using a wet polyester-tipped swab (263000, BD, USA) and was resuspended in 200 µL of nuclease-free water. All assays were conducted in the field using DECODE. The unit was powered by a Jackery Portable Power Station (500 W, 110 V) (Explorer 500, Jackery, USA). For each sample, the swab resuspension was directly transferred into a micro-drop dispenser which was subsequently used to rehydrate each pad with one droplet (27 µL). The rehydrated µPADs were sealed inside reclosable polypropylene bags (Uline, S-17954) and heated at 65 °C for 60 min inside DECODE (**Figure 12**). Two control negative (27 µL of nuclease-free water) and two control positive (27 µL of *B. fragilis* DNA, total 1 ng) devices were run together with the samples. The imaging system of DECODE took time-lapse photos of the paper strips at every minute during the heating time. The remaining swab resuspensions from each location were stored in separate 1.5 mL vials, kept on ice, and shipped back to West Lafayette in a cooler box with ice packs via FedEx Priority Overnight. The paper devices were visually inspected after 60 min. All 96 collection flag samples displayed a negative/red color below the limit of detection (500 copies/reaction) shown in Figure 11.

Conduct field trials during the harvesting seasons using µPADs and DECODE

The team conducted paper-based LAMP assays using µPADs on 100 harvester samples. The sample collection and processing procedure has been described in Objective 1. The swab resuspension of each sample was directly used to rehydrate µPADs. We added 27 µL of the resuspension using a commercial micro pipettor (3123000055, Eppendorf, USA) to each paper pad. The rehydrated µPADs were sealed inside reclosable polypropylene bags (Uline, S-17954) and heated at 65 °C for 60 min inside DECODE (**Figure 13**). Two control negative (27 µL of nuclease-free water) and two control positive (27 µL of *B. fragilis* DNA, total 1 ng) devices were run together with the samples. Throughout the heating process, the DECODE imaging equipment captured time-lapse images of µPADs every minute. The remaining swab

resuspensions were refrigerated and sent back to West Lafayette in a cooler box with ice packs via FedEx Priority Overnight. The paper devices were visually inspected after 60 min. All 96 swab samples displayed a negative/red color below the limit of detection (500 copies/reaction) shown in Figure 11.

Image analysis on µPAD results

After the images were captured with the internal camera, they underwent a series of image processing techniques beginning with importing and resizing the first image from the test run to reduce processing time. Using Python's OpenCV library, the GrabCut algorithm was utilized to draw the sample boundary for each paper pad and store these rectangular coordinates boundaries to create a mask for each sample. Once these masks were created for the samples in the first image, the program looped through the rest of the test images and created the mask for each sample in each image using the previous rectangular coordinates. The images of the samples were converted to the HSV color mode and each pixel was separated into weighted bins based on color. The color-coding function sets equally spaced and continuous HSV upper and lower boundaries for red, orange, and yellow based on the HSV colormap. After all pixels were identified and sorted into the different color bins ranging from dark red to light yellow, these bins were weighted based on a sigmoid function with a curve midpoint of 0.5, a curve steepness of 50, and limits of 0 to 1. The steepness of the curve is determined by the LoD study of this LAMP primer set. The program then output the percentage of positivity for each sample, by calculating the ratio of the number of pixels in the weighted bins to the total number of identified pixels, to display a quantitative analysis of the color change. After calculating these percentages, a quantitative analysis displaying the positive percentage throughout the duration of the test run was plotted using the OriginPro 2022b software.

Positivity percentage data over time was graphed for the samples obtained from the harvester and the collection flags (**Figure 14**). Overall, the image analysis results agree with the results seen by eye. Many tests have a high percentage of positivity in the earlier timepoints (0–20 min), affecting both the reaction and control pads. We believe this is due to reagent reconstitution, which takes time to distribute evenly across the paper device. Furthermore, spikes of increased positivity occurred for some samples throughout the experiment. These spikes were due to artifacts in imaging (caused by bubbles or glares). We averaged the data with a window of 10 min to smooth it and reduce the effects from these artifacts. The control pads (**Figure 14 A**) show what a positive reaction and a negative reaction would look like. Since none of the harvester and collection flag samples had a positive reaction (increasing reaction percent with greater than 50% reaction), they were not quantified.

Lab validation of field trial results

To validate the results generated by µPADs in the field, we conducted qPCR assays on the remaining swab resuspensions to quantify the *Bacteroidales* levels. The qPCR reaction was performed in a total volume of 25 µL, containing 12.5 µL of 2X Luna® Universal Probe qPCR Master Mix (M3004, New England Biolabs, USA) (final concentration 1X), 1 µL each of 10 µM forward and 1 reverse primer (final concentration 0.4 µM) (Table 1), 0.5 µL of 10 µM fluorescent probe (final concentration 0.2 µM) (Table 1), 9 µL of nuclease-free water, and 1 µL of template or 1 µL of nuclease-free water for NTC. The qPCR reactions were performed on a qTOWER3 Real-Time Thermal Cycler (Analytik Jena, Germany), and the thermal cycling conditions were implemented using the following program: initial denaturation at 95 °C for 1 min, followed by 45 cycles of 95 °C for 15 s, 55 °C for 15 s, and 60 °C for 30 s. **Figures 15** and **16** show the results of the qPCR assays, which agree with the results of the field experiment.

Outcomes and Accomplishments

The project team successfully conducted three visits to fresh produce operations, collecting 1,728 environmental samples and 456 harvester samples throughout the growing and harvesting operations. This data collection initiative provided valuable insights into the fecal contamination level of fresh produce and has resulted in the first-ever baseline study of *Bacteroidales* concentration as an indicator of fecal contamination in real-world scenarios. The team also devised a novel risk assessment methodology, incorporating the use of LAMP assay for detecting *Bacteroidales*, which has been demonstrated to be feasible for implementation in the field through an integrated sample-to-answer biosensor platform. This innovative approach offers a complementary solution to the current methods of environmental risk assessment, and it is anticipated that the LAMP assay could become a widely used tool for evaluating the risk of fecal contamination in fresh produce.

