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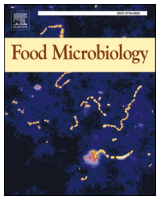

A loop-mediated isothermal amplification assay to detect *Bacteroidales* and assess risk of fecal contamination

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ABSTRACT

Fecal contamination of fresh produce from human and animal sources is a public health concern due to the risk of foodborne illnesses. The current standard laboratory procedures for microbiological analyses usually require an enrichment step that involves several hours. Molecular techniques such as polymerase chain reaction (PCR) have been used to directly detect pathogens from the samples, however, due to the low quantity of pathogen present and small volumes used for PCR, enrichment is usually required. Additionally, the need for specialized equipment and experienced workers hinders the use of these molecular techniques for field testing. Here, we developed a rapid risk-assessment assay for fecal contamination by targeting *Bacteroidales* using loop-mediated isothermal amplification (LAMP). The assay allows for naked-eye observation of reactions with as few as ~8 copies of *Bacteroidales* per cm^2 of the surface in the field. We evaluated this assay with complex field samples as well as on-site field studies. Our on-field studies demonstrated that the *Bacteroidales* LAMP assay enables us to easily and quickly (<50 min) assess the risk of fecal contamination from animal operations, with a concordance of 85.3% when compared to lab-based qPCR. These results were obtained without expensive equipment (when compared to standard laboratory procedures). These assays could be used to determine site-specific risk and help the decision-making process of fresh produce growers.

1. Introduction

For the past few decades, the incidence of food-borne illness associated with fresh produce has increased (Roth et al., 2018; Carstens et al., 2019; Machado-Moreira et al., 2019; Huang et al., 2020; Barlaam et al., 2021). The majority of foodborne pathogens linked to fresh produce (i.e., diarrheagenic *Escherichia coli*, *Salmonella enterica*) are enteric in origin, and fecal contamination can occur anywhere along the farm-to-fork chain (Alegbeleye et al., 2018; Bozkurt et al., 2021; Hoelzer et al., 2018; Qi et al., 2020). The use of poorly composted animal manures, substandard irrigation waters, wild animal encroachment, poor employee hygiene, discharge of human waste, and the spread of airborne bacteria (bioaerosols) from nearby livestock operations, are all potential points of entry while growing fresh produce (Beuchat, 2006; FDA, 2018; Gutierrez-Rodriguez and Adhikari, 2018; Heredia and García, 2018; Kumar et al., 2018).

In the majority of cases, the concentration of the enteric pathogens is relatively low (Ferone et al., 2020). Additionally, due to the high level of heterogeneity in fresh produce products, pretreatments are typically required where the pretreatment process will also dilute the pathogens (FDA, 2021; Ferone et al., 2020). Enteric pathogens can enter a viable but non-culturable state (VBNC) and maintain a low level of metabolic activity without growing on typical microbial media, therefore escaping detection using culture-based approaches (Martínez-Vaz et al., 2014; Oliver, 2005). The presence of pathogenic enteric microorganisms on fresh produce poses a possible health risk to humans. Since it is difficult to determine the presence/absence of all potential pathogens, it is common practice to only quantify the abundance of one or a few fecal

Abbreviations: PCR, Polymerase Chain Reaction; LAMP, Loop-mediated Isothermal Amplification; VBNC, Viable But Non-Culturable; FIB, Fecal Indicator Bacteria; qPCR, Quantitative Polymerase Chain Reaction; NTC, No Template Control; 16S rRNA, 16S ribosomal ribonucleic acid; LoD, Limit of Detection; gDNA, Genomic deoxyribonucleic acid; ANOVA, Analysis of Variance.

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Table 1
Sequences for selected LAMP primer set targeting *Bacteroidales*. The primer naming convention is Host.bacteria.gene.primer_set#.type_of_primer

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

indicator bacteria (FIB)—microorganisms that have been selected as indicators of fecal contamination (Brauwere et al., 2014).

Preferred laboratory procedures for FIB detection include culture-based methods (Hoadley and Cheng, 1974) and DNA-based approaches. Culture-dependent approaches require the use of a microbiology lab and have limitations in detecting the VBNC state (Li et al., 2020; Zhao et al., 2017). Another limitation of the culture-based FIB approach is the overnight incubation requirement, which delays findings and prevents early warnings and prompt implementation of contamination control or mitigation steps (Noble and Weisberg, 2005). To quickly determine microbial contamination, DNA-based methods such as the polymerase chain reaction (PCR) have been used. PCR-based approaches for monitoring FIB depend heavily on access to a laboratory, professional staff, and expensive equipment, preventing rapid in-field

Table 2
Limit of detection characterization of the assay. *B. fragilis* genomic DNA (1 μL) was added to reactions (24 μL reagents) in triplicate at different concentrations (serially diluted from 10,000 copies/reaction to 1 copy/reaction). The reactions were run in its respective thermal (cycling) condition. The Tt/Ct value (in minutes) of each reaction is reported in the table.

DNA concentration (copies/reaction)	Tt/Ct values for detecting *Bacteroidales* (in minutes)					
	LAMP (Universal. *Bacteroidales*.16S rRNA.1)			qPCR (GenBac3)		
10000	16.47	16.73	16.64	17.05	16.70	17.00
5000	17.12	17.84	17.22	17.96	17.69	17.95
1000	19.28	20.09	19.55	20.19	20.28	20.26
500	18.64	19.76	19.75	21.08	21.08	21.17
100	20.93	24.25	23.19	23.40	23.38	23.53
50	17.85	20.55	28.06	24.56	24.44	24.52
10	No Tt	43.15	No Tt	27.03	27.10	27.01
5	36.69	37.80	No Tt	27.65	27.88	27.63
1	No Tt	No Tt	44.5	30.32	30.31	30.56
0	No Tt	No Tt	No Tt	No Ct	No Ct	No Ct

