

## ABSTRACT

RAVALIYA, KRUTI DILIP. Evaluation of a Novel Microbial Source Tracking Method to Identify Fecal Contamination in the Fresh Produce Production Environment. (Under the direction of Lee-Ann Jaykus, Jay Levine and Clyde Sorenson.)

Current microbiological indicators of fecal contamination do not correlate well with the presence or absence of pathogens. Alternatives, such as library-dependent and independent methods are becoming more widely available. Library independent methods include tracking of molecular markers from organisms such as *Bacteroidales*. Characterization studies have been done within different hosts, allowing researchers to develop assays for targeting *Bacteroidales* markers in specific hosts. These studies evaluated fecal contamination in water systems; however, none have been applied to fresh produce systems. In developing assays for use in environmental samples, precautions must be taken, including identifying the appropriate primers and probe, sample preparation, assessing the efficiency of the PCR due to potential matrix-associated inhibitors, proper use of controls, and interpretation of results. The purpose of this study was to adapt existing methods for typing *Bacteroidales* species based on 16SrDNA for use in fresh produce and environmental samples, and to evaluate if they might be relevant microbiological indicators of fecal contamination. Initially, the assay was optimized for detection of total *Bacteroidales* with respect to pre-analytical sample processing methods, DNA extraction and PCR primer choice. A combination of filtration and centrifugation were chosen for sample processing, followed by DNA extraction using the MP Bio FastSpin for Soil Kit and amplification using the AllBac primer. A

homologous internal amplification control (IAC) was developed for inclusion in PCR to identify those samples demonstrating inhibition due to a variety of PCR inhibitors. The sum total of these methods were applied in a pilot study in which samples (originating from Northern Mexico) of fresh produce, harvester hand rinsates, and source and irrigation water were processed for detection of evidence of fecal contamination using the *Bacteroidales* assay and generic *E. coli*. A total of 174 samples were processed using these methods, and the general fecal contamination marker was identified in about 36%. The marker was not identified in 55% percent of the samples. By environmental sample type, the marker was detected in 30% of hand rinse samples, 45% of produce rinses, and 28% of irrigation water samples. In evaluation of marker detection based on produce type, 59% of cantaloupe samples, 31% tomato samples, and 17% pepper samples contained the AllBac general fecal marker. Inhibitors to PCR may have been present, and were identified through the use of the AllBac IAC; these indicated which samples should be diluted up to 1,000-fold, and anything further was deemed “uninterpretable.” The uninterpretable samples represented 8% of the sample pool. Average log genome equivalence copies (GEC) for all samples was 5.5. Average log GEC for hand rinses, irrigation waters, and produce rinses was 5.3, 6.0 and 6.1, respectively. Based on produce type, average log GEC for peppers, melons, and tomatoes was 4.6, 6.4, and 5.4 respectively. Average cycle threshold (Ct) value for all positive samples was 30.4. The average Ct values for hand rinse, irrigation water, and produce rinse was 31.8, 28.9, and 30.3, respectively. For produce rinses, the average Ct value for peppers,

melons and tomatoes was 31.9, 29.2, and 31.5, respectively. There was poor correlation between the presence of *E. coli* and *Bacteroidales* in hand rinse samples, and produce rinse samples, but strong correlation was identified in irrigation water samples ( $R^2 = 0.99$ ). These results suggest that of the produce types, melon is most likely to contain fecal matter, and that produce will be more likely to contain fecal matter over other types of environmental samples (water and hand rinses). Environmental samples can introduce inhibitors and should be addressed, so as not to cause false reporting of results. This data will also promote implement better contamination prevention strategies on the farm and during harvest.

Evaluation of a Novel Microbial Source Tracking Method to Identify Fecal  
Contamination in the Fresh Produce Production Environment

by  
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## **DEDICATION**

This work is dedicated to my mom, Ranjan, and my sister, Preeti. Returning to school was a big step, and they have always been so supportive of me.

## **BIOGRAPHY**

Kruti Ravaliya has attended North Carolina State University since August, 2010, and finished her MS in January, 2013. She earned a BS from the University of Massachusetts in 2007, in both Food Science and Spanish. Upon completion of her degree, she accepted a food product development scientist position with the International Food Network. While at the International Food Network, she worked on a variety of platforms, most notably an ultra-high temperature pasteurized dairy beverage. After developing the formulation from bench to production, this product was launched in specific markets. She decided then that she would return to school, to pursue an advanced degree in Food Science, with a focus in food safety. The title of her thesis, “Evaluation of Novel Microbial Source Tracking Method for the Identification of Fecal Contamination in the Fresh Produce Production Environment” was part of a larger collaboration on a USDA-funded grant, between the University of Nuevo Leon, Emory University and North Carolina State University. Upon completion of her degree at North Carolina State University, Kruti has accepted a fellowship position through Oak Ridge Institute for Science and Education (ORISE) at the Food and Drug Administration (FDA), in the Office of Food Safety/Produce Safety. Professional affiliations include the International Associate of Food Protection, the Institute of Food Technologists, the American Society for Microbiology, and the Society for Applied Microbiology. In addition to interests in Food Science, she is an avid runner and yoga enthusiast.

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## INTRODUCTION

Each year in the United States, 9.4 million cases of foodborne illness are reportedly caused by known disease agents (Scallan et al, 2011a). These agents include the following: *Campylobacter* spp., *Cryptosporidium* spp., *Cyclospora catenansensis*, Shiga-toxin-producing *Escherichia coli* (STEC) O157:H7, STEC non-O157:H7, *Listeria monocytogenes*, nontyphoidal *Salmonella* spp., *Salmonella enterica* serotype Typhi, *Shigella* spp. *Yersinia enterocolitica*, *Brucella* spp., *Clostridium botulinum*, *Trichinella* spp., hepatitis A virus, *Giardia intestinalis*, *Vibrio cholerae*, *V. vulnificus*, *V. parahemolyticus*, and other *Vibrio* spp., *Mycobacterium bovis*, *Bacillus cereus*, *Clostridium perfringens*, enterotoxigenic *E. coli* (EPEC), *Staphylococcus aureus*, and *Streptococcus* spp. Of the 9.4 million cases of foodborne illness associated with known pathogens, the majority of them are attributed to norovirus, non-typhoidal *Salmonella* spp., *C. perfringens*, and *Campylobacter* infections. These illnesses result in roughly 56,000 hospitalizations annually. Although most food-related illnesses are not life threatening, non-typhoidal *Salmonella* spp., *T. gondii*, and *Listeria monocytogenes* infections contribute to more than 1,400 deaths each year.

Unspecified agents also contribute to this burden of gastroenteritis. These agents include organisms or compounds (chemicals) that are known to cause illness, for which there may be insufficient data to estimate their contribution to the disease burden; agents that are not yet known to cause foodborne illness; and/or agents for which

pathogenicity/toxicity has not yet been proven. Scallan et al (2011b) estimated that approximately 38 million cases of food-related gastroenteritis are reported annually due to unspecified agents.

The economic ramifications of foodborne illness are significant, and are estimated to be about \$78 billion in 2010. This value takes into account economic loss due to sick leave, medical costs (e.g., medical treatment, hospital costs), and loss of quality of life (including pain and suffering). The three pathogens of highest economic impact, in terms of annual dollars, are non-typhoidal *Salmonella* spp., *Campylobacter* spp., and norovirus (Scharff, 2012).

Fresh produce is one of many commodity groups that can cause foodborne disease. However, concern about the safety of these products has increased dramatically in the past two decades. This is a function of both increases in fresh produce consumption, and relative increases in the frequency of fresh produce-associated foodborne illness. Clearly, consumption of fresh produce in the United States has increased; consumption in 1970 was at 574 pounds per capita, while the same figures for 1997 were 711 pounds per capita. This 24% increase can be attributed to factors such as the increasing recognition of fresh produce as a “healthy food” alternative; changes in nutritional guidelines and recommendations; and increased availability of a variety of fresh produce items year-round (Sivapalasingam et al., 2004). This increase in

consumption has been accompanied by an increase in fresh produce-associated foodborne disease. For example, from the years 1973 to 1987, fresh produce was responsible for 0.5-4.2% of all reported foodborne disease outbreaks; the number of outbreaks doubled between the years of 1973 and 1987, and then again between 1988 and 1991. This has been accompanied by an increase in the number of reported cases, which also doubled from 1973 to 1987, and then again from 1988 to 1991 (Johnston, et al., 2005). According to a study done by the Center for Science in the Public Interest (CSPI), fresh produce was responsible for 14.8% of foodborne illness outbreaks and 21% of all cases between the years 1990 and 2005. The annual cost of foodborne illness outbreaks attributed to fresh produce in the United States is estimated to be \$38.6 billion (Scharff, 2010).

Fresh produce can be divided into two categories: fresh fruits and vegetables, defined within the Code of Federal Regulations (CFR) and fresh-cut produce, which is defined according to the International Fresh-cut Produce Association. According to CFR 46.2, fresh fruits and fresh vegetables “*include all produce in fresh-form generally considered as perishable fruits and vegetables, whether or not packed in ice or held in common or cold storage (Agriculture CFR 46.2 1963).*” The International Fresh-cut Produce Association defines fresh-cut produce as “*fruits or vegetables that have been trimmed and/or peeled and/or cut into 100% usable product that is bagged or pre-packaged to offer consumers high nutrition, convenience and flavor while still maintaining freshness*”

([www.fresh-cuts.org](http://www.fresh-cuts.org)). Within these two categories, there are many different types of fresh produce; major groups having significant association with foodborne illness include leafy greens, berries, melons, and fresh herbs. Relatively speaking, Anderson et al. (2011) ranked the “riskiest” fresh produce commodity-pathogen pairs, finding leafy greens and *E. coli* O157:H7 to rank highest, followed by tomatoes/leafy greens/melons and *Salmonella enterica*, and crucifers/melons and *E. coli* O157:H7.