Summary of Findings and Recommendations

The following were the most important findings:

1. We were the first to report baseline studies surveying the background concentration of *Bacteroidales* as a fecal contamination indicator in fresh produce fields and also adjacent to animal feeding operations. As expected, the concentrations of *Bacteroidales* in the sampled commercial fields were very low (0 – 2.00 copies/cm^2). On the other hand, fields adjacent to animal feeding operations have *Bacteroidales* concentrations over 10^4 copies/cm^2.
2. The harvester samples showed that *Bacteroidales* concentrations on harvesters were higher (0 – 100 copies/cm^2) compared to those observed in the fields. However, this concentration should still be considered as being within acceptable safety limits.
3. Host-specific *Bacteroidales* constitute a small proportion of the total *Bacteroidales* population and are thus not always present when fecal contamination occurs, particularly at low levels of contamination. When choosing a host-specific marker, there is a trade-off between specificity and sensitivity; where a decreased cross-reaction rate between various hosts might also affect the sensitivity of the assay. Therefore, for studies attempting to detect fecal contamination, we strongly recommend using not only host-specific *Bacteroidales* markers but also a universal *Bacteroidales* marker to reduce the incidence of false negatives.
4. We developed a fully-integrated LAMP testing platform which included components such as heating, imaging, fluid delivery, and paper-based LAMP assay, and deployed it on a commercial lettuce farm. The unit, operating in the back (trunk) of a car, was powered by a portable power station. Our platform enabled *in-situ* identification of fecal contamination within 60 min of sampling. We are the first to implement a portable paper-based LAMP testing platform in fresh produce farms. It serves as an enabler for establishing future nucleic acid amplification tests (NAATs) as part of standard growing and harvesting practices during fresh produce production.

References cited:

Allende, A., Truchado, P., Lindqvist, R., & Jacxsens, L. (2018). Quantitative microbial exposure modelling as a tool to evaluate the impact of contamination level of surface irrigation water and seasonality on fecal hygiene indicator E. coli in leafy green production. *Food Microbiology*, *75*, 82–89.

Bernhard, A. E., & Field, K. G. (2000a). A PCR assay to discriminate human and ruminant feces on the basis of host differences in Bacteroides-Prevotella genes encoding 16S rRNA. *Applied and Environmental Microbiology*, *66*(10), 4571–4574.

Bernhard, A. E., & Field, K. G. (2000b). A PCR Assay To Discriminate Human and Ruminant Feces on the Basis of Host Differences in Bacteroides-Prevotella Genes Encoding 16S rRNA. *Applied and Environmental Microbiology*, *66*(10), 4571–4574. https://doi.org/10.1128/AEM.66.10.4571-4574.2000

Brauwere, A. de, Ouattara, N. K., & Servais, P. (2014). Modeling Fecal Indicator Bacteria Concentrations in Natural Surface Waters: A Review. *Critical Reviews in Environmental Science and Technology*, *44*(21), 2380–2453. https://doi.org/10.1080/10643389.2013.829978

Carstens, C. K., Salazar, J. K., & Darkoh, C. (2019). Multistate outbreaks of foodborne illness in the United States associated with fresh produce from 2010 to 2017. *Frontiers in Microbiology*, *10*, 2667.

Converse, R. R., Blackwood, A. D., Kirs, M., Griffith, J. F., & Noble, R. T. (2009). Rapid QPCR-based assay for fecal Bacteroides spp. As a tool for assessing fecal contamination in recreational waters. *Water Research*, *43*(19), 4828–4837. https://doi.org/10.1016/j.watres.2009.06.036

Davidson, J. L., Wang, J., Maruthamuthu, M. K., Dextre, A., Pascual-Garrigos, A., Mohan, S., Putikam, S. V. S., Osman, F. O. I., McChesney, D., Seville, J., & Verma, M. S. (2021). A paper-based colorimetric molecular test for SARS-CoV-2 in saliva. *Biosensors and Bioelectronics: X*, *9*, 100076. https://doi.org/10.1016/j.biosx.2021.100076

Denis, N., Zhang, H., Leroux, A., Trudel, R., & Bietlot, H. (2016). Prevalence and trends of bacterial contamination in fresh fruits and vegetables sold at retail in Canada. *Food Control*, *67*, 225–234. https://doi.org/10.1016/j.foodcont.2016.02.047

Francois, P., Tangomo, M., Hibbs, J., Bonetti, E.-J., Boehme, C. C., Notomi, T., Perkins, M. D., & Schrenzel, J. (2011). Robustness of a loop-mediated isothermal amplification reaction for diagnostic applications. *FEMS Immunology & Medical Microbiology*, *62*(1), 41–48.

Gómez-Doñate, M., Casanovas-Massana, A., Muniesa, M., & Blanch, A. R. (2016). Development of new host-specific Bacteroides qPCR s for the identification of fecal contamination sources in water. *Microbiologyopen*, *5*(1), 83–94.

Hoadley, A. W., & Cheng, C. M. (1974). The Recovery of Indicator Bacteria on Selective Media. *Journal of Applied Bacteriology*, *37*(1), 45–57. https://doi.org/10.1111/j.1365-2672.1974.tb00413.x

Kildare, B. J., Leutenegger, C. M., McSwain, B. S., Bambic, D. G., Rajal, V. B., & Wuertz, S. (2007). 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal Bacteroidales: A Bayesian approach. *Water Research*, *41*(16), 3701–3715.

Kobayashi, A., Sano, D., & Okabe, S. (2013). Effects of temperature and predator on the persistence of host-specific Bacteroides-Prevotella genetic markers in water. *Water Science and Technology*, *67*(4), 838–845. https://doi.org/10.2166/wst.2012.626

Lamendella, R., Domingo, J. W. S., Oerther, D. B., Vogel, J. R., & Stoeckel, D. M. (2007). Assessment of fecal pollution sources in a small northern-plains watershed using PCR and phylogenetic analyses of Bacteroidetes 16S rRNA gene. *FEMS Microbiology Ecology*, *59*(3), 651–660. https://doi.org/10.1111/j.1574-6941.2006.00211.x

Mascorro, J. A., HERNÁNDEZ-RANGEL, L. G., HEREDIA, N., & GARCÍA, S. (2018). Bacteroidales as Indicators and Source Trackers of Fecal Contamination in Tomatoes and Strawberries. *Journal of Food Protection*, *81*(9), 1439–1444. https://doi.org/10.4315/0362-028X.JFP-18-073

Mieszkin, S., Furet, J.-P., Corthier, G., & Gourmelon, M. (2009). Estimation of Pig Fecal Contamination in a River Catchment by Real-Time PCR Using Two Pig-Specific Bacteroidales 16S rRNA Genetic Markers. *Applied and Environmental Microbiology*, *75*(10), 3045–3054. https://doi.org/10.1128/AEM.02343-08