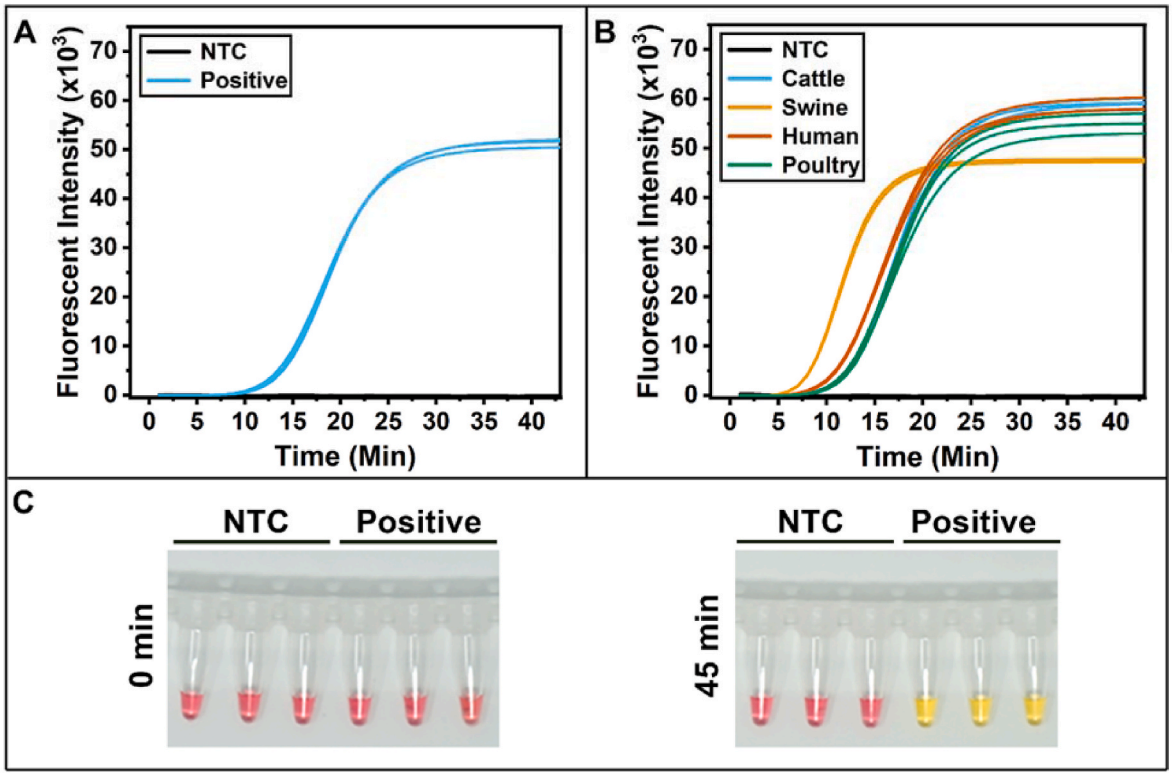

Fig. 1. Characterization of LAMP primer (Universal.*Bacteroidales*.16s rRNA.1). A) Fluorometric result from LAMP primer set using genomic DNA extract from pure culture of *Bacteroides fragilis*. 1 μL of *B. fragilis* DNA extract was added to the reaction mix to result in a final concentration 1 ng of total DNA (1×10^6 copies of 16S rRNA) per reaction. B) Fluorometric performance of LAMP primer set using stool extractions. 1 μL of stool DNA extract was added to the reaction mix to result in a final concentration 1 ng of total DNA per reaction. Both A and B had a reaction volume of 25 μL and used NEB WarmStart LAMP 2X Master Mix. C) Colorimetric result from LAMP primer set using genomic DNA extract from pure culture. 1 μL of *B. fragilis* DNA extract was added to the reaction mix to result in a final concentration 1 ng of total DNA per reaction. Before and after the reaction, samples were imaged via a flatbed scanner. The three samples on the left are NTC and the three samples on the right are positive samples. Fluorometric reactions were run in a qTower^3 G with a ramp rate of 1 °C/s. Colorimetric reactions were run with Anova Culinary Precision Cooker (ANTC01; Anova, USA) at 149 °F (65 °C). NTC: no template control where 1 μL of nuclease-free water was added to the reaction mix instead of DNA extract.

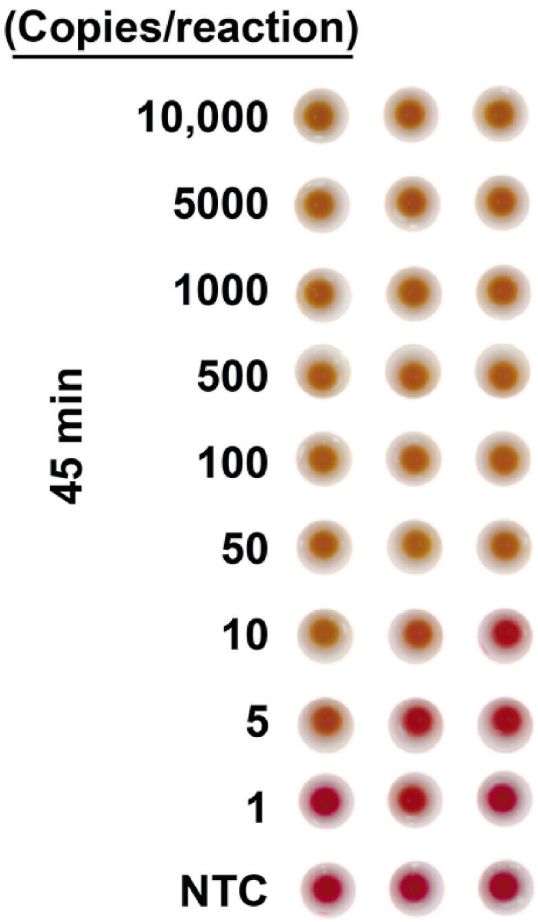

Fig. 2. Limit of detection characterization of LAMP (Universal.*Bacteroidales*.16S rRNA.1) colorimetric assay. Serially diluted (1000 copies/reaction to 1 copy/reaction) samples of *B. fragilis* genomic DNA (1 μL) were added to reactions (24 μL reagents) in triplicates. NTC indicates no template control where 1 μL of nuclease-free water was added to the reaction mix instead of resuspension. Reactions had a final volume of 25 μL and used NEB 2x colorimetric master. Reactions were run with a 12-quart container (B07RM787V2; Amazon, USA) filled with ultrapure water and an Anova Culinary Precision Cooker (ANTC01; Anova, USA) set to 149 °F (65 °C).

assessment of contamination.

FIB, such as *Escherichia coli*, *Enterococcus faecalis,* and *Bacteroidales,* are commonly used to assess microbial water quality (Allende et al., 2018; Kundu et al., 2018; Topalcengiz and Danyluk, 2022; Truchado et al., 2016). *Bacteroidales* are a good target since they are confined to warm-blooded animals and are a major component of gut microflora (Bernhard and Field, 2000). Furthermore, as obligate anaerobes, *Bacteroidales* are unable to proliferate in standard atmospheric conditions, therefore they would not grow excessively to exaggerate their levels when evaluating the possibility of fecal contamination using concentration of *Bacteroidales*. Molecular techniques such as PCR and quantitative PCR (qPCR) are currently applied to detect *Bacteroidales*. The PCR-based assays target either highly conserved regions of the 16S gene (Kildare et al., 2007) or variable regions representing individual hosts (Gómez-Doñate et al., 2016). *Bacteroidales* assays have been extensively used as general indicators of microbiological water quality (Fan et al., 2020; Haramoto and Osada, 2018; Malla et al., 2018; Miura et al., 2021; Pham and Kasuga, 2020; Somnark et al., 2018). These methods are advantageous because of their high levels of precision, specificity, and sensitivity (Drozd et al., 2013; Holcomb and Stewart, 2020; Schriewer et al., 2010). Recently, a few studies have also attempted to use *Bacteroidales* as a target to detect possible fecal contamination in fresh produce (Harris et al., 2017; Mascorro et al., 2018; Ordaz et al., 2019; Prince-Guerra et al., 2020; Ravaliya et al., 2014).

