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Use of *Bacteroidales* Microbial Source Tracking To Monitor Fecal Contamination in Fresh Produce Production

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In recent decades, fresh and minimally processed produce items have been associated with an increasing proportion of food-borne illnesses. Most pathogens associated with fresh produce are enteric (fecal) in origin, and contamination can occur anywhere along the farm-to-fork chain. Microbial source tracking (MST) is a tool developed in the environmental microbiology field to identify and quantify the dominant source(s) of fecal contamination. This study investigated the utility of an MST method based on *Bacteroidales* 16S rRNA gene sequences as a means of identifying potential fecal contamination, and its source, in the fresh produce production environment. The method was applied to rinses of fresh produce, source and irrigation waters, and harvester hand rinses collected over the course of 1 year from nine farms (growing tomatoes, jalapeño peppers, and cantaloupe) in Northern Mexico. Of 174 samples, 39% were positive for a universal *Bacteroidales* marker (AllBac), including 66% of samples from cantaloupe farms (3.6 $\log_{10}$ genome equivalence copies [GEC]/100 ml), 31% of samples from tomato farms (1.7 $\log_{10}$ GEC/100 ml), and 18% of samples from jalapeño farms (1.5 $\log_{10}$ GEC/100 ml). Of 68 AllBac-positive samples, 46% were positive for one of three human-specific markers, and none were positive for a bovine-specific marker. There was no statistically significant correlation between *Bacteroidales* and generic *Escherichia coli* across all samples. This study provides evidence that *Bacteroidales* markers may serve as alternative indicators for fecal contamination in fresh produce production, allowing for determination of both general contamination and that derived from the human host.

The incidence of food-borne disease associated with fresh produce has been on the rise for the last few decades (1, 2). Particular groups of fresh produce items appear to cause the vast majority of produce-associated outbreaks, including leafy greens, tomatoes, melons, and fresh herbs (3). Most of the food-borne pathogens associated with fresh produce are enteric (fecal) in origin, and contamination with fecal matter can happen anywhere along the farm-to-fork chain. During fresh produce production, common points of entry include the use of improperly composted animal manures, the production waters used for irrigation, pesticide, or fungicide application, and wild or domestic animal encroachment (4). During harvest and packing/processing, the hands of ill pickers or packers can serve as a source of contamination, as can waters used for chilling, rinsing, decontamination, or icing, as well as poor facility sanitation (5).

Because testing for specific pathogens is expensive and time-consuming, and because pathogen contamination occurs infrequently and, when present, at low concentrations, the industry and regulators rely on enumeration of fecal indicator bacteria such as *Escherichia coli* (6). However, measuring *E. coli* contributes little to our knowledge of the source of contamination due to its presence in both human and animal feces, the possibility of non-fecal sources, and persistence and growth in the environment (7). To address this issue, the environmental microbiology field has investigated a variety of microbial source tracking (MST) techniques to identify and quantify the dominant source(s) of fecal contamination. Among the most promising are those targeting members of the order *Bacteroidales*, which are considered appropriate because they are limited to warm-blooded animals, are a dominant component of gut microflora, and are unable to proliferate in the environment (8). Molecular assays for *Bacteroidales* have been designed to target either highly conserved regions of the 16S gene, leading to the production of universal PCR assays, or variable regions representing specific hosts (9). To date, *Bacteroidales* assays have been utilized in a range of water environments (10–13); however, to our knowledge, there have been no efforts to use the *Bacteroidales* MST approach to monitor potential fecal contamination sources in the fresh produce production environment.

Being able to identify the source of fecal contamination during fresh produce production is critical for the development of relevant pathogen control strategies. As part of a larger field epidemiological study, the purpose of which was to identify, quantify, and prevent pathogen contamination along the fresh produce farm-to-fork chain, this study was done to ascertain the utility of *Bacteroidales* MST methods as a means of indicating fecal contamination and its source. The method was applied to a subsample of produce items and relevant environmental samples collected from various farms in Northern Mexico during the 2011 harvest season. Samples of irrigation waters, as well as rinses collected from fresh produce and the hands of farm workers, were analyzed for a universal *Bacteroidales* marker (AllBac), three human-specific markers (HF183, BFD, BVulg), and a bovine-specific marker (BoBac).