Milbury, C. A., Zhong, Q., Lin, J., Williams, M., Olson, J., Link, D. R., & Hutchison, B. (2014). Determining lower limits of detection of digital PCR assays for cancer-related gene mutations. *Biomolecular Detection and Quantification*, *1*(1), 8–22. https://doi.org/10.1016/j.bdq.2014.08.001

Mohan, S., Pascual-Garrigos, A., Brouwer, H., Pillai, D., Koziol, J., Ault, A., Schoonmaker, J., Johnson, T., & Verma, M. S. (2021). Loop-Mediated Isothermal Amplification for the Detection of Pasteurella multocida, Mannheimia haemolytica, and Histophilus somni in Bovine Nasal Samples. *ACS Agricultural Science & Technology*, *1*(2), 100–108. https://doi.org/10.1021/acsagscitech.0c00072

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*, *28*(12), e63.

Ordaz, G., Angel Merino-Mascorro, J., Garcia, S., & Heredia, N. (2019). Persistence of Bacteroidales and other fecal indicator bacteria on inanimated materials, melon and tomato at various storage conditions. *International Journal of Food Microbiology*, *299*, 33–38. https://doi.org/10.1016/j.ijfoodmicro.2019.03.015

Pascual-Garrigos, A., Maruthamuthu, M. K., Ault, A., Davidson, J. L., Rudakov, G., Pillai, D., Koziol, J., Schoonmaker, J. P., Johnson, T., & Verma, M. S. (2021). On-farm colorimetric detection of Pasteurella multocida, Mannheimia haemolytica, and Histophilus somni in crude bovine nasal samples. *Veterinary Research*, *52*(1), 126. https://doi.org/10.1186/s13567-021-00997-9

Perkins, G., Lu, H., Garlan, F., & Taly, V. (2017). Chapter Three - Droplet-Based Digital PCR: Application in Cancer Research. In G. S. Makowski (Ed.), *Advances in Clinical Chemistry* (Vol. 79, pp. 43–91). Elsevier. https://doi.org/10.1016/bs.acc.2016.10.001

Shanks, O. C., Atikovic, E., Blackwood, A. D., Lu, J., Noble, R. T., Domingo, J. S., Seifring, S., Sivaganesan, M., & Haugland, R. A. (2008). Quantitative PCR for Detection and Enumeration of Genetic Markers of Bovine Fecal Pollution. *Applied and Environmental Microbiology*, *74*(3), 745–752. https://doi.org/10.1128/AEM.01843-07

Siefring, S., Varma, M., Atikovic, E., Wymer, L., & Haugland, R. A. (2008). Improved real-time PCR assays for the detection of fecal indicator bacteria in surface waters with different instrument and reagent systems. *Journal of Water and Health*, *6*(2), 225–237. https://doi.org/10.2166/wh.2008.022

Tambalo, D. D., Fremaux, B., Boa, T., & Yost, C. K. (2012). Persistence of host-associated Bacteroidales gene markers and their quantitative detection in an urban and agricultural mixed prairie watershed. *Water Research*, *46*(9), 2891–2904. https://doi.org/10.1016/j.watres.2012.02.048

Thio, S. K., Bae, S., & Park, S.-Y. (2021). A portable dielectrophoretic platform for loop-mediated isothermal amplification (LAMP) based E-coli detection. *The 25th International Conference on Miniaturized Systems for Chemistry and Life Sciences (MicroTAS 2021)*.

Wang, J., Dextre, A., Pascual-Garrigos, A., Davidson, J. L., Maruthamuthu, M. K., McChesney, D., Seville, J., & Verma, M. S. (2021). Fabrication of a paper-based colorimetric molecular test for SARS-CoV-2. *MethodsX*, *8*, 101586. https://doi.org/10.1016/j.mex.2021.101586

Wang, J., Ranjbaran, M., Ault, A., & Verma, M. S. (2023). A loop-mediated isothermal amplification assay to detect Bacteroidales and assess risk of fecal contamination. *Food Microbiology*, *110*, 104173. https://doi.org/10.1016/j.fm.2022.104173

Zhao, X., Zhong, J., Wei, C., Lin, C.-W., & Ding, T. (2017). Current Perspectives on Viable but Non-culturable State in Foodborne Pathogens. *Frontiers in Microbiology*, *8*. https://www.frontiersin.org/articles/10.3389/fmicb.2017.00580

APPENDICES

Publications and Presentations

Journal articles

Ranjbaran, M., **Verma, M.S.*** (2022). Microfluidics at the interface of bacteria and fresh produce. *Trends in Food Science and Technology* 128:102-117. DOI: 10.1016/j.tifs.2022.07.014

Wang, J., Davidson, J., Kaur, S., Dextre, A., Ranjbaran, M., Kamel, M., Athalye, S., **Verma, M.S. *** (2022). Paper-based biosensors for the detection of nucleic acids from pathogens. *Biosensors* 12:1094. DOI:10.3390/bios12121094

Wang, J., Ranjbaran, M., Ault, A., **Verma, M.S.*** (2023). A loop-mediated isothermal amplification assay to detect *Bacteroidales* and assess risk of fecal contamination. *Food Microbiology* 110:104173. DOI:10.1016/j.fm.2022.104173

Wang, J., Ranjbaran, M., **Verma, M.S.*** (2023). *Bacteroidales* as a fecal contamination indicator in fresh produce industry: A baseline measurement. *Under Review*.

Ranjbaran, M., Kaur, S., Wang, J., **Verma, M.S.*** (2023). A drop dispenser for simplifying on-farm detection of foodborne pathogens. *In Preparation*.

Wang, J., Ranjbaran, M., Kaur, S., **Verma, M.S.*** (2023). Enabling nucleic-acid testing of fresh produce in farm using portable paper-based biosensors. *In Preparation*.

*Corresponding author

Presentations

Verma, M.S. (2022). Field evaluation of microfluidic paper-based analytical devices for microbial source tracking [poster]. *Center for Produce Safety Research Symposium*, San Diego, USA.

Budget Summary

This project was awarded $390,670 in grant funds, and all funds were spent.