The majority of foodborne pathogens of concern in fresh produce are associated with fecal matter (human or animal) contamination, with the exception of perhaps *Listeria monocytogenes*, an environmentally ubiquitous foodborne pathogen. Fresh produce can come into contact with fecal material along the entire farm-to-fork chain. Contamination is dependent on the type of produce item and its growing, harvesting, packing/processing, and handling circumstances. Animal fecal material is a common source of contamination, potentially occurring through the use of improperly composted manures, or because of wild or domestic animal encroachment. Fecally-contaminated irrigation or wash/chill water can also be a vehicle for introducing pathogens (Bhagwat et al., 2004). As produce is harvested and processed, it comes into contact with human handlers, who can contribute to the contaminant load with human-specific pathogens, such as norovirus and *Shigella*, as well as other non-human-specific pathogens. Poor equipment sanitation is another potential source of contamination. Once pathogens contaminate fresh produce, mishandling, such as temperature abuse,

can result in pathogen proliferation (Sivapalasingam et al., 2004). Clearly, there are many ways that fresh produce can become contaminated with foodborne pathogens that eventually reach the consuming public.

#### EPIDEMIOLOGY OF FRESH PRODUCE ASSOCIATED FOODBORNE DISEASE

A wide variety of produce items have been implicated in recent outbreaks of foodborne disease, including cantaloupe, herbs, and leafy greens. Associated pathogens include norovirus, *E. coli* O157:H7, and *Salmonella* spp (Berger et al., 2010). Several of the more significant recent outbreaks are discussed in detail in the paragraphs below; others are summarized in Table 1.

**Table 1: Foodborne Pathogens and their Vehicles of Transmission**

Year	Commodity	Pathogen	# of illnesses	# of Deaths	# of States involved	Reference
2012 - ongoing	Mangoes	<i>Salmonella</i> Braenderup	121	0	15	cdc.gov
2012 - ongoing	Cantaloupes	<i>Salmonella</i> Typhimurium, <i>Salmonella</i> Newport	270	3	26	cdc.gov
2012	Raw Clover Sprouts	<i>Escherichia coli</i> O26	29	0	11	cdc.gov
2011	Romaine Lettuce	<i>Escherichia coli</i> O157:H7	60	0	10	cdc.gov
2011	Turkish Pine Nuts	<i>Salmonella</i> Enteritidis	43	0	5	cdc.gov
2011	Cantaloupes	<i>Listeria monocytogenes</i>	147	33	28	cdc.gov
2011	Papayas	<i>Salmonella</i> Agona	106	0	25	cdc.gov
2011	Alfalfa Sprouts, Spicy Sprouts	<i>Salmonella</i> Enteritidis	25	0	5	cdc.gov
2011	Cantaloupes	<i>Salmonella</i> Panama	20	0	10	cdc.gov
2011	Hazelnuts	<i>Escherichia coli</i> O157:H7	8	0	3	cdc.gov
2010	Alfalfa Sprouts	<i>Salmonella</i> serotype I 4,[5],	140	0	26	cdc.gov
2010	Mamey Fruit Pulp	<i>Salmonella</i> Typhii	9	0	2	cdc.gov
2010	Alfalfa Sprouts	<i>Salmonella</i> Newport	44	0	11	cdc.gov
2010	Romaine Lettuce	<i>Escherichia coli</i> O145	26	0	5	cdc.gov
2010	Alfalfa Sprouts	<i>Salmonella</i> Saintpaul	235	0	14	cdc.gov
2008	Raw Produce	<i>Salmonella</i> Saintpaul	1442	2	43	cdc.gov
2008	Cantaloupes	<i>Salmonella</i> Litchfield	51	0	16	cdc.gov

\*This strain of *Salmonella* occurs commonly in the United States, so some cases may not be associated with this outbreak

In the summer of 2011, over 3800 cases of pathogenic *E. coli* infection were reported in Europe (mostly Germany), of which 845 patients went on to develop hemolytic uremic syndrome (HUS), with 18 fatalities. A few unique features of this outbreak included the high rate of HUS (20% of patients), and the fact that nearly 70% of the patients who suffered from HUS were older (median age 42 years) and female (Frank, et al., 2011). Initially, the produce items in question were thought to be cucumbers, leaf lettuce and tomatoes, however further evaluation revealed sprouts as the vehicle of transmission. The causative agent was enteroaggregative Shiga-toxin—producing *E. coli* O104:H4, a unique strain not previously associated with this sort of outbreak. The strain was particularly interesting in that it did not possess the *eae* gene considered necessary for adhesion to the gastrointestinal lining. It seems that the evolution of this unique strain was associated with mutations and isolated events of insertion and deletion (Mellman et al., 2011). Particular lots of fenugreek seeds imported from Egypt were implicated as the likely transmission vehicle. How those seeds became contaminated is unknown, although it can be assumed that the seeds must have been contaminated with human or animal fecal matter. Generally, this sort of contamination occurs at the pre-harvest phase, however contamination at other points throughout the production chain could not be excluded (Gault, et al., 2011; EFSA Document).

In 2008, an outbreak of *Salmonella enterica* serotype Saintpaul occurred in association with fresh, raw produce. This outbreak, which spanned 43 states, was one of the largest *Salmonella* outbreaks ever identified, with 1500 individuals affected (Behravesh et al., 2011). In a complicated trace-back exercise, tomatoes that were imported from Mexico

were initially identified as the vehicle of transmission. However, upon further investigation, Serrano and Jalapeño peppers were implicated. The same *Salmonella* strain was traced to a farm in Nuevo Leon, Mexico, from which the peppers originated. Ultimately, *Salmonella* serovar Saintpaul was isolated from the produce as well as the associated irrigation water (Mody et al., 2011, <http://www.cdc.gov/salmonella/saintpaul/jalapeno/index.html>).

Another well-documented produce-associated outbreak occurred in the U.S. in 2006, due to *E. coli* O157:H7 contamination of bagged, fresh spinach. This outbreak spanned 26 states, but was traced back to one processing facility in California, associated with four Californian ranches. Over 200 cases and three deaths were reported. Ultimately, the pathogen was traced back to domesticated livestock, such as pigs and cattle, as well as wild pigs. Feral pigs were noted to be the most abundant wildlife on the ranches, with clear evidence of their intrusion into farm fields and fecal deposition in the crop fields (Jay, et al., 2007).

In 2011, an outbreak of *Listeria monocytogenes* associated with cantaloupe melons resulted in 146 cases of invasive listeriosis, spanning 28 states. Thirty deaths occurred due to this outbreak, and one miscarriage. Initially identified by PulseNet, environmental samples collected from a cold room associated with the cantaloupe packing shed were positive for *L. monocytogenes*. Interestingly, multiple serotypes of *L.*

*monocytogenes* were involved in this outbreak. Ultimately, improper sanitation in the cold storage area, lack of a chill step, and poor packing facility design were all identified as contributory factors for contamination (Laksanalamai et al., 2012).

Most recently, an outbreak of norovirus associated with consumption of frozen strawberries occurred in Germany. As of mid-October, 2012, roughly 11,000 people, mainly schoolchildren, were reported to have become ill, resulting in at least 30 hospitalizations. The allegedly contaminated, frozen strawberries were imported from China. Although the ultimate source of contamination has yet to be identified, there is speculation that a contaminated food handler played a role (Barfblog.com, accessed 03 November 2012; <http://www.foodproductiondaily.com/Quality-Safety/Outbreak-of-norovirus-in-Germany-over>, accessed 03 November 2012).

#### METHODS TO IDENTIFY FECAL CONTAMINATION

The vast majority of produce-associated pathogen contamination is associated with fecal contamination. Identification of pathogen contamination in fresh produce, and along the fresh produce farm-to-fork chain, is not easy. There are two options: identify the presence of a particular pathogen, or identify generic fecal contamination. The former approach is complicated: (i) pathogen prevalence is low (usually <1%) and pathogen concentrations, when present, are also low; (ii) most pathogens of concern have low infectious doses and their distribution along the farm-to-fork chain is usually

not uniform; (iii) sample sizes required to effectively capture low levels of pathogens are large; therefore, it is necessary to enrich pathogens prior to detection, which is both time-consuming and not amenable to in-field testing; and (iv) several important pathogens cannot be cultivated with ease. Furthermore, detection assays are pathogen-specific, meaning that a large number of assays would need to be applied to test for the presence of multiple pathogens (Field and Samadpour, 2007).

As an alternative, assays have been developed to identify fecal indicator organisms. A microbiological indicator is defined as *“a single or group of microorganisms or alternatively, a metabolic product whose presence in a food or the environment at a given level is indicative of a potential quality, hygiene, and/or safety problem”* (Jaykus and McClure, 2000). Since the ecological niche for these organisms is fecal matter, their presence on a food product or in water suggests exposure to some source of fecal pollution. While not diagnostic per se, because enteric pathogens are derived from fecal matter, the presence of the fecal indicators is suggestive of the potential for pathogen contamination. Hence, the presence of fecally-associated bacteria can be used as a proxy for the presence of enteric pathogens.

An indicator organism should fulfill a variety of criteria. Specifically, (i) the indicator and pathogen should originate from the same source (i.e., gastrointestinal tract); (ii) the indicator should not be able to grow outside the host environment; (iii) the indicator

should be more resistant to disinfection, and more persistent than the pathogen; (iv) indicator concentrations should be higher than pathogen concentrations; (v) indicator prevalence should be correlated with pathogen prevalence; (vi) indicator concentrations should correlate with the degree of fecal contamination; and (vii) indicators should be easy to detect and quantify (Griffin, et al, 2001).

The most commonly used fecal indicators are fecal coliforms, generic *E. coli*, and total *Enterococcus*. For example, the U.S. Environmental Protection Agency (EPA) recommends using *E. coli* as an indicator of fecal contamination in recreational waters, and members of the *Enterococcus* genus as indicators of fecal contamination in both freshwater and saltwater systems (Anderson, et al 2005). In general, water quality management methods rely more heavily on indicator testing rather than direct pathogen testing.

A number of different indicators are used in food systems, including the aerobic plate count (APC), total coliforms, total *Enterococcus*, fecal coliforms, and *E. coli*. From a regulatory perspective, these have been applied to foods such as pasteurized milk, molluscan shellfish, and meat and poultry. For example, the Grade A Pasteurized Milk Ordinance (PMO) provides standards for raw milk products destined for pasteurization, as well as Grade A pasteurized milk that is bulk shipped (Pasteurized Milk Ordinance, US Dept of Health and Human Services, Public Health Service, Food and Drug

Administration, 2009). This regulation was implemented in the early 20<sup>th</sup> century, and has evolved over time; currently, raw milk that will be pasteurized cannot have an APC greater than 100,000 colony forming units (cfu)/ml if derived from a single farm, or in the case of comingling milk from multiple producers, the APC cannot exceed 300,000 cfu/ml. Pasteurized milk and milk products may not have an APC greater than 20,000 cfu/ml and coliforms may not exceed 10 cfu/ml. These standards should provide sufficient information about hygiene and quality of the product, but do not provide any information about safety.