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MATERIALS AND METHODS

Study area. The study area comprised the Mexican states of Nuevo León and Coahuila on the United States-Mexico border. This region is a major agricultural area that regularly exports to the United States and has high production volumes of some crops that are considered at elevated risk for contamination with enteric pathogens: cantaloupes, tomatoes, and jalapeño peppers (14). Nine farms and three packing sheds participated in this study: four farms produced cantaloupes, four produced jalapeño peppers, and three produced tomatoes (two of which were also included as jalapeño farms). Institutional review board approval was received by the lead institution (Emory University) covering the duration of the study (approval number IRB00035460). Before beginning the study, an observational survey of the fields was conducted to assess potential sources of contamination. This survey, as well as existing knowledge of the geographic area, suggested that humans and cows were potentially significant sources of fecal contamination.

Sample collection. Samples were collected from May to December 2011. During each sampling event, 6 to 9 samples consisting of fresh produce rinses obtained before harvest, during harvest, and during packing or just prior to distribution, of hand rinses from the pickers/packers, and of water from the irrigation source and/or field irrigation lines were collected. Each type of sample had a different sample collection protocol, described below. All samples were placed on ice after collection, driven to the laboratory at the Universidad Autónoma de Nuevo León (UANL), and stored at 4°C until shipment, on ice, to North Carolina State University (NCSU). Samples were received at NCSU within 48 h of harvest for peppers and tomatoes and within 72 to 96 h for melons. All samples were held at 4°C until processing, which occurred within 24 to 72 h of receipt.

Irrigation water. Water samples were collected from the well used for irrigation water and from the irrigation lines on the field. Well water samples were collected by first disinfecting the pump with 200 ppm hypochlorite. The pump was allowed to run for 30 s before three 1.5-liter water samples were collected in Whirl-Pak bags (Nasco, Ft. Atkinson, WI). Irrigation water samples were collected as close as possible to the harvest row where the drip tape deposited irrigation water or from the center of the distribution system when this was not possible and were collected in the same manner as well water. All three of the well or in-field irrigation water samples were combined to create a composite sample of ~ 4.5 liters, which was then redivided into smaller subsamples for specific microbiological testing (microbiological indicators, bacterial pathogens, norovirus, or MST).

Produce rinses. Multiple produce items were combined to create a single produce rinse sample. Specifically, 2 melons, 18 tomatoes, or 14 jalapeño peppers were rinsed to comprise one sample. For preparation of the rinses, half of each batch of produce was placed in a Whirl-Pak bag containing 500 ml 0.15% sterile peptone water (PW), shaken for 30 s, massaged for 30 s, and shaken again for 30 s. The first half of the produce batch was removed and replaced with the second half, and the process was repeated. This process was done three times with three different produce batches, and the rinses were combined to create a composite sample of 1,500 ml. The composite sample was divided into smaller subsamples for microbiological testing. Sample collection was done for produce collected at each sampling point (preharvest, harvest, distribution, and packing).

Hand rinses. Before sample collection, researchers obtained written consent from farm managers and oral consent from farm workers to collect a hand rinse sample. The worker placed his or her hand in a Whirl-Pak bag containing 750 ml PW. The worker was asked to shake the hand for 30 s, and then the hand was massaged for an additional 30 s. The first hand was removed from the buffer, the second hand was placed in the same bag, and the process was repeated. Three individual hand rinse samples (representing the hands of three pickers or packers, 750 ml each) were combined to create a composite sample of 2,250 ml that was divided into smaller subsamples for specific microbiological testing.

***Escherichia coli* screening.** Samples were analyzed for generic *E. coli* at UANL using a membrane filtration method. For each sample, a range of volumes (effective original sample volume, 1 μl to 250 ml) were vacuum filtered through a 47-mm, 0.45-μm-pore-size S-Pack filter (Millipore, Billerica, MA). After filtration, the filter was removed and placed onto a Rapid'*E. coli* 2 agar plate (Bio-Rad, Hercules, CA), which was inverted and incubated at 44°C for 24 h.

The average concentration (number of CFU per volume filtered) of *E. coli* in each sample was determined and standardized to CFU per 100 ml. For statistical analyses, samples below the assay lower limit of detection were reported as 0.5 CFU per greatest volume filtered, and samples above the upper limit of detection were reported as 1 + 250 CFU (upper limit of quantification) per smallest volume tested. The data on the prevalence and concentration of *E. coli* for these samples are presented by N. Heredia, F. E. Bartz, L.-A. Jaykus, J. S. Leon, and S. Garcia (unpublished data).