Tables 1–2 and Figures 1–16 (see below)

Table 1: Sequences for qPCR primers and probes used in this project

Target	Primer	Sequence (5' - 3')	Reference
Universal *Bacteroidales*	GenBac3.FP	GGGGTTCTGAGAGGAAGGT	Siefring et al., 2008
	GenBac3.RP	CCGTCATCCTTCACGCTACT	
	GenBac3.Probe	/FAM/CAATATTCC/ZEN/TCACTGCTGCCTCCCGTA/IABkFQ/	
Cattle-specific *Bacteroidales*	CowM2.FP	CGGCCAAATACTCCTGATCGT	Shanks et al., 2008
	CowM2.RP	GCTTGTTGCGTTCCTTGAGATAAT	
	CowM2.Probe	/FAM/AGGCACCTA/ZEN/TGTCCTTTACCTCATCAACTACAGACA/IABkFQ/	
Pig-specific *Bacteroidales*	Pig-2-Bac41F	GCATGAATTTAGCTTGCTAAATTTGAT	Mieszkin et al., 2009
	Pig-2-Bac163Rm	ACCTCATACGGTATTAATCCGC	
	Pig-2Bac113MGB	/FAM/TCCACGGGA/ZEN/TAGCC/IABkFQ/	
Human-specific *Bacteroidales*	HF183F	ATCATGAGTTCACATGTCCG	Bernhard & Field, 2000b; Converse et al., 2009
	BFDRev	CGTAGGAGTTTGGACCGTGT	
	BFDFAM	/FAM/CTGAGAGGA/ZEN/AGGTCCCCCACATTGGA/IABkFQ/	
Poultry-specific *Bacteroidales*	qCD362_F	AATATTGGTCAATGGGCGAGAG	Kobayashi et al., 2013
	qCD464_R	CACGTAGTGTCCGTTATTCCCTTA	
	qCD394_P	/FAM/TCCTTCACG/ZEN/CTACTTGG/IABkFQ/	

Table 2: Sequences for selected LAMP primer set targeting *Bacteroidales*.

Primer	**Sequence (5' - 3')**
Universal.*Bacteroidales*.16S rRNA.1.F3	TGCGGGTATCGAACAGGATT
Universal.*Bacteroidales*.16S rRNA.1.B3	GGTAAGGTTCCTCGCGTATC
Universal.*Bacteroidales*.16S rRNA.1.FIP	TTAACGCTTTCGCTTGGCCACAGTAGTCCGCACGGTAAACG
Universal.*Bacteroidales*.16S rRNA.1.BIP	GTACGCCGGCAACGGTGAAAACATGTTCCTCCGCTTGTG
Universal.*Bacteroidales*.16S rRNA.1.LF	GGCCGAACAGCGAGCAT
Universal.*Bacteroidales*.16S rRNA.1.LB	CAAAGGAATTGACGGGGGC

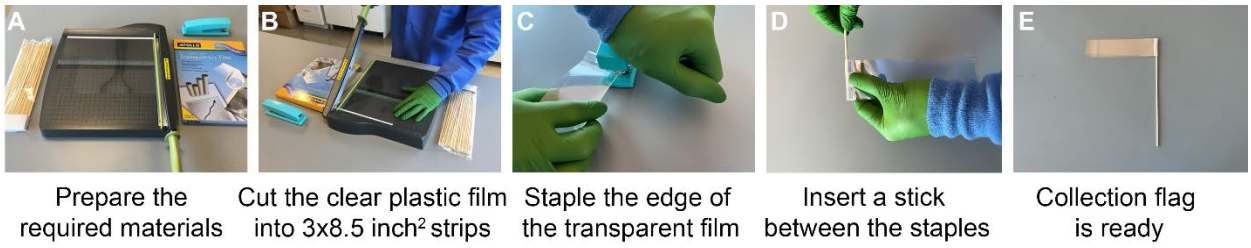

Figure 1: Fabrication of collection flags. A) To assemble the collection flags, we used bamboo skewers (29.8 cm), transparent film (Apollo Plain Paper Copier Transparency Film), a stapler, and a paper-cutter. B) The transparent film was pre-cut into 7.62 × 21.59 cm (3 × 8.5 inch) strips. C) All four pieces of the film were stapled together at the edge (approximately 0.5 cm in length) to form a loop. D) A bamboo skewer was inserted through the loop to fix all four pieces of film to the skewer. E) The fabrication of collection flag is completed. Before deployment on fields, the four films were spread out to collect more dust/ aerosols sample.

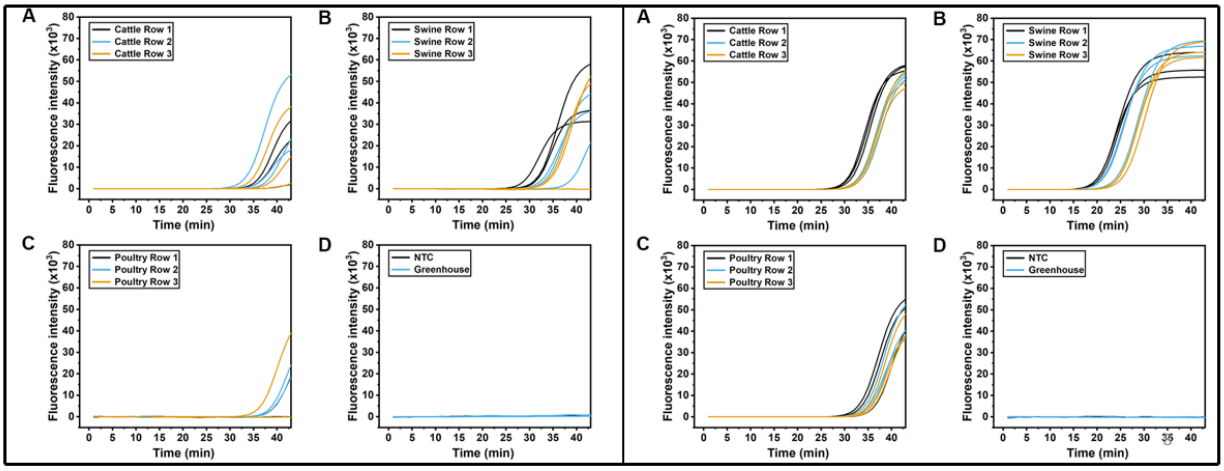

Figure 2: Comparing two collection methods using qPCR. Left) qPCR assays using lettuce leaves swab resuspension solution; 1 µL of resuspension was added to the reaction mix. Right) qPCR assay using collection flag swab resuspension solution; 1 µL of resuspension was added to the reaction mix.