Compared to PCR, loop-mediated isothermal amplification (LAMP) (Notomi et al., 2000) enables simpler detection of microorganisms in environmental samples (Lee et al., 2019; Ramya et al., 2018; Thio et al., 2021). Due to the inherent characteristic of LAMP *Bst* DNA polymerase, only a single temperature (in the range of 60–65 °C) is required for the reaction to be conducted (as opposed to cycling of temperature, which is required for PCR). The LAMP reaction could be carried out in the field using a cost-effective, simple heat source, such as an incubator or a water bath (Pascual-Garrigos et al., 2021), in contrast to the expensive thermocyclers needed by traditional PCR methods. Furthermore, the *Bst* polymerase is resistant to common PCR inhibitors found in unpurified environment samples, enabling direct measurements (Francois et al., 2011). As a result, LAMP has been widely used as a point-of-care assay for applications in food safety and diagnostics of human and animal health (Davidson et al., 2021; Wang et al., 2021; Mohan et al., 2021; Pascual-Garrigos et al., 2021; Niessen et al., 2013; Lalle et al., 2018; Niessen et al., 2013, 2013). Incorporating a colorimetric dye (e.g., EBT, phenol red) in LAMP assays enables color changes that are visible to the naked eye (Tanner et al., 2015). Jiang et al. developed a human-associated *Bacteroides* detection device based on fluorescent-LAMP for monitoring human fecal contamination in water (Jiang et al., 2018). However, this approach requires a relatively long assay time (80 min) and a transilluminator to visualize the fluorescence. Khodaparast et al. reported another *Bacteroides* LAMP assay for rapid detection of fecal contamination in environmental water. Similar to the work by Jiang et al., the LAMP assay is a fluorometric assay that is also specific to contamination from human sources. However, the fluorescent results were obtained using an 8000 USD portable fluorometer. Here, we present the first demonstration of an *in-situ* LAMP *Bacteroidales* detection assay that accounts for multiple sources of fecal contaminations to support risk assessment in fresh produce. We define risk assessment as the procedure or method of identifying and characterizing risk factors that have the potential to impair fresh produce safety before determining appropriate ways to eliminate or control the risk (Manuele FA, 2016; Rausand M, 2013). We have verified the inclusivity of the assay for detecting the main sources of potential fecal contamination (human, cattle, swine, and poultry). The colorimetric LAMP assay allows for a quick and simple visual "yes/no" result readout without the use of expensive instruments or highly experienced operators, which can be used to support the risk assessment process in fresh produce safety.

In this study, we provide the following four advancements: (i) we designed LAMP primers for *Bacteroidales* and characterized their performance using genomic DNA from *Bacteroides fragilis* pure culture and extracted fecal DNA, (ii) we evaluated the LAMP assay for quantifying FIB by comparing it with qPCR results, (iii) we implemented a new method for collecting bioaerosols using plastic sheets and wooden sticks, combined to form collection flags, and demonstrated that they are more consistent compared to sampling lettuce leaves directly, and (iv) we modified the LAMP assay and combined it with collection flags for supporting field-deployable risk assessment of fecal contamination.

2. Materials and methods

2.1. Genomic DNA preparation and fecal DNA extraction

B. fragilis (ATCC® 25285™) was grown overnight (37 °C, 4% H_2, 5% CO_2, 91% N_2, <20 ppm O_2) in Chopped Meat Carbohydrate Broth (BD297307; BD, USA). Genomic DNA was extracted from *B. fragilis* with Purelink Genomic DNA Mini Kit (K182001; Invitrogen, USA) according to the manufacturer's protocol.

Stool samples (from cattle, swine, and poultry) were collected using a disposable utensil while steaming. For each host, stool samples were collected from three individuals and pooled in sterilized 50 mL