Bacterial concentration and DNA extraction. For irrigation water, samples of 250 ml were filtered through a 47-mm, 0.45-μm-pore-size Millipore S filter. The filter was stored at −20°C until DNA extraction. Hand rinse and produce rinse samples were processed by centrifuging two 50-ml aliquots at 8,000 × *g* for 10 min and then sequentially filtering the supernatants through a 47-mm, 0.45-μm-pore-size Millipore S filter. The pellet was resuspended in 300 μl of DNase-free water and stored in the same 50-ml tube with the filter at −20°C until DNA extraction.

DNA extractions were performed on bacterial concentrates using the FastSpin kit for soil (MP Bio, Solon, OH) with a minor modification. Briefly, the filter was removed from the 50-ml tube, cut in half, rolled, and placed into the lysing matrix E tube along with 300 μl of the resuspended soil pellet. DNA concentrates were stored at −80°C before PCR analysis.

Real-time PCR for *Bacteroidales*. Detection of the universal, conserved 16S rRNA gene marker for *Bacteroidales* was performed using the AllBac primers and probe (Integrated DNA Technologies, Coralville, IA; Table 1). The 25 μl of quantitative PCR (qPCR) master mix contained 1× PCR buffer (Life Technologies, Grand Island, NY), 200 nM deoxynucleoside triphosphate (dNTP) mix, 1.5 mM $MgCl_2$, 200 nM primers, 80 nM probe, 200 nM internal amplification control (IAC) probe, 1.0 U Platinum *Taq* polymerase, 2.5 μl experimental DNA, and DNase-free water to bring to volume. qPCR cycling conditions were 95°C for 2 min, followed by 45 cycles of 95°C for 15 s, 53°C for 30 s, and 72°C for 30 s, performed on a Cepheid SmartCycler (Sunnyvale, CA).

Samples positive for the AllBac marker were also assayed for bovine- and human-specific 16S rRNA gene *Bacteroidales* markers. Detection of a bovine-specific marker was performed using the BoBac primers and probe (Table 1) using the same master mix concentrations and qPCR cycling conditions as described for AllBac, except with a 57°C annealing step. Multiple human-specific qPCR assays were used, including the HF183, BFD, and BVulg assays (Table 1). These assays were carried out using the same master mix concentrations and qPCR cycling conditions as the AllBac assay.

An IAC was synthesized following the protocol of Hoorfar et al. (15) for each qPCR assay. The competitive IAC was amplified with the same primers as the target but was identified using a fluorescent probe complementary to the IAC (Table 1). When amplified in the presence of the target, IAC threshold cycle (C_T) values of 29 to 31 were considered supportive of adequate PCR amplification (16). If the IAC failed to be amplified, or was amplified at C_T values above 31, the sample was diluted 10-fold, up to 1,000-fold, and reamplified. If the IAC failed to amplify at the highest sample dilution, the sample was designated "uninterpretable."

Quantification of AllBac and BFD *Bacteroidales* assays. Plasmid DNA standards for the AllBac and BFD assays were created following the protocol of Silkie and Nelson (17). To create the standard curves, 10-fold serial dilutions of the plasmid DNA were amplified in triplicate 6 times over several days. Both standard curves showed a high degree of linearity, with a coefficient of determination (R^2) of 0.995 for AllBac and 0.969 for BFD. The dynamic range of the qPCR assays was 0.6 to 8.6 $\log_{10}$ genome equivalent copies (GEC)/μl for AllBac and 1.1 to 6.8 $\log_{10}$ GEC/μl for BFD. Using these standard curves, the assay lower limit of quantification (LLOQ) was 2.3 $\log_{10}$ GEC per sample for the AllBac assay and 2.9 $\log_{10}$