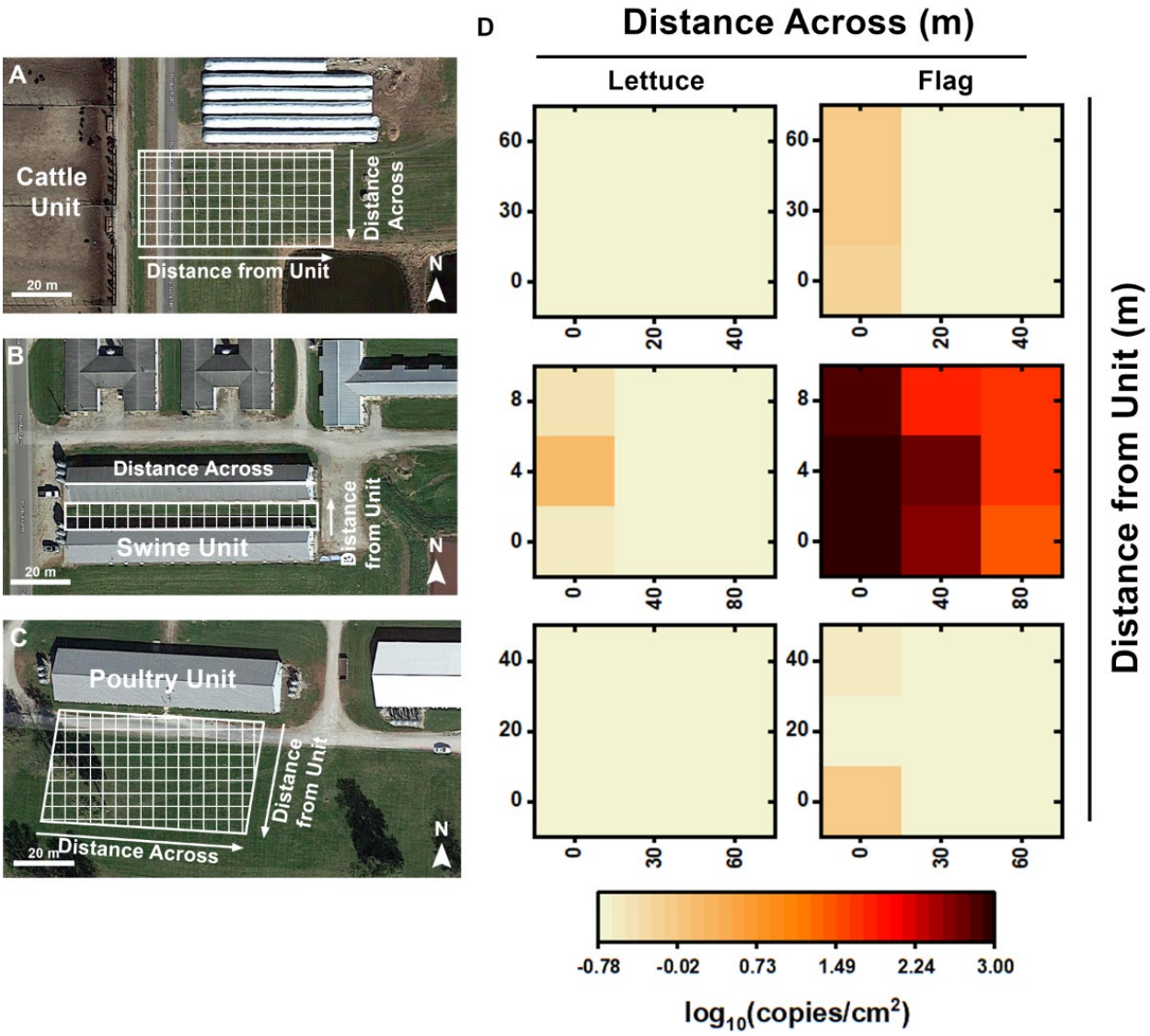

Figure 3: Background levels of fecal contamination around animal operations. A, B, and C) Satellite images of the sampled area. D) Heat map of the fecal contamination level in the sampled area using qPCR assays.

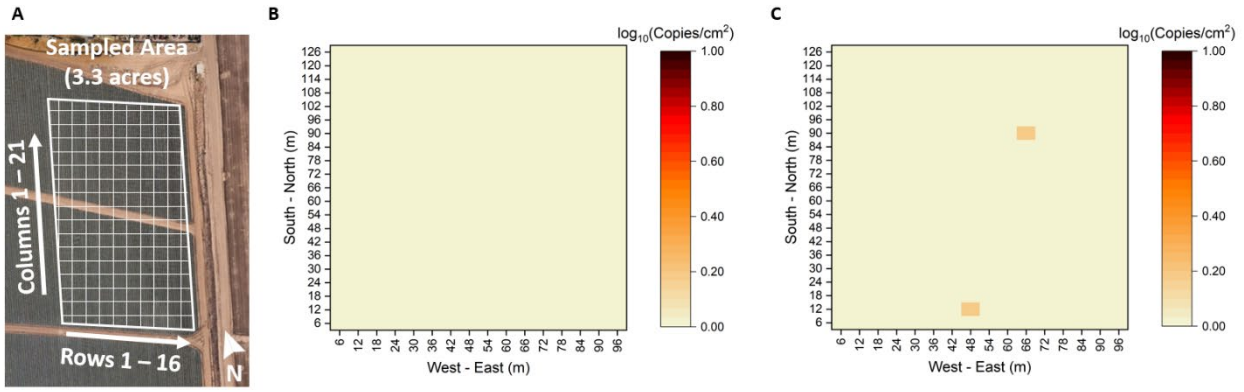

Figure 4: Risk of fecal contamination mapping using qPCR (May 2021). The Ct value of each qPCR reaction was converted to $\log_{10}$ (copies/cm^2) via a linear fit to log-transformed concentrations.