The majority of indicators used by the food industry are applied to determine the presence or absence of fecal contamination. In this regard, the major indicators used are fecal coliforms, generic *E. coli*, and total *Enterococcus*. Members of the *Enterococcus* genus are Gram-positive cocci that are salt tolerant, and grow well at 45°C. They are also quite resistant to environmental stresses and typical food processing practices, such as freezing, drying, thermal processing. Two species in particular (*E. faecalis* and *E. faecium*) are highly exclusive to and prevalent in fecal matter.

The most commonly used fecal indicators, however, are the fecal coliforms and *E. coli*. Historically speaking, the use of fecal coliforms dates back to 1915 (Kornacki and Johnson, 2001), and the establishment of the National Shellfish Sanitation Program (National Shellfish Sanitation Program, Guide for the Control of Molluscan Shellfish, Food and Drug Administration, 2009). This cooperative program was developed to

provide guidelines on the safe production of molluscan shellfish, with a focus on preventing enteric bacterial contamination. The program has established guidelines for the quality/safety of shellfish harvesting waters based on the levels of microbial indicators of fecal contamination. Standards dictate that to harvest shellfish, median fecal coliform levels of harvest waters must not exceed 70 total per sample, or 14 cfu per 100 ml, within appropriate confidence levels, and depending on the method of analysis. While implementation of these standards has resulted in the desired reduction in enteric bacterial disease associated with molluscan shellfish, there is no relationship between the levels of fecal coliforms and those of *Vibrio* spp., or the presence of human enteric viruses. Hence, fecal coliforms are not good indicators for these pathogens (BioMerieux, Jaykus and McClure, 2000).

More recently, the U.S. Department of Agriculture – Food Safety Inspection Service (FSIS) promulgated the 1996 Pathogen Reduction and HACCP Rule, in which guidelines and criteria were established to confirm the effectiveness of processing controls in animal slaughter practices, in preventing fecal contamination. Although it was generally concluded that no single set of tests could be used as a sole indicator of adequate process control, *E. coli* standards were put into place (Table 1.2). For slaughterhouses, different sampling frequencies were recommended based on factors such as annual production volume (small vs. large processors) and type of producer (cattle, swine, chickens, turkeys).

**Table 2 : *E coli* Standards for Pathogen Reduction in Meat and Poultry Processing Facilities**

Species	Lower Limit of Marginal Range	Upper Limit of Marginal Range	# of Samples Tested	Maximum # permitted in marginal range
Cattle	Negative	100 cfu/cm <sup>2</sup>	13	3
Swine	10 cfu/cm <sup>2</sup>	10,000c cfu/cm <sup>2</sup>	13	3
Chickens	100 cfu/ml	1000 cfu/ml	13	3
Turkeys	N/A	N/A	N/A	N/A

#### FECAL COLIFORMS AS MICROBIOLOGICAL INDICATORS

At the basic level, most fecal indicator organisms belong to the *Enterobacteriaceae* family. Members of this family are Gram-negative, asporogeneous rods that are fermenters of glucose and lactose. They are widely found throughout the environment, and are sensitive to methods that are commonly used to inactivate microorganisms in foods, including heating, freezing, drying, and reduced water activity. A variety of *Enterobacteriaceae* are associated with different environments, including soil, plants, and the gastrointestinal tract of warm-blooded animals. In food systems, the presence of *Enterobacteriaceae* in general indicates poor sanitation, gross under-processing, and/or cross-contamination, depending upon the application. As such, *Enterobacteriaceae* are general indicators of filth.

Within the *Enterobacteriaceae* family, the coliform subgroup consists of genera and species that ferment lactose (but not glucose) and produce gas and acid on Violet Red Bile Agar (VRBA) after 48 hours of incubation at 35°C. The presence of coliforms in raw

foods is expected as they are environmentally ubiquitous. However, when isolated from highly processed foods or sanitized and cleaned surfaces, they, too, provide evidence of post-processing contamination. In the U.S., coliforms are generally used as a hygiene indicators, however, in Europe, members of the entire *Enterobacteriaceae* family are used for this purpose (Mossell and Struijk, 1995) European standards are based on *Enterobacteriaceae* rather than coliforms for the following reasons: (i) inclusion of lactose and glucose fermenters (total *Enterobacteriaceae*) increases assay sensitivity; (ii) coliforms are poorly defined and testing methods are variable; and (iii) there are some lactose-negative pathogenic members of the *Enterobacteriaceae* family, meaning that the absence of lactose-fermenting coliforms does not indicate the absence of these pathogens.

Since many foodborne human pathogens are of animal or human fecal origin, indicators specific to fecal material are commonly used. Fecal coliforms are members of the *Enterobacteriaceae* family that ferment lactose and produce gas and acid at 45°C for 48 hours in EC broth. Since these organisms can tolerate higher temperatures, they are also referred to as “thermotolerant coliforms.” Initially, the higher temperature was thought to discriminate those coliforms that are fecally-associated, however this has since been disproven (Kornacki and Johnson, 2001; BioMerieux). The fecal coliform group consists of the *Klebsiella*, *Enterobacter*, *Citrobacter*, and *Escherichia* genera.

Members of this group are found in mammalian feces at levels as high as  $10^9$  cells per gram (Leclerc et al., 2001).

#### GENERIC *E. COLI* AS A MICROBIOLOGICAL INDICATOR

Despite the usefulness of the fecal coliform group, most believe that it is more useful to use *E. coli* as an indicator of fecal contamination. *E. coli* can be discriminated from the fecal coliforms based on the IMViC test panel. This stands for (i) Indole (the ability to produce indole by metabolism of tryptophan); (ii) Methyl Red (the ability to ferment glucose and produce a substantial amount of acid indicated by a pH dye, Methyl Red); (iii) Vogues-Proskauer reaction which involves the production of 2,3 butanediol and possibly acetoin from the metabolism of glucose; and (iv) the use of Citrate as the sole carbon source. Generic *E. coli* has been used historically as an indicator of fecal contamination, and is readily quantifiable. An EPA study done in the U.S., that attempted to correlate presence and levels of indicator organisms with enteric disease in East Coast swimmers, found the best correlations when using *Enterococcus* and *E. coli* (Ishii and Sandowsky, 2008). A variety of methods are available to detect and quantify *E. coli*.

According to the Bacterial Analytical Method, a Most Probable Number (MPN) method is the “gold standard.” In MPN, lauryl tryptose broth with 4-methylumbelliferyl-b-D-glucuronide (LST-MUG) is inoculated with serial dilutions of the sample in replicate (usually 3-5 dilutions replicated in 3-5 tubes each). After incubation at 35°C, each tube

is scored based on the production of gas and fluorescence. During the reaction, *E. coli* produce glucuronidase, which hydrolyzes MUG, to yield a fluorogenic compound. This can be detectable by exposure to UV-light at 366 nm. Tubes showing evidence of gas production and fluorescence are subcultured to Brilliant Green Lactose Broth (BGLB) and EC broths, which are used to discriminate between coliforms and fecal coliforms. Finally, to discriminate between fecal coliforms and *E. coli*, a loopful of samples showing evidence of growth on EC broth is plated to Levine's eosin-methylene blue agar (L-EMB). Positives on L-EMB agar will appear green, with a metallic sheen. Those that are positive from L-EMB agar can be transferred to Plate Count Agar slants (PCA) and confirmed for *E. coli*. A plating method is also available, which uses Violet Red Bile Agar (VRBA). This agar has a pH indicator, so as lactose is fermented to lactic acid, coliform-positive colonies will appear pink. However for specific, rapid detection of *E. coli*, 3M offers a concise Petrifilm® product, containing a gel of VRBA. Positive *E. coli* colonies on this product will appear blue because of a precipitated blue product due to the beta-glucuronidase reaction. Another rapid method includes the TEMPO® system by BioMerieux. This method miniaturizes the previously described MPN technique using LST-MUG, and can be done on one "card," where the media is distributed over three dilutions. The growth and subsequent fluorescence and gas production is measured and MPN is calculated using values from each of the dilutions. In this method, *E. coli* can be enumerated in roughly 24 hours.

There are a number of advantages to using *E. coli* as a fecal indicator, not the least of which is that it is very easy to detect and quantify, with both standardized and rapid assays widely available. Disadvantages include conflicting information regarding its natural prevalence and persistence in the environment. For example, it has been reported that *E. coli* can survive in environmental settings for long periods of time (Lopez-Torres, et al., 1987) and that it can be found in rain forest environments not having a clear source of fecal pollution (Lopez-Torres, et al., 1987). Others have demonstrated that *E. coli* was capable of reproducing under simulated environmental conditions, with growth rates affected by variables such as concentration of organic matter (Desmarais et al., 2002). However, competing studies done by Ishii et al. (2006), found that *E. coli* from warm-blooded mammals was unable to survive well outside of the host, particularly in soils. There may also be particular strains that are more adept at survival outside of the gastrointestinal tract. Other limitations to using *E. coli* include (i) that it is not well correlated to the presence of enteric pathogens (Horman et al., 2004; Winfield and Groisman, 2003); (ii) that it does not correlate any better than other indicators (fecal coliforms, total coliforms, *Clostridium perfringens*, F-specific coliphage – these will be discussed below) to the presence of pathogens (Wu et al., 2011); and (iii) that it is easily inactivated (Hurst et al., 2002). Another important limitation is that *E. coli* cannot be used to discriminate between hosts. In other words, the presence of *E. coli* suggests recent fecal contamination, but does not tell us anything about the source of that fecal pollution. This sort of information is critical to understanding how to

eliminate routes of contamination in water and food supplies. In short, without understanding how fecal contamination occurs, it is difficult to implement effective control strategies (Field et al., 2003; Scott et al., 2002; Simpson et al., 2002).