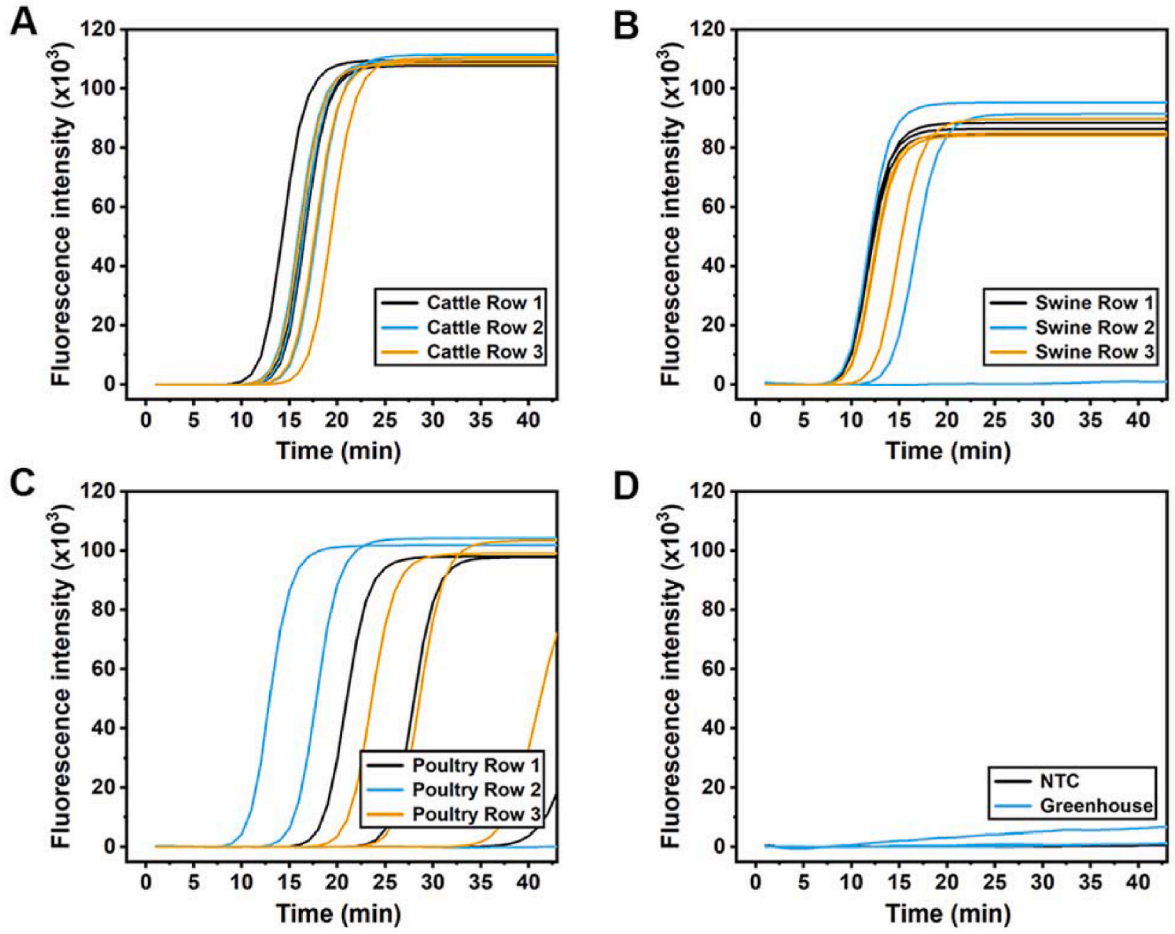

Fig. 3. Fluorometric LAMP (Universal.*Bacteroidales*.16s rRNA.1) assays using lettuce leaves swab resuspension solution. 1 μL of resuspension was added to the reaction mix. NTC indicates no template control where 1 μL of nuclease-free water was added to the reaction mix instead of resuspension solution. Reactions had a final volume of 25 μL and used NEB WarmStart LAMP 2X Master Mix. Reactions were run on a qTower^3 G at 65 °C with a ramp rate of 1 °C/s.

centrifuge tubes. The tubes were immediately stored in an icebox. After returning back to the lab, samples were mixed with 15% glycerol and stored at −80 °C until nucleic acid extraction. The human fecal matter was purchased from Lee Biosolutions (991–18; Lee Biosolutions, USA). The genomic DNA of human and animal stool samples were extracted with Fast DNA Stool Mini Kit (51604; QIAGEN, Germany) according to the manufacturer's protocol in the lab.

2.2. Quantitative PCR (qPCR)

The qPCR reaction was performed in a total volume of 25 μl, containing 12.5 μl 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 primers (final concentration 0.4 μM) (Table S1 (Siefring et al., 2008),), 0.5 μl of 10 μM fluorescent probe (final concentration 0.2 μM) (Table S1), 9 μl nuclease-free water, and 1 μL of template or 1 μL of nuclease-free water for no template control (NTC). The qPCR reactions were performed on a qTOWER^3 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.

2.3. LAMP primer design and screening

Multiple sequence alignment was performed using the NCBI Multiple Sequence Alignment Viewer (MSA). A conservative region on the *Bacteroidales* 16S ribosomal RNA gene was found by aligning the first 1000 hits of NCBI BLAST using the algorithm somewhat similar sequences (blastn) (NCBI, 2019). The LAMP primer set was designed based on the conservative region using PrimerExplorer V5 (http://primerexplorer.jp/lampv5e/index.html) with the default parameters (Table 1, S2).

The LAMP primer set was tested with both fluorometric and colorimetric LAMP assays. 1 ng of *B. fragilis* pure culture DNA (176,975 copies) extract was used as the template for the primer screening. NTC had 1 μL of nuclease-free water instead of *B. fragilis* DNA.

2.4. LAMP

The fluorometric LAMP reaction was performed in a total of 25 μL comprising 12.5 μL WarmStart LAMP 2X Master Mix (E1700; New England Biolabs, USA) (final concentration 1X), 0.5 μL Fluorescent dye 50X (B1700AVIAL; New England Biolabs, USA) (final concentration 1X), 2.5 μL 10X LAMP 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), 8.5 μL nuclease-free water, and 1 μL of template or 1 μL of nuclease-free water for NTC. The colorimetric LAMP reaction was performed in a total of 25 μL comprising 12.5 μL WarmStart® Colorimetric LAMP 2X Master Mix (M1800; New England Biolabs, USA) (final concentration 1X), 5 μL of 5 μM SYTO™ 9 Green Fluorescent Nucleic Acid Stain (S34854; Invitrogen, USA) (final concentration 1 μM), 2.5 μL 10X LAMP 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), 4 μL nuclease-free water, and 1 μL of template or 1 μL of nuclease-free water for NTC.