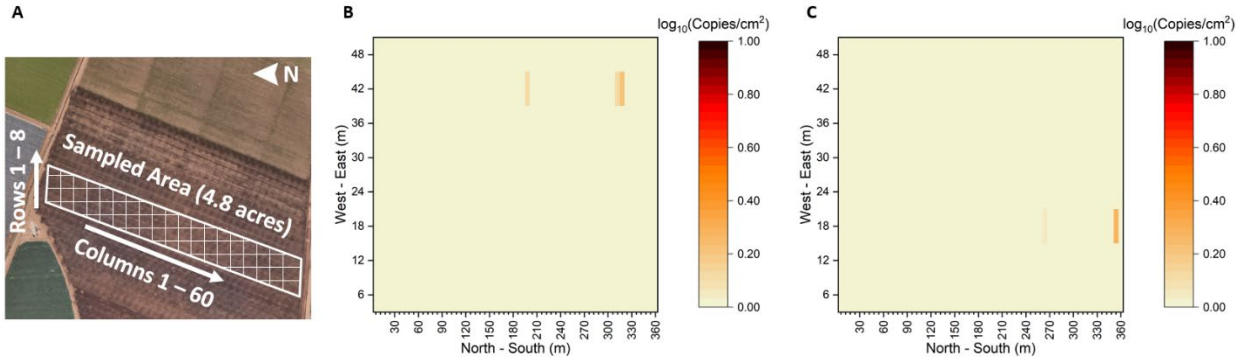

Figure 5: Risk of fecal contamination mapping using qPCR (August 2021). The Ct value of each qPCR reaction was converted to $\log_{10}$ (copies/cm^2) via a linear fit to log-transformed concentrations.

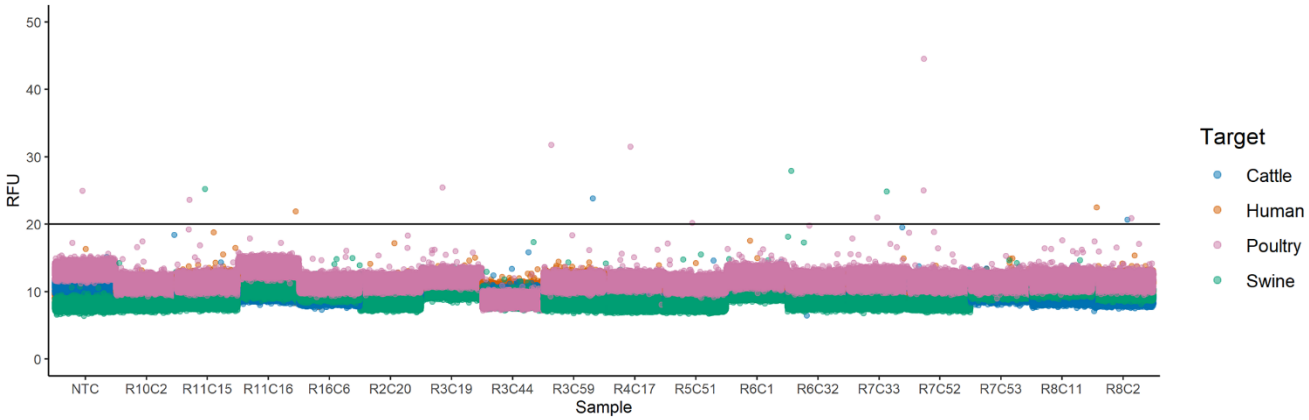

Figure 6: Scatter plot of microbial source tracking result. Each of the 17 samples with a concentration greater than 1 copy/ reaction was selected for microbial source tracking. The experiment was carried out in a dPCR method. Each sample was tested with four different host-specific qPCR primer-probe sets (cattle-specific *Bacteroidales*, swine-specific *Bacteroidales*, human-specific *Bacteroidales*, and poultry-specific *Bacteroidales*). On the scatter plot, the fluorescence intensity of each partition was displayed. Based on the NTC, the fluorescence threshold was determined to be 20 RFU.

Figure 7: Fresh-pack lettuce harvester and sampling site.

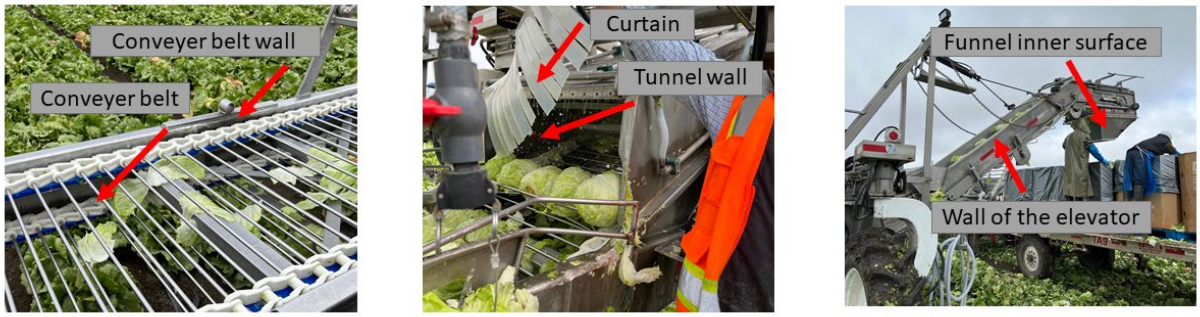

Figure 8: Processed lettuce harvester and sampling sites.

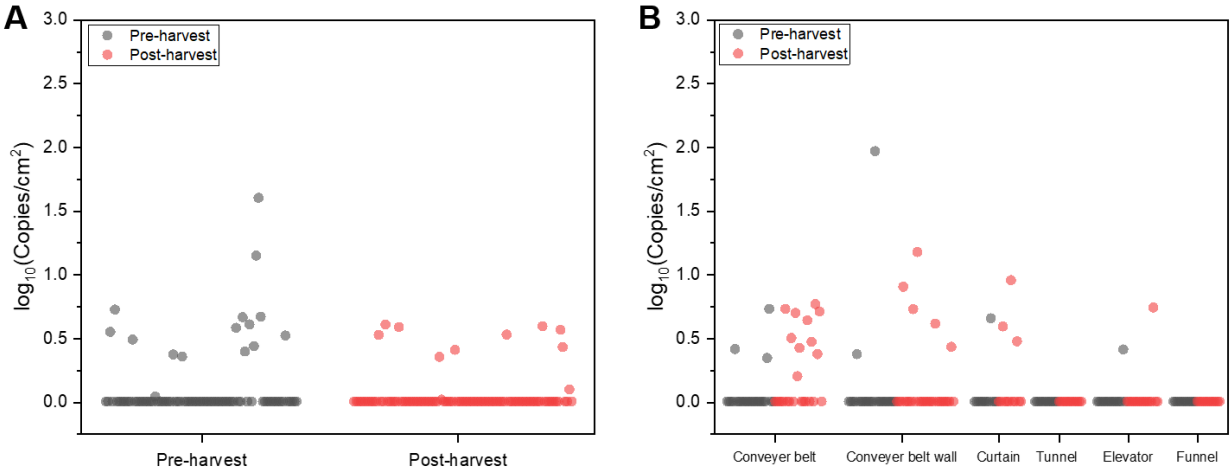

Figure 7: Quantified background levels of fecal contamination with A) fresh-pack lettuce and B) processed lettuce harvester samples, using the lab run qPCR data.