TABLE 1 Primers and probes used in this study

Marker	Assay	Oligonucleotide name	5′→3′ sequence[a]	Reference or source
General fecal contamination	AllBac	AllBac296f	GAGAGGAAGGTCCCCCAC	25
	AllBac	AllBac412r	CGCTACTTGGCTGGTTCAG	
	AllBac	AllBac375Bhqr	(FAM)-CCATTGACCAATATTCCTCACTGCTGCCT-(BHQ-1)	
Bovine	BoBac	BoBac367f	GAAG(G/A)CTGAACCAGCCAAGTA	25
	BoBac	BoBac467r	GCTTATTCATACGGTACATACAAG	
	BoBac	BoBac402Bhqr	(FAM)-TGAAGGATGAAGGTTCTATGGATTGTAAACTT-(BHQ-1)	
Human	HF183	HF183	ATCATGAGTTCACATGTCCG	16
	HF183	BFDRev	CGTAGGAGTTTGGACCGTGT	
	HF183	BFDFAM	(FAM)-CTGAGAGGAAGGTCCCCCACATTGGA-(BHQ-1)	
	BFD	BFDFor	CGTTCCATTAGGCAGTTGGT	26
	BFD	BFDRev	CGTAGGAGTTTGGACCGTGT	
	BFD	BFDFAM	(FAM)-CTGAGAGGAAGGTCCCCCACATTGGA-(BHQ-1)	
	Bvulg	BVulgF1	CATCATGAGTCCRCATGTTCA	16
	Bvulg	BFDRev	CGTAGGAGTTTGGACCGTGT	
	Bvulg	BFDFAM	(FAM)-CTGAGAGGAAGGTCCCCCACATTGGA-(BHQ-1)	
IAC		IACProbe	(TET)-ATCTCAGTTCGGTGTAGGTCGTTCGCTCC-(BHQ-1)	This study

[a] FAM, 6-carboxyfluorescein; BHQ-1, black hole quencher 1; TET, 6-carboxy-2′,4,7,7′-tetrachlorofluorescein succinimidyl ester.

GEC per sample for the BFD assay. Results were reported as GEC per 100-ml sample processed. For statistical analyses, samples below the LLOQ were reported as 1.2 $\log_{10}$ GEC per 100 ml for the AllBac assay and 1.5 $\log_{10}$ GEC per 100 ml for the BFD assay.

Statistical analyses. Statistical analyses were performed using the JMP version 10 software package (SAS Institute, Inc., Cary, NC). The concentrations of *Bacteroidales* markers and *E. coli* within samples were $\log_{10}$ transformed before statistical analyses. Pearson chi-square analyses were performed to compare the presence of *Bacteroidales* markers between produce types and between sample types and to compare the presence of *Bacteroidales* markers to the presence of *E. coli*. A Kruskal-Wallis one-way analysis of variance (ANOVA), followed by a Tukey's multiple comparison test, was performed to compare *Bacteroidales* marker concentrations between sample types and between produce types. Spearman rank analyses were utilized to determine correlations between *Bacteroidales* marker concentrations in different sample types and to determine correlations between *Bacteroidales* and *E. coli* concentrations within samples. A logistic regression was performed to determine correlations between the presence of *Bacteroidales* markers and the concentration of *E. coli* in samples.

RESULTS

A total of 174 samples, including produce rinses, hand rinses, and irrigation waters, were processed from 9 farms and 3 packing sheds sampled during the harvest season of 2011.

Prevalence of AllBac marker. Thirty-seven percent (65/174) of samples required dilution of DNA extracts to obtain AllBac qPCR results that were considered interpretable (Table 2). Of these, 83% (54/65) were successfully amplified after dilution. The remaining 17% (11/65) of inhibited samples required dilution in excess of 10,000-fold and were reported as "uninterpretable."

The universal AllBac marker was identified in 39% (68/174) of the samples. Based on sample type, 47% (40/85) of produce rinses, 34% (17/50) of hand rinses, and 28% (11/39) of irrigation water samples were positive for *Bacteroidales* (Table 2). There was a significant difference in the prevalence of AllBac by sample type ($P < 0.03$). Fifty-four percent (37/68) of AllBac-positive samples were from cantaloupe farms. In fact, 66% (37/56) of samples from cantaloupe farms were positive for AllBac, compared to 31% (24/78) of samples from tomato farms and 18% (7/40) of samples from jalapeño farms (Table 2). There was a statistically significant difference in the prevalence of AllBac by produce type ($P < 0.0001$).