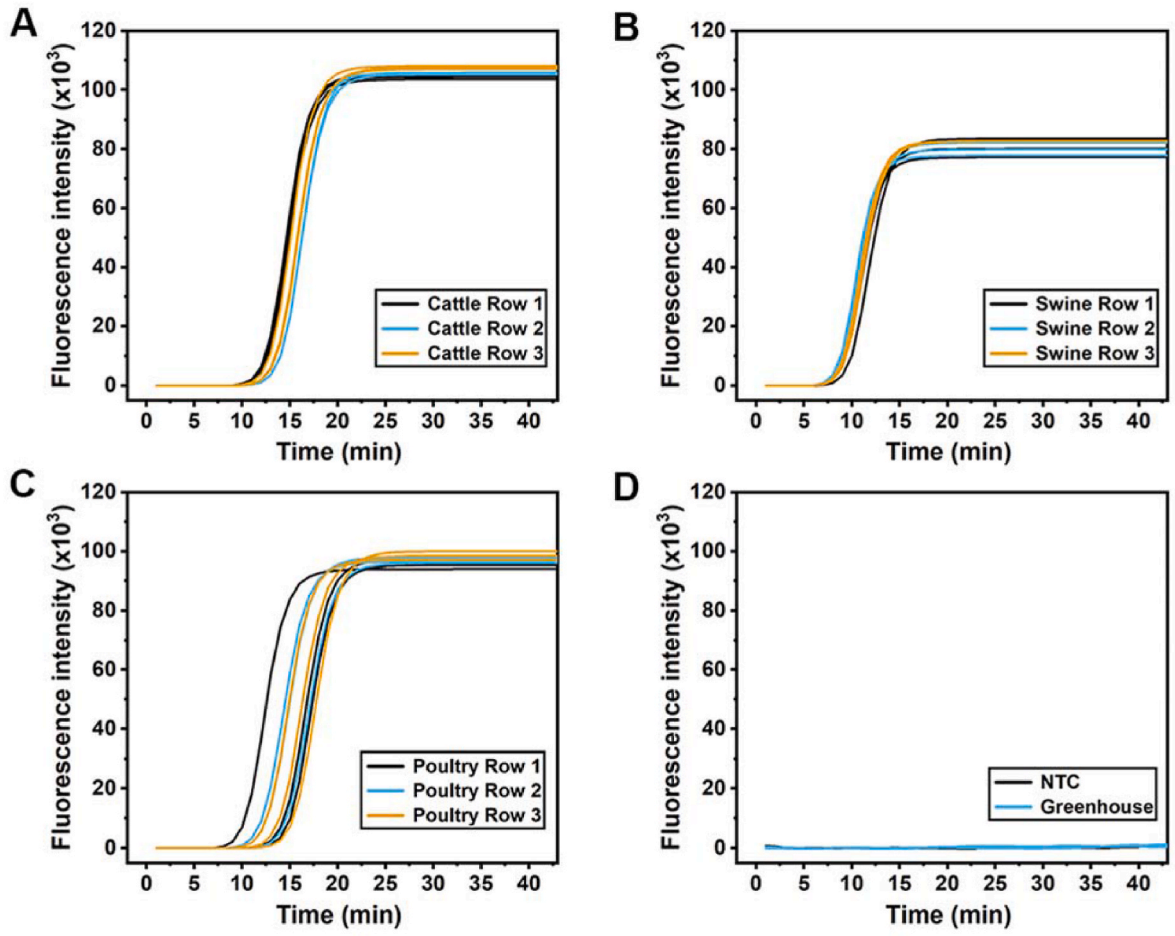

Fig. 4. Fluorometric LAMP (Universal.*Bacteroidales*.16s rRNA.1) assays using collection flag swab resuspension solution. 1 μL of resuspension was added to the reaction mix. NTC indicates no template control where 1 μL of nuclease-free water was added to the reaction mix instead of resuspension. Reactions had a final volume of 25 μL and used NEB WarmStart LAMP 2X Master Mix. Reactions were run on a qTower^3 G at 65 °C with a ramp rate of 1 °C/s.

2.5. Sensitivity and specificity

The sensitivity of LAMP and qPCR were measured using quantified *B. fragilis* DNA. The *B. fragilis* DNA was quantified using a Quant-iT™ PicoGreen™ dsDNA Assay (P7589; Invitrogen, USA) according to manufacturer's instructions. Serial dilutions were made to determine the limit of detection (LoD) of both LAMP and qPCR. All reactions were done in triplicates.

We conducted *in-silico* specificity studies to verify the conservation of the LAMP primers with specific taxa of interest and to predict the cross-reactivity of the primer set (Tables S3 and S4). *In-silico* sequence identity analyses were conducted by performing a BLAST search of the sequence LAMP primers spanned on (from the 5′ end of F3 to the 3' end of complimentary B3) against sequences available in the NCBI Nucleotide database for the specific taxa. The nucleotide sequence of the sections that do not have a LAMP primer is converted to "N" indicating any nucleotide is acceptable. The parameters and results of the BLAST are shown in Table S3. The identities of the best BLAST hit were calculated by the number of nucleotide matches (including all N) divided by the total length of the sequence (213 base pairs).

2.6. Host inclusivity of LAMP

Stool DNA extract from different hosts (cattle, swine, poultry, and human) were used to test the host inclusivity of the LAMP assay. The stool extracts were diluted to 1 ng/μL and were used as the template for this experiment.

2.7. Measurement of FIB in leafy greens and collection flags

The detection of artificially contaminated leafy greens was evaluated. Romaine lettuce (B01N5NG0OY; Amazon, USA) was grown in a greenhouse (20 °C) at Purdue University. The details of lettuce cultivation are described elsewhere (SF Gate Contributor, 2020). Briefly, the lettuce seed was germinated in soil at 20 °C inside a germination tray. After 14 days, lettuce seedlings were transplanted into individual plastic pots with each pot containing 2–3 lettuce seedlings. Three weeks after transplanting, 1 teaspoon of Miracle Gro All Purpose Plant Food (B000F6XGZ0, Amazon, USA) was sprinkled on the soil around the base of each plant and then watered to dissolve the fertilizer. The plants were watered every 2–3 days throughout the growing process. The mature lettuce (~60 days) was placed around animal feeding operation facilities in Indiana, USA.

Collection flags were placed next to the lettuce plants. The collection flags were assembled 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. Fig. S1 illustrates the fabrication procedure.

Ten 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 (Fig. 5 A, B, and C). Both the plants and collection flags were encoded with a unique identifier and the