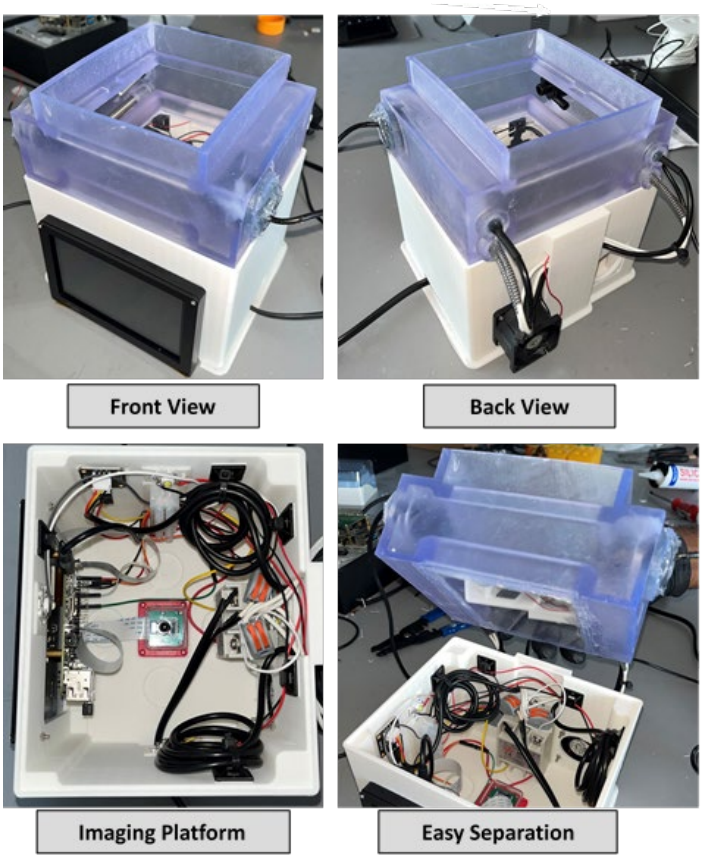

Figure 8: Design and fabrication of the portable heater.

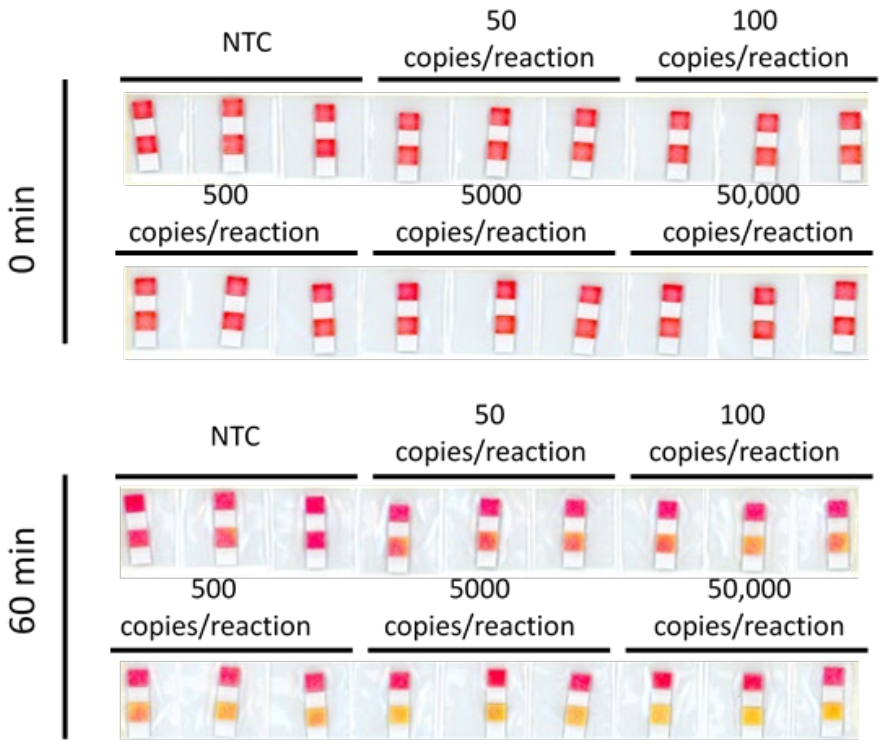

Figure 9: Limit of detection experiment with µPADs.

Figure 10: Field trial during the growing season using µPADs and DECODE.

Figure 11: Field trial during the harvesting season using µPADs and DECODE.