Because *E. coli* is not an ideal indicator, scientists have searched for alternative cultivable microorganisms that might have better correlation to pathogen presence, and/or the ability to allow the user to discriminate between types of fecal contamination. Two in particular show some degree of promise: *Clostridium perfringens* and F<sup>+</sup>-specific RNA coliphage (male-specific coliphage). These are described briefly below.

#### *CLOSTRIDIUM PERFRINGENS*

*Clostridium perfringens*, a Gram-positive, rod-shaped sporeforming bacteria, is environmentally ubiquitous and also found in the gastrointestinal tract of many animals. *Clostridium perfringens* has been touted as a potentially useful indicator largely because it appears to be quite resistant to environmental stresses. Nonetheless, its utility as an indicator is questionable. For example, Desmarais et al (2001) found *C. perfringens* in deep and remote soils in a Florida embankment that was not impacted by fecal contamination, suggesting that it was a natural environmental inhabitant, and not associated with recent fecal deposition. On the other hand, other studies suggest that it can be used in conjunction with a second indicator (such as Enterococci or F<sup>+</sup>

coliphage) to predict fecal contamination in recreational waters with relatively high predictive value (Viau, et al., 2011; Curiyel-Ayala et al., 2012).

#### MALE-SPECIFIC COLIPHAGE (F+-SPECIFIC RNA COLIPHAGE)

Coliphages are viruses that infect Gram-negative bacteria, specifically *E. coli*. Coliphages are generally categorized into two groups based on their attachment sites for initiation of infection: (i) those that attach to the host bacterial cell wall (called somatic coliphages); and (ii) those that attach to the host cell by means of a pilus of “male” host strains (called F-specific coliphages). Within the latter category, there are two classes. The first constitutes those F-specific coliphages belonging to the *Leviviridae* family. These are small, icosahedral, single stranded RNA coliphages, also called F+ RNA coliphages. The second class, called F+ DNA coliphages, belong to the *Inoviridae* family and are small, filamentous, single-stranded DNA viruses (Long et al., 2005).

Coliphages tend to be heartier than many bacterial indicators, with greater environmental persistence, making them better indicators for some applications, particularly for the presence of enteric viruses (Brion et al., 2002). Four genogroups of F+RNA coliphages have been identified through nucleic acid sequence analysis. Groups I and IV are more closely associated with *E. coli* populations in non-human animals; group II isolates are associated with human and porcine fecal *E. coli* populations; and group III isolates are most closely associated with human fecal matter (Griffin et al., 2001).

Because of this apparent host specificity, many investigators have sought to use F+ RNA coliphages as an alternative indicator, potentially to aid in the identification of fecal contamination by source; most of this work has been done in water and sewage systems. Long et al. (2005) showed that F-specific RNA coliphages were absent in many individual fecal specimens obtained from a variety of animals (e.g., cow, sheep, horse, pig), but they were found abundantly in wastewater influents, suggesting their utility for evaluating fecal contamination at the population level but not the individual level. Similar results were observed by Griffin et al. (2000), who were able to isolate F+-specific RNA coliphages from lagoon waters in a wildlife park, but not in individual animal fecal samples. Because of this, it may prove more useful to composite samples when looking for F+ coliphages.

One issue associated with the use of coliphages as indicators of fecal contamination is their levels and prevalence in contaminated samples. In cases of point source pollution (i.e., wastewater influents) and relatively “dirty” matrices (such as lagoons), the densities of F+ coliphages tend to be in the range of  $10^4$ - $10^5$  pfu/100 ml (Mara and Oragui, 1981; Havelaar and Hogeboom, 1984; Donnison and Ross, 1994; Long et al., 2005).

When used in environmental studies in developing countries (such as Bangladesh), researchers have confirmed a positive correlation between generic *E. coli*, F+ coliphage

and the presence of pathogenic *E. coli* (Ferguson et al., 2012). In regards to F+ coliphage in the produce production environment, a study done by Endley et al. (2003) found that coliphage were more likely to predict the presence of *Salmonella enterica* serovar. Typhimurium, than generic *E. coli*. F+ coliphages have also been isolated from food processing facilities, such as in broiler processing, where both the F+ RNA and F+ DNA coliphage was detected (Espinosa and Pillai, 2002). They have also been detected in a variety of produce items, including cilantro, parsley and carrots, and were found in higher concentrations than *E. coli* (Pillai, 2006). Clearly, further research remains to be done in order to understand the utility of male-specific coliphages as alternative indicators of fecal contamination. At the current time, they may be best used in conjunction with other validated methods, to potentially deliver more specific information. Indicator assay advantages and disadvantages are summarized in Table 3.

**Table 3: Advantages and Disadvantages of Common Indicators (Scott et. al., 2004)**

Assay	Advantages	Disadvantages
Fecal Coliform/Fecal Streptococcus ratio	Easy to perform; may be useful for recent contamination	Variable survival rates of fecal streptococci can alter ratio
<i>Bifidobacterium</i> sp.	Sorbitol fermenters may be human specific	Low numbers present in environment; variable survival rates; culture methods are not well-defined
<i>B. fragilis</i> HSP40 bacteriophage	Very human specific; easy to perform	Not present in sewage in some areas
F+ RNA bacteriophage	Groups are well-correlated with source; easy to perform	Unreliable in marine and tropical waters due to variable survival rates
Human enteric viruses	Human-specific; direct monitoring for pathogen circumvents need to use indicators	Low numbers in the environment; labor-intensive; more sensitive methods needed
MAR	Rapid; can be used to discriminate isolates from multiple animal sources	Requires a reference database; may be geographically-specific; isolates that show no antibiotic resistance cannot be typed
PFGE	Extremely sensitive to minute genetic differences	May be too sensitive to broadly discriminate for source tracking
BOX-PCR	Rapid; easy to perform	Reproducibility is a concern; reference database is required; may be geographically specific
Ribotyping	Highly-reproducible; some methods useful for classifying isoaltes from multiple sources	Labor-intensive; reference database required; may be geographically-specific; variations in methodology exist
<i>Bacteroides-Prevotella</i> Molecular Marker	Does not require culturing of organism; PCR method is rapid, easy to perform	Little is known about survival and distribution in water systems; currently not applicable to all animals
Caffeine	Useful for assessing impact from human sewage	Minute quantities in the environment make sensitivity an issue; requires expensive analyses
Fecal Sterols and/or Stanols	Some sterols/stanols have greater specificity for humans and/or animals	Present naturally in sediments; requires expensive analyses; low prevalence makes sensitivity an issue

## MICROBIAL SOURCE TRACKING

Microbial source tracking (MST) is an emerging tool used in environmental microbiology to identify and, in some cases, quantify the dominant **source(s)** of fecal contamination, usually in waters. A variety of MST techniques are being used to identify the origins of fecal contamination. The underlying principle is that one can identify unique targets and/or discernible traits, which can be attributed to different host sources. As such, MST is not used to predict the presence of pathogens as is the case with indicators, but rather to identify where fecal contamination has originated.

There are two general approaches to MST, those being “library dependent” and “library independent” methods. The former is culture-based and entails the application of phenotypic and/or genotypic methods to discriminate the sources of strains; consequently, it requires a large databank of well-characterized strains of fecal origin upon which the characteristics of unknown or test strains can be compared. Library independent methods are typically used to classify a sample based on whether it does or does not contain detectable fecal contamination from a particular source. This approach is appealing because it does not require an extensive library, can oftentimes be done in the absence of an isolate, and can usually be performed relatively quickly. For further information on various MST approaches, the interested reader is referred to Savichtcheva and Okabe (2006), Yan and Sadowsky (2007), Stoeckel and Harwood (2007), and Field and Samadpour (2007).

## LIBRARY-DEPENDENT MST METHODS

There are many library-dependent MST methods, and they overlap with methods that are commonly used in molecular epidemiology. These are described by other sources in much greater detail (Farber et al., 1996; Meays et al., 2004), and are summarized in Table 3.

Ribotyping and pulsed field gel electrophoresis (PFGE) are probably the best characterized (Meays et al., 2004). Ribotyping employs oligonucleotide probes with complementarity to ribosomal RNA genes (rDNA), which are both abundant and highly conserved across bacterial genera, and even families. Briefly, the procedure involves isolation of chromosomal DNA from a bacterial isolate, followed by restriction endonuclease digestion, and separation by gel electrophoresis. The DNA is then transferred to a membrane and probed with labeled rDNA sequences corresponding to *E. coli*. The resulting “fingerprint” can be compared to other fingerprints in an existing library to determine strain relatedness.

Pulsed field gel electrophoresis, the “gold-standard” for identification of microorganisms by the U.S. Centers for Disease Control and Prevention (CDC) in association with its PulseNet program, is another important library-dependent method. PFGE uses “infrequent cutter” restriction enzymes that, when applied to isolated bacterial DNA, result in large fragments that can only be resolved using a special form of electrophoresis based on the application of electrical field pulses, alternating in

orientation. Again, the resulting “fingerprint” is compared to others in an extensive library of PFGE patterns. Note that both ribotyping and PFGE are almost exclusively applied to foodborne pathogens, so to be useful, one would need to have a pathogen isolate (e.g., *Salmonella*, *E. coli* O157:H7). Even then, and with access to a comprehensive library, there is still little information about contamination *source*, at least with respect to particular sources of fecal contamination.

The source of contamination could potentially be addressed using *Escherichia coli* genotyping. *Escherichia coli*, a normal inhabitant of the gut flora of vertebrates displays host-specific differential rates of colonization. Decades of work establishing the population structure of the species reveals that most strains fall into one of four main phylogenetic groups designated A, B1, B2, and D (Hommais et al., 2005). In 2000, Clermont and colleagues developed a so-called universal method for *E. coli* identification, which can rapidly assign strains into one of these four phylogenetic groups. This triplex PCR assay targets a 279 bp fragment of the *chuA* gene; a 211 bp fragment of the *yjaA* gene; and a 152 bp fragment of TSPE4.C2, a noncoding region of the genome. The presence and absence of combinations of these three amplicons is used to designate phylogenetic groups (Clermon et al., 2000). A link between phylogeny and pathogenicity has been previously reported (Juareguy et al., 2008). Further, the phylogenetic groups A, B1, and D tend to predominate in gut microflora with notable geographically-associated differences in population structure (Duriez et al., 2001; Escobar-Paramo, 2004). There is even evidence for a human-specific *E. coli*

clone (Clermont et al., 2008). Finally, recent work by Higgins et al. (2007) confirmed differential *E. coli* genotype distribution on the basis of sample type; of particular note were differences for waters showing differential levels of fecal contamination. The utility of this method in MST remains unknown.