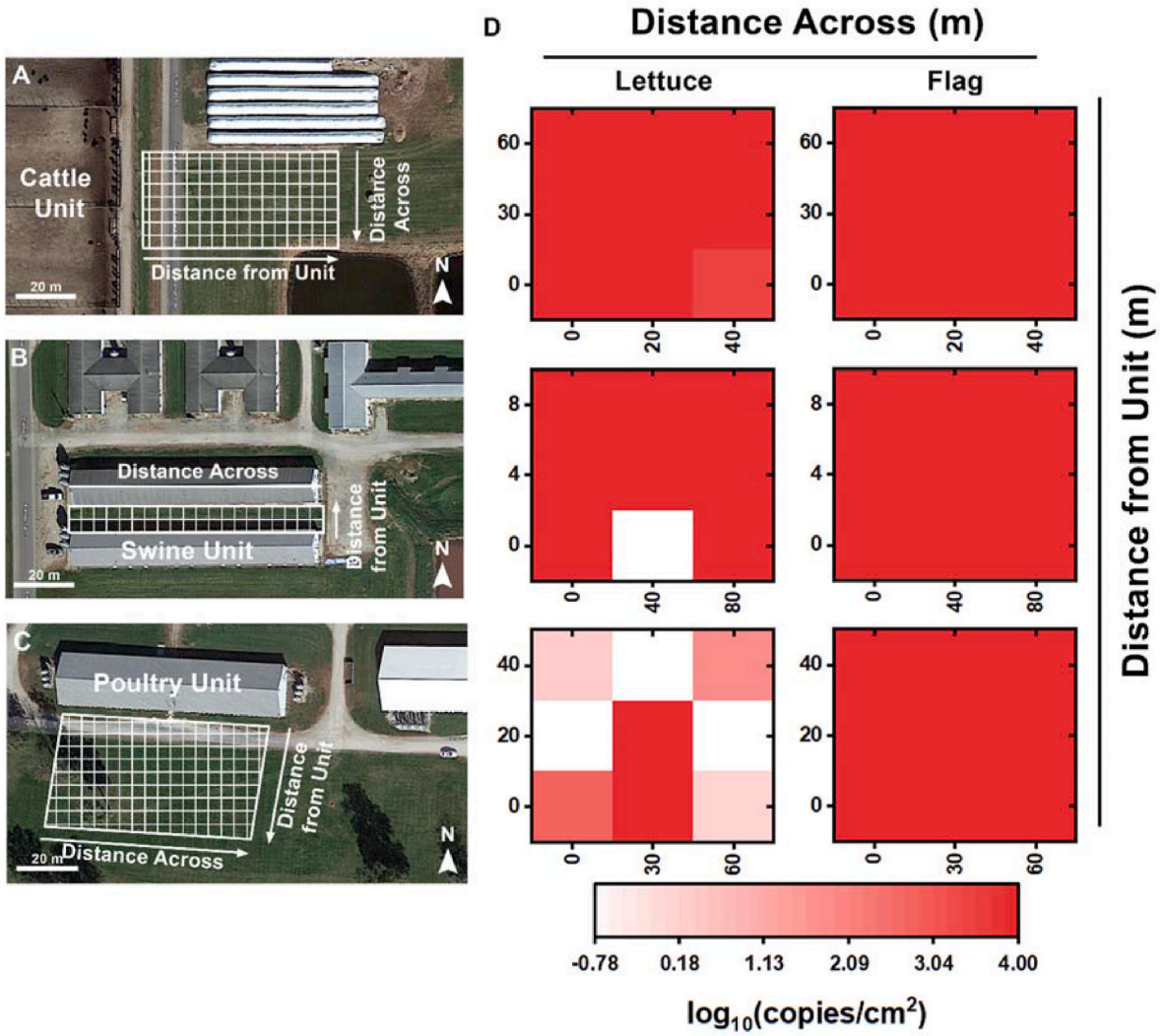

Fig. 5. Risk of fecal contamination mapping using LAMP (Universal.Bacteroidales.16s rRNA.1). The Tt value of each LAMP reaction was converted to $\log_{10}$ (copies/cm^2) via a linear fit to log-transformed concentrations (Fig. S6 A).

location associated with the plant/flag's identifier was recorded. A group of ten lettuce 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 United States Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM) for isolating specific pathogens from fresh vegetable samples, 25 g lettuce (approximately four leaves) or four pieces of transparency films were swabbed using a wet polyester-tipped swab (263000, BD BBL, USA). Each swab was resuspended in 200 μL molecular biology grade water. The resuspension was directly used for qPCR and LAMP assays without performing DNA extraction.

2.8. LAMP assay deployed on-site

LAMP reactions were prepared in individual domed PCR tubes (AB0337; Thermo Fisher, USA). The LAMP reactions were performed in a total of 25 μL comprising 12.5 μL WarmStart® Colorimetric LAMP 2X Master Mix (M1800; New England Biolabs, USA) (final concentration 1X), 2.5 μL 10X LAMP 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), 9 μL nuclease-free water, and 1 μL of resuspension (previously described in the earlier section 2.7 Measurement of FIB in leafy greens and collection flags) or 1 μL of nuclease-free water for NTC. A 12-quart container (B07RM787V2; Amazon, USA) was filled with bottled drinking water and an Anova Culinary Precision Cooker (ANTC01; Anova, USA) set to 149 °F (65 °C) was attached as reported previously (Pascual-Garrigos et al., 2021). The tubes were submerged in the water using a PCR tube holder designed and 3D-printed in-lab with a Form 3B 3D printer (Formlabs, MA) using high-temperature resin v2 (Pascual--Garrigos et al., 2021). The tubes were removed from the water after 60 min.

For the on-site experiment, the reagents were prepared in the lab, and the addition of sample was done on-site using a 0.5–10 μL single-channel pipette (3123000020; Eppendorf, Germany) with no additional measures to avoid contamination. The experiment on-site happened no more than 30 min after swabbing the sample from collection flags. The swabbing procedure was the same as explained in section 2.7 Measurement of FIB in leafy greens and collection flags. The resuspension was directly used for LAMP assays on-site without performing DNA extraction. The samples were maintained on ice before being brought to the lab to perform in-lab LAMP and qPCR for the concordance study. The resuspended samples were used for in-lab LAMP and qPCR without a separate DNA extraction step.

Time-lapse video of the tubes was taken from 0 to 60 min using a HERO8 Black digital camera (GoPro, USA). Endpoint images of the tubes were taken at 0 and 60 min using a Sony Alpha a7II mirrorless digital camera (B00R1P93SC, Amazon, USA). All images obtained were adjusted by using the white balance tool on Adobe Lightroom to obtain a

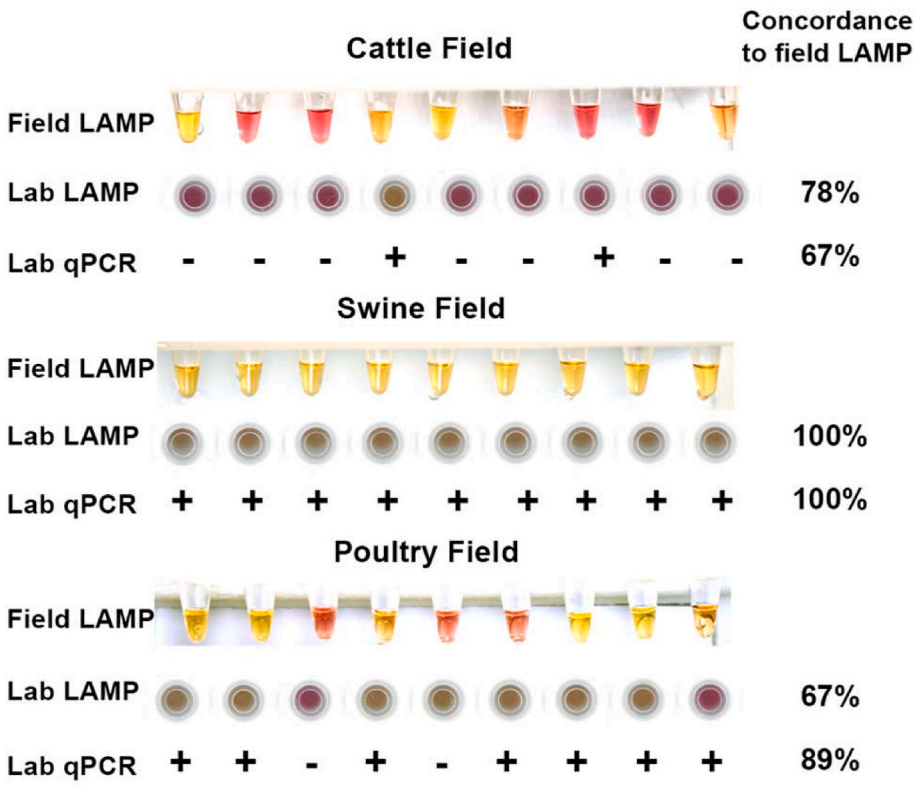

Fig. 6. On-site colorimetric LAMP (Universal.*Bacteroidales*.16s rRNA.1) assays comparison to lab LAMP and qPCR. 1 μL of swab resuspension was added to the reaction mix. Reactions had a final volume of 25 μL. The LAMP assay used NEB 2X colorimetric master. The qPCR used NEB 2X Luna® Universal Probe qPCR Master Mix. On-site LAMP reactions on-site were run in individual domed PCR tubes (AB0337; Thermo Fisher, USA) with an Anova Culinary Precision Cooker at 65 °C. Lab confirmations were run on a qTower^3 G with a ramp rate of 1 °C/s and 8 °C/s for LAMP and qPCR respectively. For the qPCR tests, any fluorescent values that were greater than the highest background intensity were considered positive (+) amplifications, conversely negative (−) amplifications.

relatively uniform and consistent background.