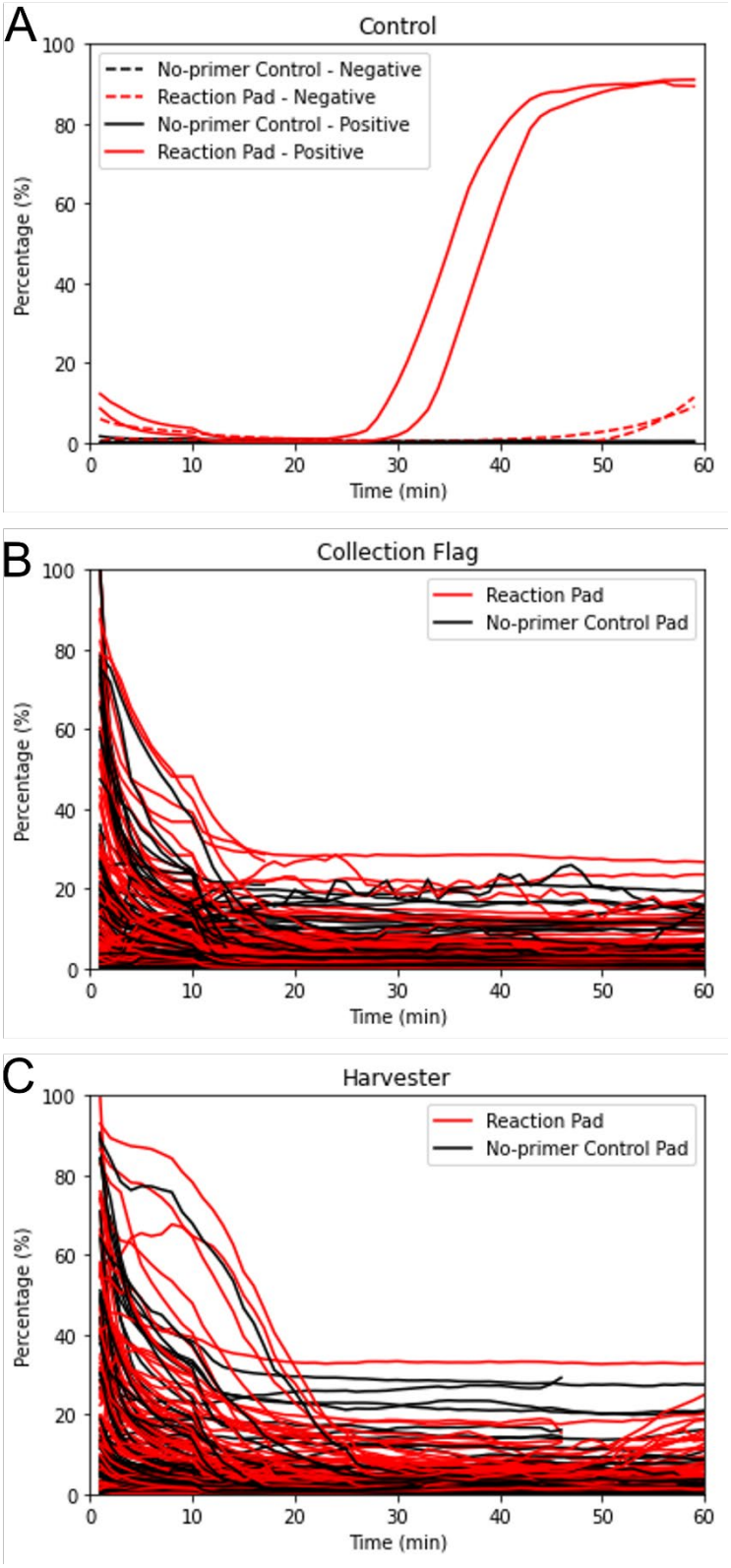

Figure 12: Image analysis results of A) control test (positive control has DNA template while negative control uses water), B) collection flag samples, C) harvester samples. The y-axis represents reaction percent and x-axis represents time. The reaction percentage over time data is smoothened by a rolling moving average analysis with a window size of 10. Reaction pad has primers while no-primer control does not have primers.

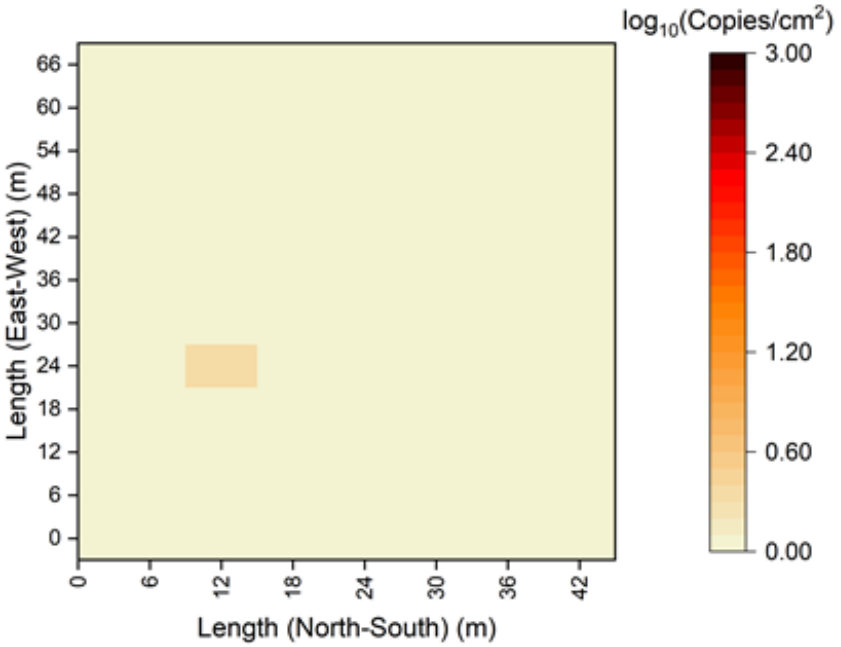

Figure 13: Lab validation for collection flag samples run in the field.

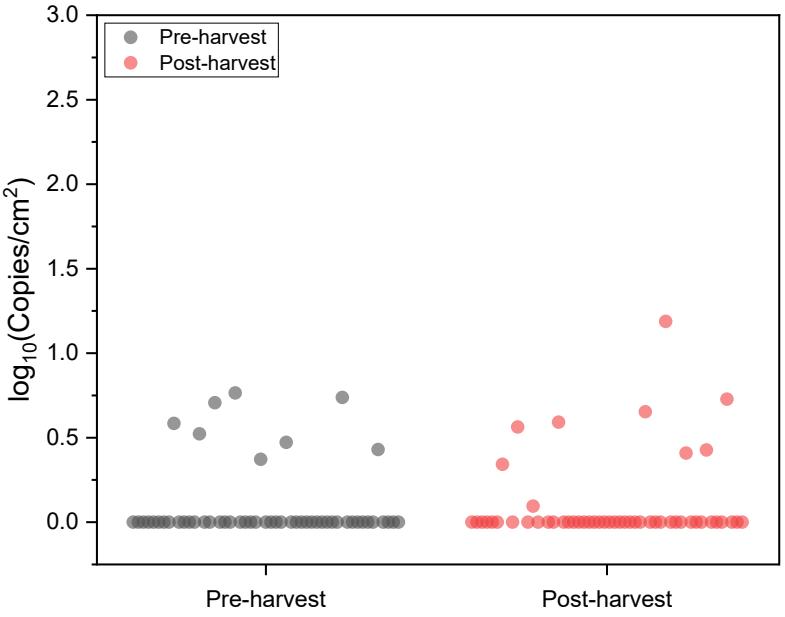

Figure 14: Lab validation for harvester samples run in the field.