**Table 4: Common Library-dependent Methods Used for the Identification of Microbial Populations, Advantages and Disadvantages (Farber et al., 1996; Meays et al., 2004)**

Method	Description	Advantages	Disadvantages	References
Ribotyping	Southern blot of genomic DNA cut with restriction enzymes, probed with ribosomal dequences; discriminates species	Highly-reproducible; classify isolates from multiple sources	Complex; expensive; labor intensive; geographically specific; database required, variations in methodology	Samadpour and Chechowitz (1995), Farber (1996), Tynkkyen et al. (1999), Parveen et al. (1999), Farag et al. (2001), Hartel et al. (2002), and Samadpour (2002), Scott et al. (2003)
Pulse-field Gel Electrophoresis (PFGE)	DNA fingerprinting with rare-cutting restriction enzymes coupled with electrophoretic analysis; discriminates species	Extremely sensitive to minute genetic differences; highly reproducible	May be too sensitive to broadly discriminate source; long assay time, limited simultaneous processing; database required	Tynkkyen et al. (1999), Simmons et al. (2000), Hager (2001b), and King and Stansfield (2002)
Denaturing-gradient Gel Electrophoresis (DGGE)	Electrophoresis analysis of PCR product based on melting properties of the amplified DNA sequences; discriminates species	Works on Isolates	Not well-developed; technically demanding; limited simultaneous processing; not good on environmental isolates; database required	Farnleitner et al (2000), Buchan et al. (2001), and Chee-Sanford et al. (2001)
Repetitive DNA Sequences (Rep-PCR)	PCR used to amplify palindromic DNA sequences coupled with electrophoretic analysis; discriminates species	Simple and Rapid	Reproducibility is a concern; cell culture is required; a large database is required; variability increases as the database increases	Dombek et al. (2000), and Holloway (2001)
Length heterogeneity PCR(LH-PCR)	Separates PCR products for host specific genetic markers based on length	Does not require culturing or a database	Expensive equipment; technically demanding	Suzuki et al. (1998), and Bernhard and Field (2000a,b)
Terminal Restriction fragment length polymorphism analysis (T-RFLP)	Uses restriction enzymes coupled with PCR in which only fragments containing a fluorescent tag are detected	Does not require culturing or a database	Expensive equipment; technically demanding	Bernard and Field (2000a,b)
Host-specific 16S rDNA	Combine LH-PCR and T-RFLP methods on fecal anaerobes ( <i>Bacteroides</i> and <i>Bifidobacterium</i> ); discriminates human and cattle, other markers are being developed	Does not require culturing or a database; indicator of recent pollution	Only tested on human and cattle markers; limited simultaneous processing; expensive equipment; technically demanding; little known about survival of <i>Bacteroides</i> spp. in environment	Bernard and Field (2000a,b)

Library-dependent methods have notable advantages, including ease of standardization and hence, reproducibility. As they largely rely on the availability of a pure culture isolate, the same isolate can be available for additional follow-up work if so desired. However, these methods can be quite labor intensive and expensive; and, of course, they require access to well-characterized libraries before being able to be effectively used. In addition, because of a high degree of regional diversity in microbial populations, libraries corresponding to one geographic region may not be applicable to other regions. Further, the majority of library-dependent MST assays available are for humans and domesticated animals, but not wildlife or birds. (Field and Samadpour, 2007).

#### LIBRARY-INDEPENDENT MST METHODS

Library-independent (and hence, culture-independent) methods have many advantages over culture-dependent methods, not the least of which is the ability to sample the entire population, without culture bias. These assays are useful because they usually require significantly less analysis time, sometimes with time-to-results of only two to three hours. Since these assays do not require a library, the researcher can evaluate the entirety of the genetic material present for a variety of markers, making library independent methods more flexible.

One type of library-independent molecular-based method that is widely used is 16S rDNA sequence typing. Certain regions of 16S bacterial rDNA genes (also known as

markers) are highly conserved (allowing for detection of general fecal contamination), and other regions are highly variable (having a high degree of host-specificity) (Layton et al., 2006). In 16S rDNA sequence typing, PCR methods are developed for amplification of these genes. Universal assays are able to amplify the rDNA of a wide range of organisms, while other sets of PCR primers are used to target the variable regions of these genes; these regions tend to display the host specificity (Dick, et al., 2004).

Based on the observation that many of the fecal-associated anaerobic bacteria have host specificity, rDNA assays have been used for library independent MST. Since anaerobes constitute roughly 30% of all fecal bacteria, they are oftentimes present in higher concentrations than are the fecal coliforms or enterococci. In years past, fecal anaerobes have not been widely used as microbiological indicators due to difficulties associated with their cultivation. This situation has changed over the last two decades with the introduction of molecular techniques. Of particular interest are *Bacteroidales*, a fecal anaerobic group for which there is substantial evidence of host specificity (Bernhard and Field, 2000).

*Bacteroidales*, an order within the phylum *Bacteroidetes*, contains at least four different families: *Bacteroidaceae*, *Prevotellaceae*, *Porphyromonaceae*, and *Rikenellaceae* (Coyne and Comstock, 2008). *Bacteroides* is composed of obligate anaerobic non-sporeformers that make up about 30% of bacterial isolates characterized from fecal material of warm-

blooded animals (mammals), as well as some birds. The most populous *Bacteroides* species in the human colon are *B. vulgatus*, *B. distasonis*, and *B. thetaiotaomicron*, and the concentration of each is about  $10^{10}$  CFU per gram, dry weight feces. Many other strains are less populous, but still high in concentration ( $10^9$  per gram, dry weight feces). Many species within the *Bacteroidales* order have yet to be cultured. However, some have, and there are clearly clusters having high degrees of host specificity, for example, for cattle, swine, and humans (Mieszkin et al., 2010, Jenkins et al., 2009; Lamendella et al., 2009; Ju-Yong et al., 2009; Mieszkin et al., 2009).

There are some major features of *Bacteroidales* that make targeting this order for MST a promising library-independent method. For example, *Bacteriodes* appear to have a relatively high degree of environmental persistence, with reported survival for as long as 14 days at 4°C, even in the presence of predators. Even at warmer temperatures more representative of environmental waters (14°C), they remained detectable for 4-5 days (Kreader, 1998). In addition, as strict anaerobes, they are unable to proliferate outside of the host environment. It is generally recognized that they are a more suitable indicator for fecal contamination than are the fecal coliforms (Bernhard and Field, 2000; Bernhard and Field, 2000b; Layton et al., 2006; Kreader, 1995; Dick and Field, 2004). Perhaps most importantly, members of *Bacteroides* are highly-host specific, and appear to be present in the environment at only low levels outside of the host species (Field et al., 2003).

Culture-independent, library-independent methods do have some disadvantages, though. For instance, not all markers may be present in all individuals. Compositing samples from individuals, or sampling from sewage systems eases this limitation, however prevents distinction of host source. As is the case for culture-dependent methods, different localities will have different microbial populations, and even though libraries are not required for the culture-independent methods, the assays must be validated for the locale in which they are being used (Field and Samadpour, 2007). Aside from the detection limits of the methods themselves, other issues such as sampling volume arise. Library-independent, culture-independent methods rely on processing whole samples and collecting information on the profile of the genetic material within the entire sample, so sample volume is a critical consideration for representation purposes. Since library-independent methods almost always rely on the application of molecular approaches to “dirty” samples, preparing the sample for analysis such that matrix-associated inhibition is minimized can also be a challenge. Likewise, interpretation of results obtained from environmental samples may be difficult. Molecular-based tools require controls, and if the marker is present in low concentrations, the control can prevent detection of the marker (Hoorfar, et al., 2004). Finally, the correlation of these alternative markers to currently established fecal indicators is not well characterized (Field and Samadpour 2007).

## APPLICATIONS OF BACTEROIDALES MST METHODS TO ENVIRONMENTAL STUDIES

While members of *Bacteroidales* have been purported as potential indicators of fecal contamination for many years, the need to maintain anaerobic conditions to facilitate their proliferation was a deterrent to their widespread use. With the advent of molecular-based detection approaches, *Bacteroidales*-based assays have become more practical. Of particular interest are applications in which *Bacteroidales* host specificity has been capitalized upon for MST purposes. Such assays work by employing PCR to amplify variable DNA regions that show host specificity. Identifying these regions is the crux of how *Bacteroidales* microbial source tracking assays work. The regions that are conserved can be referred to as universal markers, or those found in all *Bacteroidales*. Regions that are variable can be host-specific, creating the possibility for assay development that can determine what kind of fecal matter is present by amplifying markers that are specific to certain animals. Previous work done by Bernard and Field (2000b) characterized host-specific regions that were specific to humans and ruminants on the 16S rRNA genome. The polymerase chain reaction (PCR) was used to selectively amplify these regions, and allow the researcher to directly detect the markers of choice.

A variety of studies have been undertaken to characterize the *Bacteroidales* populations in specific host species, including humans, cows, dogs, horses, Canada geese, chicken, elk, gulls, and cats. Much of the early work dealt with identifying differences between human fecal pollution and fecal pollution derived from other animals (Layton et al.,

2006; Bernhard and Field, 2000; Bernhard and Field, 2000b; Seurinck et al., 2004). Over the last 10 years, qPCR amplification assays have been developed for *Bacteroides* targets specific to humans (Layton et al., 2006; Kildaire et al., 2007; Seurinck et al., 2005; Bernhard and Field, 2000; Green et al., 2011; Seifring et al., 2008; Ju-Yong et al., 2009), pigs (Okabe et al., 2007; Mieszkin et al., 2009), cattle (Bernard and Field, 2000b; Kildaire et al., 2007; Layton et al., 2006; Shanks et al., 2006; Bernhard and Field, 2000; Reischer et al., 2006; Ju-Yong et al., 2009), dogs (Kildaire et al., 2007), horses (Simpson et al., 2004; Dick et al., 2004b; Layton et al., 2006), and wild bird fecal materials (Green et al., 2012; Dick et al., 2005). Most studies support this high degree of host specificity, but there are exceptions (Dick et al., 2004; Ju-yong et al., 2009). Assays have been applied to trace the origins of fecal contamination in different water systems, such as recreational water (Elmir et al., 2009; Stapleton et al., 2009; Green et al., 2010; Layton et al., 2006) and drinking water (Sokolova et al., 2012; Layton et al., 2006), as well as within estuarine, marine, and river waters (Walters and Field, 2008; Elmir et al., 2009; Dick et al., 2010; Green et al., 2010; Seurinck et al., 2005). Other studies have sought to use the *Bacteroidales* marker detection method as applied to raw sewage and wastewater treatment plant effluents (Shanks et al., 2010).