3. Results

3.1. Screening of primers

Three sets of LAMP primers were designed based on the conservative region found via NCBI Multiple Sequence Alignment (Table S2). The LAMP primer sets were screened with a fluorometric LAMP assay using stool DNA extracts from different hosts (Fig. S2). We decided that Universal.*Bacteroidales*.16S rRNA.1 (Table 1) was the appropriate primer set because it amplifies the target *Bacteroidales* from all hosts without providing any false-positive amplification in the NTC. In addition, a colorimetric (endpoint) LAMP assay was performed for the appropriate primer set (Fig. 1C). For all positive samples, both a fluorescence augment (Fig. 1A, B) and a color change (Fig. 1C) were observed within 45 min. Fluorometric and colorimetric data for all negative samples were also consistent. No false positives were observed within all data. Universal.*Bacteroidales*.16S rRNA.1 was used for further testing (see Table 1).

3.2. Sensitivity and specificity of the LAMP assay

The fluorescence intensities were extracted for the 45-min time point for both LAMP and qPCR reactions (Figs. S3 and S4). Any fluorescent intensity values that were greater than the highest background intensity (20% of the maximum reaction intensity) were considered successful amplifications. The lowest DNA concentrations that had successful amplification for all three replicates of a given reaction were classified as the LoD for the assay. Comparison of sensitivity between LAMP and qPCR showed that qPCR has a better LoD (1 copy/reaction) than LAMP (50 copies/reaction). The Tt values (time required for the fluorescent intensity to reach/exceed defined reaction threshold) for LAMP and Ct values (number of cycles required for fluorescent intensity to reach/exceed defined reaction threshold) for qPCR were calculated using software qPCRsoft 4.1 (baseline correction: 5, auto threshold) (Analytik Jena, Germany), and reported in Table 2. Linear regression analysis was used to fit correlations between Tt/Ct values and $\log_{10}$(concentrations) (Fig. S5). The same LoD experiment was repeated with colorimetric LAMP assay using the Anova Culinary Precision Cooker setup at the temperature of 149 °F (65 °C). The colorimetric LAMP assay showed the same LoD as the fluorescent LAMP assay (Fig. 2).

We conducted *in-silico* sequence-specificity analyses to check whether LAMP primers would react with seven bacterial species known to be associated with the lettuce leaves surface microbiota based on a previous report (Rastogi et al., 2012) and the NCBI strain isolation source. *In-silico* sequence-specificity analysis were conducted by performing a BLAST search of the sequence LAMP primers spanned on (from the 5′ end of F3 to the 3’ end of complimentary B3) against sequences avaible in NCBI Nucleotide database for the specific taxon of interest. Table S4 shows the overall sequence identity calculated by computing the maximum sequence identity of all hits for a single primer against an individual organism. We expect some cross-species similarity since the LAMP primers were designed based on the 16S ribosomal RNA gene, which is a highly conserved gene among diverse bacteria species. The *in-silico* sequence identity study revealed that the sequence identity rate is <50% for the seven microorganisms we tested (*Xanthomonas* (TXID 338), *Escherichia coli* (TXID 562), *Pantoea agglomerans* (TXID 549), *Bacillus subtilis* (TXID 1423), *Salmonella* (TXID 590), *Pseudomonas fluorescens* (TXID 294), *Massilia* (TXID 149698)). Thus, we do not anticipate these targets will significantly cross-react with the primer set. These results are in agreement with the experimentally tested greenhouse controls, where we did not see any amplification.

3.3. Host inclusivity of the LAMP assay

Stool DNA extracts from four hosts (cattle, swine, poultry, humans) were spiked in LAMP reactions with the Universal.*Bacteroidales*.16S rRNA.1 primer set to simulate different sources of fecal contamination. The LAMP assay detected *Bacteroidales* from all hosts’ stool DNA

extractions in 15 min with comparable Tt values (Fig. 1B). The detection in swine samples was slightly faster (one-way analysis of variance (ANOVA), $p < 0.01$). There was no amplification in the negative controls. The experiment indicates that the LAMP primer has high inclusivity among *Bacteroidales* from different hosts.

3.4. Measurement of FIB in leafy greens and collection flags

The resuspended samples were used directly for molecular amplification assays. Figs. 3 and 4 show the fluorometric LAMP data using swabs from lettuce leaves and collection flags, respectively. To confirm the results, qPCR was performed on the same samples (Figs. S6–S7). Both outcomes appear to be similar. *Bacteroidales* were not detected in the negative control group, indicating that neither the lettuce nor the collection flag samples were naturally contaminated with *Bacteroidales* or contaminated during the handling process.

We also demonstrate that collection flag samples (Fig. 4 and S7) have higher consistency than lettuce swab samples (Fig. 3 and S6). 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. This could be due to the rough foliage topography, which makes consistent swabbing challenging. Thus, we decided to use collection flags for on-site assay characterization.

To construct a fecal contamination risk evaluation map, we converted the Ct/Tt value of each qPCR/LAMP reaction to $\log_{10}$ (copies/cm^2) via a linear fit to log-transformed concentrations(Figure S5). Fig. 5 and S8 demonstrate that there are more than 10^3 copies of *Bacteroidales* per cm^2 around animal operations. However, there is a poor agreement between the concentrations reported by qPCR and LAMP. The concentration of *Bacteroidales* determined by LAMP is generally higher than that determined by qPCR. Most concentrations from qPCR are less than 10^3 copies/cm^2, which is particularly notable given that the majority of the LAMP response indicated values over 10^4 copies/cm^2 of *Bacteroidales* per cm^2. Although we have not confirmed the reason for these differences, we hypothesize two possibilities that could be investigated in the future: i) LAMP is less sensitive to inhibitors in the swab samples compared to qPCR and thus, is able to amplify the targets better leading to a higher count; or ii) the target regions used for LAMP cover a broader variety of *Bacteroidales* compared to qPCR and thus, lead to higher estimates. Nevertheless, two assays revealed that higher concentrations were present around the swine unit in our experiments compared to the cattle and poultry units.