Concentration of AllBac marker. The geometric mean concentration of the AllBac marker for all samples was 2.1 $\log_{10}$ GEC per 100 ml, ranging from 1.2 to 10.1 $\log_{10}$ GEC per 100 ml (Fig. 1). There was no significant difference in AllBac marker concentration by sample type ($P > 0.05$), but a significant difference was observed in AllBac concentration by produce type ($P < 0.0001$). A *post hoc* Tukey's multiple comparison test indicated that AllBac concentrations in samples from melon farms (geometric mean = 3.6 $\log_{10}$ GEC/100 ml; standard deviation [SD] = 2.8) were significantly different from samples from tomato farms (geometric

TABLE 2 Percent of samples positive, negative, and uninterpretable for the AllBac *Bacteroidales* marker

Produce type	Sample type	% (no.) of samples			
		Positive	Negative	Uninterpretable	Total
Jalapeño pepper	Produce rinse	24 (4)	76 (13)	0 (0)	100 (17)
	Hand rinse	14 (2)	86 (12)	0 (0)	100 (14)
	Irrigation water	11 (1)	89 (8)	0 (0)	100 (9)
	All sample types	18 (7)	83 (33)	0 (0)	100 (40)
Cantaloupe	Produce rinse	74 (20)	26 (7)	0 (0)	100 (27)
	Hand rinse	47 (8)	47 (8)	6 (1)	100 (17)
	Irrigation water	75 (9)	25 (3)	0 (0)	100 (12)
	All sample types	66 (37)	32 (18)	2 (1)	100 (56)
Tomato	Produce rinse	39 (16)	51 (21)	10 (4)	100 (41)
	Hand rinse	37 (7)	37 (7)	26 (5)	100 (19)
	Irrigation water	6 (1)	89 (16)	6 (1)	100 (18)
	All sample types	31 (24)	56 (44)	13 (10)	100 (78)
All produce	Produce rinse	47 (40)	48 (41)	5 (4)	100 (85)
	Hand rinse	34 (17)	54 (27)	12 (6)	100 (50)
	Irrigation water	28 (11)	69 (27)	3 (1)	100 (39)
	All sample types	39 (68)	55 (95)	6 (11)	100 (174)

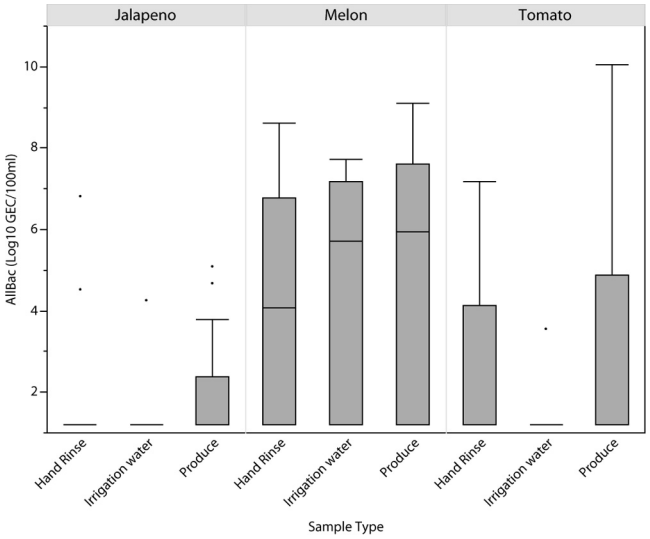

FIG 1 Box and whisker plot depicting the concentration (in $\log_{10}$ GEC/100 ml) of the AllBac marker on hands, in irrigation water (source and in-field), and on produce samples, sorted by produce type. The lower boundary of the box indicates the 25th percentile, the line within the box represents the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above the box indicate the 90th percentiles. Points represent outliers. A statistically significant difference was detected in AllBac marker concentration by produce type ($P < 0.0001$) but not by sample type ($P > 0.05$).

mean = 1.7 $\log_{10}$ GEC/100 ml; SD = 2.2) or samples from jalapeño farms (geometric mean = 1.5 $\log_{10}$ GEC/100 ml; SD = 1.4).

A Spearman rank analysis indicated that there was a positive correlation between AllBac concentrations for both produce and hands (Rho = 0.44; $P = 0.02$) and for produce and irrigation water (Rho = 0.59; $P = 0.001$).