In developing new *Bacteroidales* assays and applying them to real-world samples, many issues must be considered, including (i) choosing the correct primers; (ii) identifying the appropriate methods to prepare the sample prior to assay; (iii) assessing the efficiency of qPCR with respect to matrix-associated inhibitors; (iv) inclusion of

appropriate controls (particularly for quantification purposes); and (v) overall interpretation of results. Each of these will be discussed in greater detail below.

#### PRIMER CHOICE

A variety of primers are available for general *Bacteroidales* as well as host-specific *Bacteroidales* typing. These are summarized in Table 1.5. The most commonly used primer sets are AllBac, BacUni, and Bac32F/Bac708R (general *Bacteroidales*), HF183 (Human-specific), BoBac (Bovine-specific), and PigBac1F/PigBac1R (Pig-specific). These assays are more widely used because they appear to have a higher success rate based on citation frequency in the literature relative to other assays. Major considerations when designing a qPCR assay to detect host-specific fecal material are marker specificity (broad reactivity for general fecal markers – markers within the highly conserved regions, and high specificity for species-specific markers – markers within the variable regions) and the need for the target to be relatively abundant and uniformly distributed throughout the system being evaluated. As perhaps expected, the host-specific genes are usually not as easily detected, nor as highly prevalent, as are the general *Bacteroidales* markers. Clearly, development of assays should take into account relative abundance of particular markers and fecal indicator organisms (Shanks et al., 2007).

**Table 5: Commonly Used Primers for Host-specific Assays**

Host-target	Assay	Function	Sequence (5' → 3')	Amplicon Size	Reference
General Bacteroidales	AllBac	Forward Primer (AllBac296f)	GAGAGGGAAGGTCCCCAC	106	Layton et al., 2006; Mieszkin et al., 2009; Ju-Yong et al., 2009; Mieszkin et al., 2009b
		Reverse Primer (AllBac412r)	CGCTACTTGGCTGGTTGAG		
		Probe (AllBac375Bhqr)	(FAM)-CCATTGACCAATATTCCTCACTGCTGCCT(BHQ-1)		
	16S Uni	Forward (16SUni-F)	TCCTACGGGAGGCAGCAGT	466	Nadkarni et al., 2002; Silkie and Nelson, 2009
		Reverse (16SUni-R)	GGACTACCAGGGTATCTAATCCTGTT		
		Probe (16SUni-P)	6FAM-CGTATTACCGCGCTTGCTGGCAC-TAMRA		
	BacUni	Forward (BacUni520f)	CTGTATCCGGATTATTGGGTTTA	170	Kildaire et al., 2007; Okabe et al., 2007; Silkie and Nelson, 2009; Shriewer et al., 2010
		Reverse (BacUni690r1)	CAATCGGAGTCTTCGTGATATCTA		
		Reverse (BacUni690r2)	AATCGGAGTCTTCGTGATATCTA		
		Probe (BacUni656p)	6-FAMTGGTGTAGCGGTGAAA-MGB		
	Bac	Forward (Bac32F)	AACGCTAGCTACAGGCTT	690	Bernard and Field, 2000; Lamendella et al., 2009; Mieszkin et al., 2009b; Reischer et al., 2006; Mieszkin et al., 2009
		Reverse (Bac708R)	CAATCGGAGTCTTCGTG		
		Forward (BacR_F)	GCGTATCCAACCTTCCCG		
		Reverse (BacR_R)	CATCCCATCCGTTACCG		
		Probe (BacR_P)	FAM-CTTCCGAAAGGAGATT-NFQ-MGB		
Btheta	Forward	CGTCCATTAGGCAGTTGGT	110	Blackwood and Noble, 2005; Shanks et al., 2007	
	Reverse	ACACGGTCCAAACTCCTACG			
	Probe	FAM-CTGAGAGGAAGGTCCCCACATTGGA-TAMRA			

Table 5, Continued

Pig-specific Bacteroidales and Prevotella	PigBac1	Forward (PigBac1F)	CGGGTTGTAAACTGCTTTTATGAAG		Okabe et al., 2007; Lamendella et al., 2009
		Reverse (PigBac1R)	CGCTCCCTTTAAACCCAATAAA		
	Pig-Bac2	Forward (qBac41F)	TACAGGCTTAACACATGCAAGTCG	145	Okabe et al., 2007; Mieszkin et al., 2009
		Reverse (qPS183R)	CTCATACGGTATTAATCCGCCTTTT		
	Pig-1-Bac	Forward (Pig-1-Bac32Fm)	AACGCTAGCTACAGGCTTAAC	129	Mieszkin et al., 2009
		Reverse (Pig-1-Bac108R)	CGGGCTATTCTGACTATGGG		
		Probe (Pig-1-Bac44)	FAM-ATCGAAGCTTGCTTTGATAGATGGCG(BHQ-1)		
	Pig-2-Bac	Forward (Pig-2-Bac41F)	GCATGAATTTAGCTTGCTAAATTTGAT	116	Mieszkin et al., 2009
		Reverse (Pig-2-Bac163Rm)	ACCTCATACGGTATTAATCCGC		
		Probe (Pig-2Bac113MGB)	(VIC)TCCACGGGATAGCC(NFQ-MGB)		
Cow-specific Bacteroidales	YCF	Forward (YCF79F)	GAGTGCTTGCACTTCTGTCCG		Ju-Yong et al., 2009
		Reverse (YCF168R)	GAGGTTTCCCTCGCTTATCC		
	BacCow	Forward (BacCowCF128)	CCAACYTTCCCGWTACTC	177	Bernard and Field, 2000; Kildaire et al., 2007; Silkie and Nelson, 2007; Shriewer et al., 2010; Shanks et al., 2009
		Reverse (BacCow305r)	GGACCGTGCTCAGTTCCAGTG		
		Probe (BacCow257p)	6-FAMTAGGGTTCTCTGAGAGGAAGTCCCC-TAMRA		

Table 5, Continued

Ruminant-specific Bacteroidales	Rum-2-Bac	Forward (BacB2-590F)	ACAGCCC GCGATTGATACTGGTAA	99	Mieszkin et al., 2009b; Shanks et al., 2009
		Reverse (Bac708Rm)	CAATCGGAGTTCTTCGTGAT		
		Probe (BacB2-626P)	(FAM)ATGAGGTGGATGGAATTCGTGGTGT(BHQ-1)		
	CF193	Forward (CF193)	TATGAAAGCTCCGGCG		
		Reverse (708R)	CAATCGGAGTTCTTCGTG		
Bovine Bacteroidales	BoBac	Forward Primer (BoBac367f)	GAAG(G/A)CTGAACCAGCCAAGTA	100	Layton et al., 2006
		Reverse Primer (BoBac467r)	GCTTATTCATACGGTACATACAAG		
		Probe (BoBac402Bhqf)	(FAM)-TGAAGGATGAAGTTCTATGGATTGTAACCTT(BHQ-1)		

Table 5, Continued

Human-specific Bacteroidales	BacHum	Forward (BacHum160f)	TGAGTTCACATGTCGCGATGA	81	Kildaire et al., 2007; Silkie and Nelson, 2009; Shriewer et al., 2010
		Reverse (BacHum241r)	CGTTACCCCGCCTACTATCTAATG		
		Probe (BacHum193b)	6-FAMTCCGGTAGACGATGGGGATGCGTT-TAMRA		
	HuBac	Forward Primer (HuBac566f)	GGGTTTAAAGGGAGCGTAGG	116	Layton et al., 2006;
		Reverse Primer (HuBac692r)	CTACACCACGAATTCGCGCT		
		Probe (HuBac594Bhqf)	(FAM)-TAAGTCAGTTGTGAAAGTTTGC GGCTC(BHQ-1)		
	YHF	Forward (YHF67F)	GGGGCAGCATACTTAGCTTG		Ju-Yong et al., 2009
		Reverse (YHF210R)	ATCATGTGAACATGCGGACT		
	HF183	Forward (HF183f)	ATCATGAGTTCACATGTCCG	83	Mieszkin et al., 2009; Scott et al., 2002
		Reverse (HF183r)	TACCCCGCCTACTATCTAATG		
	Human-Bac-1	qHS601F	GTTGTGAAAGTTTGC GGCTCA	134	Okabe et al., 2007; Kobayashi et al., 2012
		qBac725R	CAATCGGAGTTCTTCGTGATATCTA		
qHS 624MG		CGTAAAATTGCAGTTGA			