3.5. LAMP assay deployed on-site

The collection flags were placed around the animal operation facilities (cattle, swine, poultry) for a period of seven days and LAMP assay was conducted on the seventh day. All samples, including the positive control (1 μL of 1 ng/μL B. *fragilis* gDNA) and no template control (1 μL of purified bottled drinking water), were added on-site without any additional measures to avoid contamination (Fig. 6, S9). The same assay, as well as a qPCR assay, were conducted the next day in a lab setting using the same samples (Fig. 6). The concordance observed between LAMP assays performed on the farm and in the lab is 78%, 100%, and 67% for cattle, swine, and poultry respectively. The concordance observed between LAMP assays performed on the farm and qPCR in the lab is 67%, 100%, and 89% for cattle, swine, and poultry respectively. The lack of consistency in the cattle and poultry samples, we presume, is due to the lower concentration of DNA in the resuspended solution as compared to the swine samples. In addition, some of the samples from the cattle unit show positive amplification in the field, but not in the lab tests, which could be due to degradation of the sample during transportation (Pascual-Garrigos et al., 2021).

4. Discussion

4.1. Bacteroidales as an appropriate biomarker for assessing fresh produce fecal contamination

Members of the order *Bacteroidales* have been employed as water fecal contamination indicators (Bernhard and Field, 2000; Jenkins et al., 2009; Somnark et al., 2018) because they are restricted to warm-blooded animals, are major components of their intestinal microflora, and they do not proliferate in the environment (Bernhard and Field, 2000).

Four advantages of implementing *Bacteroidales* to evaluate fecal contamination include: i) direct detection of DNA without the need for prolonged cultivation, ii) high abundance in contaminated water and feces, iii) potential for risk assessment of multiple pathogens, and iv) non-pathogenicity of *Bacteroidales*. *Bacteroidales* are commensal bacteria found in high concentrations in feces (total concentration 10^{11} bacteria/g of stool) (Gorbach, 1996), accounting for about 30%–40% of total fecal bacteria (Mascorro et al., 2018), outnumbering facultative anaerobes, such as *Escherichia coli* and *Entercoccus faecalis* (two other commonly used FIB), by a factor of 10^3–10^4 and 10^4–10^5 respectively (Gorbach, 1996; Lunestad et al., 2016). Thus, *Bacteroidales* detection can theoretically provide at least 1000 times better sensitivity than detecting other common FIB when a technique with the same LoD is used. Although the *Bacteroidales* LAMP assay developed here is not as sensitive as qPCR, it is still sufficient for detecting bioaerosols close to animal operations, which we demonstrate to be at levels of up to 10,000 copies/cm^2. Drawing direct relationships between *Bacteroidales* and foodborne pathogens remains to be investigated because clear risk thresholds for *Bacteroidales* have not yet been established. Although we know that *Salmonella* spp. could be at a concentration of 10^3-10^4 bacteria/g of stool and *E. coli* O157 could be < 100 CFU/g of stool, how these concentrations relate to how much feces, pathogens, and *Bacteroidales* could end up on the fresh produce remains undetermined (LeJeune et al., 2006; Ohta et al., 2019).

The collection flag and swabbing method increased the practicality of *in-situ* risk assessment of fecal contamination. We have encoded each collection flag with a unique identifier and recorded the location associated with the flag's identifier so that we can generate a heat map after the LAMP assay. Referring to a calibration curve, we are able to convert the Tt values of each test into copies of *Bacteroidales* per cm^2 of the flag surface. The LoD of this approach is as low as ~8 copies/cm^2. If there are areas of suspected animal intrusion, aerosol-driven contamination, insanitary operation, or irrigation water contamination, they should show up as hot spots on the heat map. We foresee that our LAMP assay could be used as a complementary assay to current fresh produce microbiological analyses protocols.

Furthermore, *Bacteroidales* contain host-associated 16S rRNA gene sequences that could be used for tracking sources of contamination of fresh produce (Tambalo et al., 2012) whereas traditional fecal coliform indicators, such as *Escherichia coli* and *Enterococcus faecalis*, only indicate the presence of fecal contamination. *Bacteroidales*, due to their species-specific characteristics could be used for microbial source tracking (Bernhard and Field, 2000; Kildare et al., 2007; Mascorro et al., 2018; Somnark et al., 2018) although we have not implemented these methods in the current study.

4.2. LAMP as an on-site detection assay

In previous studies (Foo et al., 2020; Khan et al., 2018), the LoD of LAMP was compared to PCR-based assays (e.g., conventional PCR, nested PCR, and qPCR). Consistent with our current study, their findings revealed that LAMP has worse LoD compared to nested PCR and qPCR (Foo et al., 2020; Khan et al., 2018). On the other hand, LAMP was simpler and faster than the other assays evaluated. Here, we show that our LAMP assay could be conducted on-site with a simple

consumer-grade water bath providing a tool for supporting risk assessment of fresh produce contamination in the field (away from a centralized laboratory, Fig. S9). Colorimetric reporters change from red to yellow as the LAMP reaction occurs and the pH decreases, making the results comprehensible to the naked eye.

5. Conclusions

Here, we have developed a low-cost, rapid, and easy to use *in-situ* colorimetric assay to assess potential fecal contamination for the fresh produce industry. This assay uses a polyester-tipped swab to concentrate target DNA from the surface of a plastic collection flag allowing a LoD as low as ~8 copies of *Bacteroidales* per cm^2 of the surface. For reference, 25 g of lettuce leaves would have approximately 1300 cm^2 of surface area and we have found about 10^3-10^4 copies/cm^2 of *Bacteroidales* per cm^2 around animal operations. Our technology requires only simple consumer-grade water bath and has minimal sample processing; it does not need DNA extraction and purification. 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. The assay can determine whether *Bacteroidales* might be present around pre-harvest fresh produce, indicating whether the product is contaminated with any sources of fecal matter. Furthermore, this assay could be used as a part of the pre-season planning to determine which areas are safe for growing. Ultimately, this assay could be integrated into a microfluidic paper-based analytical device (Davidson et al., 2021; Wang et al., 2021) to determine contamination events at the grower's end in a simple workflow.

Declaration of competing interest

M.S.V. has an interest in Krishi Inc., which is a startup that is interested in commercializing technologies developed here. This work was not funded by Krishi Inc.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2022.104173.

Research data for this article is available on Mendeley Data.

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