Source-specific MST assays. No samples that tested positive for the AllBac marker were positive for the BoBac marker. Of 68 samples positive for the AllBac marker, 46% (31/68) were positive by one of three human-specific *Bacteroidales* assays (HF183, BFD, or BVulg; Table 3). The majority (75%) of samples positive for a human marker were sampled from cantaloupe farms. A Pearson chi-square analysis revealed that there was a statistically significant difference in the prevalence of human source marker by sample type ($P < 0.0001$) but not by produce type ($P > 0.05$).

The majority (81%; 25/31) of human-specific-positive samples were detected using the BFD assay; only four samples were positive using the HF183 assay and two using the BVulg assay. Given the high proportion of samples positive by the BFD marker alone, concentrations of human-specific *Bacteroidales* DNA in samples were determined based on a standard curve corresponding to that marker. The geometric mean concentration of the BFD marker in those samples was 2.4 $\log_{10}$ GEC per 100 ml, ranging from 1.5 to 9.4 $\log_{10}$ GEC per 100 ml (Fig. 2). There was no statistically significant difference in the concentrations of the BFD marker between produce types ($P > 0.05$) or between sample types ($P > 0.05$). A Spearman rank analysis indicated that a correlation existed between BFD concentrations for produce and hands (Rho = 0.39; $P = 0.04$) but not for produce and irrigation water ($P > 0.05$).

Association of *Bacteroidales* markers with *E. coli*. Multiple statistical analyses were performed to compare the presence and concentration of *Bacteroidales* markers with *E. coli* in samples, including Pearson chi-square analyses, logistic regressions, and Spearman rank correlations. No statistically significant relationships were detected between *E. coli* and the general or human-specific *Bacteroidales* marker using any statistical test ($P > 0.05$).

TABLE 3 Percent of AllBac-positive samples also positive for human-source marker (BFD, HF183, or BVulg)

Produce type	Sample type	% (no.) positive
Jalapeño pepper	Produce rinse	25 (1)
	Hand rinse	ND[a]
	Irrigation water	ND
	All sample types	14 (1)
Cantaloupe	Produce rinse	58 (11)
	Hand rinse	67 (6)
	Irrigation water	67 (6)
	All sample types	62 (23)
Tomato	Produce rinse	19 (3)
	Hand rinse	57 (4)
	Irrigation water	ND
	All sample types	29 (7)
All produce	Produce rinse	38 (15)
	Hand rinse	59 (10)
	Irrigation water	55 (6)
	All sample types	46 (31)

[a] ND, not detected.

DISCUSSION

This study investigated the utility of *Bacteroidales* MST methods as a means of identifying potential fecal contamination, and its source, in the fresh produce production environment. Thirty-

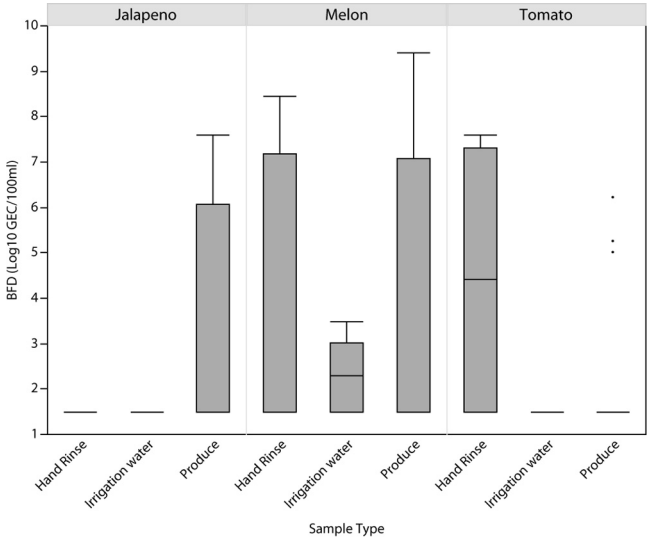

FIG 2 Box and whisker plot depicting the concentration (in $\log_{10}$ GEC/100 ml) of the BFD marker on hands, in irrigation water, and on produce samples, sorted by produce type. The lower boundary of the box indicates the 25th percentile, the line within the box represents the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above the box indicate the 90th percentiles. Points represent outliers. There was no statistically significant difference in the concentrations of the BFD marker between produce types or between sample types ($P > 0.05$).

nine percent of samples were positive for a universal *Bacteroidales* marker (AllBac), and, of these, 46% were positive for one of three human-specific markers (BFD, HF183, BVulg).