Table 5, Continued

BacCan	Dog-Specific Bacteroidales	Forward (BacCan545f1)	GGAGCGCAGACGGGTTTT	145	Kildaire et al., 2007; Silkie and Nelson, 2009; Shriewer et al., 2010
		Reverse (BacUni690r1)	CAATCGGAGTTCTTCGTGATATCTA		
		Reverse (BacUni609r2)	AATCGGAGTTCCTCGTATATCTA		
		Probe (BacUni656p)	6-FAMTGGTGTAGCGGTGAAA-MGB		
HorseBact	Horse-specific Bacteroidales	Forward (Ho622F)	TGCGTAGGCGGGAAGTCA	100	Simpson et al., 2004; Dick et al., 2005; Layton et al., 2006; Silkie and Nelson, 2009
		Forward (Ho622F-w.7)	AGCGCAGGCGGAGTGAT		
		Reverse (Ho-722R)	GAATCCATCGCCCTCTAGTGT		
		Reverse (Ho-722R-w.7)	AGTTCGCCCTTCCTCCTCCC		
		Probe (HoF2-644)	6-FAMCAGCCGTAAAATMGYCGG-MGBNFQ		
GB342		Forward	GGGGTTCTGAGAGGAAGGT	129	Shanks et al., 2006; Shanks et al., 2007
		Reverse	AGTAGCGTGAAGGATGACGG		
		Probe	FAM-CAATATTCCTCACTGCTGCCTCCCGTA-TAMRA		
Chicken and Duck Bacteroidales	Chicken/Duck- Bac	qCD362F-HU	AATATTGGTCAATGGGCGAGAG	102	Kobayashi et al., 2012
		qcD464R-HU	CACGTAGTGTCCGTTATTCCTTA		
		qBac394 MGB-HU	TCCTTCACGCTACTTGG		
	Chicken-Bac	qC160F-HU	AAGGGAGATTAATACCCGATGATG	105	Kobayashi et al., 2012
		qBac265R-HU	CCGTTACCCCGCCTACTAC		
	Duck-Bac	qBac366F-HU	TTGGTCAATGGGCGGAAG	108	Kobayashi et al., 2012
		qDuck474R-HU	GCACATCCACACGTGAGA		
qbac394 MGB-HU		TCCTTCACGCTACTTGG			

**Table 6: Summary of Recent Studies Involving *Bacteroidales***

Paper Title & References	Sample Matrix & Matrix Prep	Assay	Purpose	# of Samples	Summary of Findings
Development of Bacteroides 16S rRNA gene TaqMan-Based RT PCR assays for estimation of total, human, and bovine fecal pollution in water. <b>Layton, A. et al. (April, 2006)</b>	Various animal feces suspended in DNase-free water; extracted using FastSpin for Soil.	AllBac, BoBac, HuBac	RT PCR assays was designed to detect Bacteroides 16S rRNA genes present in all mammalian fecal samples and determine whether the quantity of Bacteroides 16S rRNA genes present in a water sample was related to the fecal concentration	3 Human, 4 Swine, 4 Canine, 4 Equine, 6 Bovine	AllBac detection limit of 1 mg/l; marker consistently found in fecal samples; human marker found in similar levels to AllBac linearly correlated to E. coli concentrations, also proportional to concentration of human, bovine and equine feces in water

Table 6, Continued

<p>Estimation of pig fecal contamination in a river catchment by qPCR using two pig-specific Bacteroidales 16S rRNA genetic markers. <b>Mieszkin, S. et. Al. (2009)</b></p>	<p>River water, pig slurry, lagoon, fecal samples. Water samples filtered and extracted using DNEasy Kit; solid samples extracted using FastSpin for Soil kit</p>	<p>Bac, AllBac, HF183, Pig-1-Bac, Pig-2-Bac, Pig-Bac2</p>	<p>To design new primers for the detection and quantification of pig-specific Bacteroidales; Validate sensitivity/specificity of new primers and TaqMan assay using target (pig-related) and non-target (other animal related) DNA; Evaluate TaqMan for detection and quantitative estimation of pig-associated fecal pollution</p>	<p>24 Adults and children, 10 cows, 10 sheep, 10 horses, 25 pig, 23 slurry samples, 14 lagoon surface, 7 compost, 24 recreation water</p>	<p>Uncultured strains of Prevotella bacteria, Pig-2-Bac is promising marke; correlates to quantity of <i>E. coli</i> through chain; fecal pollution in river water comes from multiple sources, assays are efficient</p>
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Table 6, Continued

Human-specific fecal bacteria in wastewater treatment plant effluents. <b>Wery, N. et. Al. (2009)</b>	Wastewater treatment facility effluents; samples were concentrated and extracted using QIAamp DNA Mini Stool Kit	W18-W112 <i>Bacteroidales</i> Primers	Identify fecal bacteria able to persist after wastewater treatment and that could be used as indicators of human fecal contamination	5 plants, sampled 2x (May & July 2007)	Some gut Bacteroidales were not recovered, while less dominant feces species were recovered; wastewater treatment did not induce major changes in the structure of the bacteria populations. <i>B. cacae</i> as a potential indicator
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Table 6, Continued

<p>Presence of Bacteroidales as a predictor of pathogens in surface waters of Central California Coast. <b>Schriewer, et al., 2010</b></p>	<p>River water, coastal water - ultra filtration with various buffers used</p>	<p>BacUni, BacHum, BacCow, BacCan</p>	<p>Compare the ability of Bacteroidales markers and FIB to predict the occurrence of waterborne pathogens in riverine/esutarine water; also to use statistical approaches to characterize strengths and limitations of assay</p>	<p>143 samples from 10 different sites</p>	<p>Universal marker found in all samples; human in one-third; dog/cow &lt;10%; Bacteroidales detection correlated significantly with Cryptosporidium, no significant correlation between universal Bacteroidales and pathogens, or traditional FIB</p>
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Table 6, Continued

<p>Quantitative analysis of human/cow specific 16S rRNA gene markers for assessment of fecal pollution in riverwater by qPCR. <b>Ju-Yong et al., 2010</b></p>	<p>River water, human fecal samples. Extraction from solid samples using QIAamp stool DNA Mini Kit; river water samples filtered and extracted with xanathogenate-sodim dodecyl sulfate</p>	<p>AllBac, Bac, YH, YCF</p>	<p>Recover clones that have host specific DNA markers; design host-sp primer sets from recovered sequences for development of qPCR; demonstrate that qPCR can be used to identify and quantify fecal pollution sources in water</p>	<p>8 adult, 2 child fecal samples (200 mg), 88 riverwater samples (four rivers, 11 sites, twice sampled)</p>	<p>Recovered genes from human, cow, pig fecal samples, not successful in designing pig-sp markers - closely clustered w/ human, AllBac primer was the only discriminative primer - host-sp not very sensitive, did not find host-sp primer set w/ high specificity and broad detection range, two human primer sets detected animal feces, human and cow sp primers did not show positive signals in non-target fecal samples</p>
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Table 6, Continued

<p>Relative decay of microbial source tracking markers in freshwater microcosms. <b>Dick et al., 2010</b></p>	<p>Wasterwater-spiked riverwater; extraction using MOBio Powersoil Kit</p>	<p>AllBac, BacHum, HF183, Bac, cps</p>	<p>Measure relative decay of cultivated <i>E. coli</i> and general and HF Bacteroidales 16S rRNA gene copy numbers in wastewater-spiked river water as it ages under different conditions</p>	<p>15 environments w/ 15 1-L samples each - then 40 ml of 5-10 ml inoculations</p>	<p>HF marker decay was consistent with or significantly faster than <i>E. coli</i>, various environments affect target differently, AllBac markers were more persistent than HF or <i>E. coli</i>; no significant effect from sunlight exposure on persistence of HF markers; T<sub>99</sub> value for <i>E. coli</i> higher than Bacteroidales</p>
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Table 6, Continued

<p>Performance assessment PCR-based assays targeting Bacteroidales genetic markers of Bovine fecal contamination. <b>Shanks, O.C., et. Al. (2010)</b></p>	<p>animal fecal samples (bovine, human, alpaca, canada goose, cat, chicken, white-tail deer, mule deer, elk, moose, duck, goat, horse, pelican, pig, gull, sheep, gazelle, giraffe, okapi, takin, tufted deerk, raccoon, turkey); DNA extraction using FastSpin for Soil Kit</p>	<p>CF128, CF193, Bac, BoBac, GeneBac3</p>	<p>Assess seven different PCR and qPCR assays to determine markers associated with specific types of animals, and frequencies of markers in different bovine and ruminant populations</p>	<p>431 samples</p>	<p>Broad range of specificity, DNA sequence conservation can be restrictive for animal associate assays, further research is needed for host-sp alternative markers, GenBac3 yielded highest gene concentrations</p>
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Table 6, Continued

<p>Specificity and sensitivity evaluation of novel and existing bacteroidales and bifidobacteria-sp PCR assays on feces and sewage samples and their application for microbial source tracking in Ireland. <b>Dorai-Raj et al., 2009</b></p>	<p>Sewage from wastewater treatment facilities, animal feces,</p>	<p>CF128, HF183, Bac, BT1, BT2, BV1, BV2, BiCAT, RumD1, RumD2</p>	<p>Development and evaluation of novel ruminant-sp PCR assays and the use of these assays for MST on contaminated water samples collected from rural water supplies in Ireland, also evaluation of existing host-sp PCR assays on Irish fecal/sewage reference samples and the application of the assays for MST on Irish, naturally contaminated water samples.</p>	<p>33 human sewage, 74 ruminant (cow, sheep, deer, goat), 44 non-ruminant (horse, donkey, dog, goose, chicken, pet pig, farmed pig)</p>	<p>Ruminant-sp PCR assays show good specificity, sensitivity; all tests were done with conventional PCR, should be redone with qPCR</p>
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Table 6, Continued

<p>Concentrations of host-sp/generic fecal markers measured by qPCR in raw sewage and fresh animal feces. Silkie and Nelson, 2009</p>	<p>Animal fecal matter, raw wastewater; samples filtered and extraction with PoweMax Soil DNA isolation kit</p>	<p>16SUni, BacUni, BacHum, BacCo, BacCan</p>	<p>Determine concentrations of host-sp/generic indicator qPCR targets in fresh fecal matter from several types of hosts (human, dog, cow, horse, Canada goose), also investigate how copy number of target gene and proximity to origin replication affects qPCR results</p>	<p>67 dogs, 94 Canada geese, 85 horses, 115 cows - from 10 sites; 1 L raw wastewater from 10 WWTP</p>	<p>Host-sp markers were found in high concentrations in their respective hosts' samples, which were equal to or greater than the concentration of generic <i>E. coli</i> and <i>Enterococcus</i> markers, host specific markers were consistent in total Bacteroidales in target host feces and raw sewage</p>
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Table 6, Continued