Over half of samples that tested positive for *Bacteroidales* were obtained from farms producing cantaloupe, while only 10% were from jalapeño farms. Samples from melon farms also had the highest mean concentrations of the AllBac marker and the human-source BFD marker. These data are indicative of a higher degree of fecal contamination in the cantaloupe production environment relative to the other two products studied. Melons have been associated with many food-borne disease outbreaks, and cantaloupes are generally recognized as a produce item at elevated risk for pathogen contamination (3, 18). Irrigation water may have been a source of preharvest contamination to melons in this study, given that the concentration of AllBac on produce was positively correlated with concentrations in irrigation water. *Bacteroidales* contamination of melons may have been related to hand harvesting as well, as concentrations of the general and human markers (AllBac and BFD) on produce were positively correlated to concentrations on hands. Although the application of manure is a possible source of fecal contamination, none of the samples in this study were positive for the bovine-specific *Bacteroidales* marker.

Only 31% of tomato chain samples were positive for *Bacteroidales*. Forty percent of tomato produce rinses were positive for AllBac, and like melons, the source of this contamination may be hands or irrigation waters. Taken together, these results suggest that irrigation water and human handling may serve as risk factors for fecal contamination. However, our sample sizes were quite small, so a much more comprehensive effort would be required to further refine interpretations regarding sources of *Bacteroidales* contamination in these production environments.

Consistent with results from others working in the water quality area (13, 19, 20), there was no correlation between the presence or concentration of *E. coli* and the MST markers. Based on these findings, a monitoring strategy that incorporates both *E. coli* and MST would provide the most comprehensive information, as the two measures provide for different types of interpretation. Specifically, *E. coli* can be useful for indicating potential quality, hygiene, and/or safety problems, whereas MST markers can be indicative of sources of fecal contamination. Knowledge of the contamination source can be useful in developing targeted intervention strategies. For example, when there is evidence of human-specific contamination, ensuring availability of appropriate toilets and/or hand washing facilities, and/or increasing scrutiny relative to hand-washing compliance, would be advised. In addition, knowledge of contamination sources may provide inferences into the types of pathogens potentially present, as certain pathogens (e.g., *Shigella* spp., noroviruses) are associated exclusively with human fecal contamination, while others tend to arise more from animal sources (e.g., *Campylobacter jejuni*, *Giardia* spp.). All samples in this study were evaluated for the presence of the *E. coli* O157:H7, *Salmonella*, and norovirus pathogens, and these results will be the subject of an upcoming manuscript (Heredia et al., unpublished).

One limitation with the use of *Bacteroidales* MST assays in fresh produce production samples was the presence of matrix-associated inhibitory compounds. To minimize the effect of inhibition, we relied on sample dilution (21). However, sample inhibition and subsequent dilution may have had a major impact on our results, as more than one-third of all samples required dilution to achieve interpretable results, and many samples required dilution up to 1:1,000-fold. Dilution to these levels can result in false-negative results, and it is likely that the prevalence of *Bacteroidales* in our sample set was higher than reported. For future studies utilizing *Bacteroidales* MST assays applied to similar samples, it may be beneficial to incorporate additional methods to reduce the effects of inhibition, although these can also result in significant loss of DNA (21). For now, we recommend that a negative *Bacteroidales* assay result after sample dilution be interpreted with caution and perhaps should be classified as "presumptively negative."

Another interesting aspect of the MST assays utilized in this study was the variability in the human source markers. The BFD marker was detected in 39% (25/64) of AllBac-positive samples, whereas the HF183 marker and BVulg markers were detected in <10% of the samples. Differences in marker prevalence may be due to variability in the analytical sensitivity and specificity of each marker or differences in the geographic prevalence of each marker in the sampling region (22–24). A suite of MST markers such as used in this study may increase the likelihood of finding a specific source of contamination if present.

In conclusion, this work provides preliminary evidence that *Bacteroidales* MST methods have utility in indicating fecal contamination source in the fresh produce production environment. MST markers aided in hypothesis generation relative to potential risk factors for fecal contamination. If used more widely, these MST markers could also help in the identification of targeted intervention strategies for controlling different types of fecal contamination in production environments. While the assays are not perfect (e.g., issues regarding PCR inhibition and associated data interpretation), the method appears to have utility and merits further study as we continue to search for useful indicators of fecal contamination to ensure the safety of our food supply.

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