<p>Phylogenetic analysis of Bacteroidales 16S rRNA gene sequences from human and animal effluents and assessment of ruminant fecal population by real-time PCR. <b>Meiszkin et al., 2009</b></p>	<p>Effluents (sewage, bovine manure, pig slurry), feces (human, bovine, pig, wild bird)</p>	<p>Bac, AllBac, Rum-2-Bac</p>	<p>Evaluate host-sp distribution of bacteroidales 16S rRNA gene sequences from human and animal-related effluents and feces and to define a ruminant-sp marker</p>	<p>82 feces (adult, cow, sheep, horses, pigs, wild birds), 52 effluent</p>	<p>some members of <i>Bacteroidales</i> isolated had host-sp distributions, bovine-sp cluster identified</p>
<p>Identifying human and livestock sources of fecal contamination in Kenya w/ host-sp bacteroidales assay. <b>Jenkins, M.W., et. Al (2009)</b></p>	<p>cow, donkey, human fecal samples, raw sewage, riverwater; solid and filtered samples extracted using DNEasy Tissue Kit</p>	<p>BacUni, BacHum, HF183, BacCow</p>	<p>To test whether qPCR assays for detection of host-sp fecal Bacteroidales 16S rRNA markers in water samples in USA and Europe could be used in Kenya to investigate sources of fecal pollution on surface riverwater where fecal pollution is widespread and potential point and non-point sources from humans and animals are both diverse and numerous, also to apply the validated assays to conduct preliminary source tracking and multi-species qPCR assays</p>	<p>15 cows, 10 donkeys, 12 humans, 5 raw sewage, 18 water samples from 12 locations</p>	<p>BacUni-UCD, BacCow-UCD, HF183 performed well, HF183 demonstrated 100% sensitivity; BacCow-UCD demonstrated high efficacy and discrimination against donkeys; correlation with FIB were poor; BacUni-UCD detected all</p>

Table 6, Continued

<p>Rapid decay of host-sp fecal bacteroidales cells in seawater as measured by qPCR w/ propidium monoazide. <b>Bae and Wuertz, 2009</b></p>	<p>seawater, treated with PMA; DNA extracted using FastSpin for Soil Kit</p>	<p>BacUni, BacHum, BacCan, BacCow</p>	<p>To determine the survival and persistence in seawater of host-sp fecal Bacteroidales cells and their DNA using PMA qPCR, also to test that sunlight is the single most important contributor to survival of cells and DNA</p>	<p>15 total samples (4 cattle, 11 dogpark)</p>	<p>Method can detect recent fecal contam and enhances usefulness of source tracking; cells had 2-log red. Time of 28 hrs for whole cells as opposed to DNA which had 177 hr; natural sunlight did not affect survival of cells and DNA</p>
<p>Quantitative evaluation of Enterococci and Bacteroidales released by adults and toddlers in marine water. <b>Elmir et al., 2009</b></p>	<p>Marine bathing water, sand</p>	<p>HF8, BacUCD862</p>	<p>To measure shedding of enterococci and Bacteroidales using various methods, and to evaluate shedding from adults and toddlers</p>	<p>Large pool field study: 20, sampled 2x, 5 L samples; Small pool field study: 14 toddlers 5 L samples collected</p>	<p>Bacteroidales releases were variable with human UCD marker more frequently detected among the individual toddlers and generally observed at higher levels within the adults, relative to HF8</p>

Table 6, Continued

<p>Evaluation of swine-sp PCR assays used for fecal source tracking and analysis of molecular diversity of swine-sp Bacteroidales populations. <b>Lamendella et al., 2009</b></p>	<p>Riverwater, wellwater, fecal matter, lagoon matter, manure matter</p>	<p>Bac, PigBac1, PF163,</p>	<p>To determine host-sp frequency of detection, and detection limits of currently available swine-associate PCR-based MST assays</p>	<p>215 fecal, 4 manure, 3 waste lagoon, 10 riverbasin water, 16 wellwater</p>	<p>General <i>Bacteroidales</i> assay had lower detection limits than swine-associated markers in fecal and environmental samples; detection limits were lower in feces than lagoon and manure; most of the lagoon bacteria were <i>Bacteroides</i> or <i>Parabacteroides</i>, not <i>Prevotella</i>-like (more prevalent in feces and manure); <i>Prevotella</i>-targeted assays cross-reacted w/ nontarget fecal sources.</p>
<p>Survival and Persistence of human and ruminant -sp fecal bacteroidales in freshwater microcosms. <b>Walters et al., 2009</b></p>	<p>human and cow feces</p>	<p>HF134, HF183</p>	<p>To measure marker persistence and cell survival of HF134, HF183, CF128, CF193, using methods to detect RNA</p>	<p>15 human, 15 cow</p>	<p>Bacteroidales survived 6 days; survival and persistence of human and ruminant fecal markers is similar to <i>S. typhimurium</i>, human-sp markers is comparable in to infectious enteric viruses.</p>

Table 6, Continued

<p>Discrimination of viable and dead fecal bacteroidales by qPCR w/ propidium monoazide. <b>Bae, S. et al., 2009</b></p>	<p>human feces, WWTP in/effluent; DNA extracted using FastDNA Spin kit for Soil</p>	<p>BacUni, BacHum</p>	<p>To evaluate the applicability of PMA-qPCR methods to detect culturable <i>Bacteroides fragilis</i>; to determine the feasibility of PMA-qPCR analysis for environmental samples containing different concentrations of solids; to validate the utility of the PMA-qPCR method for detection of fecal <i>Bacteroidales</i> bacteria in defined live and heat-treated mixtures of human feces and in WWTP influent and effluent</p>	<p>1 human fecal sample, 1 L WWTP influent, 1 L WWTP effluent</p>	<p>Differences for samples with and without PMA treatment, 2.5 log reduction between influent and effluent water in <i>Bacteroidales</i> DNA, but human-sp was still present in effluent water without PMA, none were present in effluent water treated with PMA,</p>
<p>A PCR Assay to Discriminate Human and Ruminant Feces on the Basis of Host Differences in <i>Bacteroides-Prevotella</i> Genes Encoding 16S rRNA. <b>Bernhard et al., 2000</b></p>	<p>Sewage, human/animal feces; extraction using BeadBeater method</p>	<p>Bac</p>	<p>Develop a rapid, inexpensive method of diagnosing the source of fecal pollution in water.</p>	<p>6 1-L water, samples, 13 Human, 3 sewage, 19 Cow, 3 cat, 3 deer, 3 dog, 3 duck, 3 elk, 1 goat, 1 llama, 3 pig, 3 seagull, 4 sheep</p>	<p>11/13 human fecal samples were detected with the HF8 gene cluster (HF 183 included), genes from CF151/123 cluster were detected in cows and not humans or sewage samples</p>

Table 6, Continued

<p>Improving the performance of an end-point PCR assay commonly used for the detection of <i>Bacteroidales</i> pertaining to cow feces. <b>Lui et al., 2011</b></p>	<p>Human fecal samples, filtered and DNA extracted from filter, using QIAamp DNA Stool Mini Kit</p>	<p>Bac, CF128</p>	<p>To investigate if <i>Bacteroidales</i> communities in cow populations in Hong Kong carry target gene sequence CF128F and whether CF128F/Bac708R shows low specificity in Honk Kong and what might be the cause</p>	<p>180 fecal samples from cow, goat, human pig horse, dog, cat rat, rabbit</p>	<p>All fecal samples successfully amplified using universal <i>Bacteroidales</i> primers (Bac32F/Bac708R). Others at 95% and 80%. No false positives were observed.</p>
<p>Quantitative PCR Method for Sensitive Detection of Ruminant Fecal Pollution in Freshwater and Evaluation of This Method in Alpine Karstic Regions. <b>Rescher et al., 2006</b></p>	<p>Freshwater samples, filtered, and DNA extracted using Ultra Clean Soil DNA Kit w/ Bead Beating</p>	<p>BacR</p>	<p>Establish a method for sensitive quantification of ruminantfecal pollution in spring water and groundwater from alpine karstic regions important for public water supplies</p>	<p>&gt; 300 fecal samples; some water samples</p>	<p>Amplification specific for ruminants was observed. Unable to determine absolute quantitative results - - 20 to 10<sup>7</sup> marker copies per reaction volume. Inhibition was observed; used 100-fold dilution for PCRs of fecal samples. Marker concentration varied throughout sample collection area.</p>

Table 6, Continued

Quantitative PCR for detection and enumeration of genetic markers of bovine fecal pollution. Shanks et al., 2007	Fecal and Wastewater samples; DNA extracted using FastSpin for DNA kit	Btheta, BG342	Development of two qPCR assays for the enumeration of bovine specific markers.	204 fecal samples (16 species), 5 WW samples	Absence of PCR inhibitors in all bovine samples according to IAC. Host-specific detection, 98-100%
Evaluating the operation utility of Bacteroidales quantitative pcr-based MST approach in determining the source of faecal indicator organisms at a UK bathing water. <b>Stapleton et al., 2009.</b>	Freshwater, industrial effluents, sewage; DNA was extracted, GITC-based method	AllBac, HF8, Bac	Assess whether the specific Bacteroidales MST technique offered by the EA is capable of producing operationally useful results in a system where base-line data was already available.	39 river, bay water, industrial effluents, bathing water and sewage samples	concentrations of markers varied across sample types. Two sewage samples were removed from the study due to having higher levels of human-sp markers than universal.
Differential decay of human faecal Bacteroides in marine and freshwater. <b>Green et al., 2011</b>	Marine and freshwater samples, spike with sewage, and light and dark treatments; samples filtered and DNA extracted using All Prep DNA/RNA Micro Kit	Bsteri, Buni, GenBac, HF183, HF132, HumM2	Understanding the decay of culturable and molecular markers from E coli and Bacteroides, in marine and freshwater microcosms, in sunlight and dark treatments	60 water samples	Molecular markers persisted longer in marine waters, some markers exhibited significant difference between light and dark treatments

Table 6, Continued

<p>Comparison of Bacteroidales-Prevotella 16S rRNA genetic markers for fecal samples from different animal species. <b>Fogarty and Voytek, 2005.</b></p>	<p>Fresh fecal samples from chicken, cattle, horses, deer, geese, seagulls, dogs, pigs and humans; DNA extracted using QIAamp Stool Kit</p>	<p>Bac, CF128, HF183</p>	<p>Compare <i>Bacteroides-Prevotella</i> populations from nine host species collected at multiple geographical locations and to determine if unique populations could be identified for each host species.</p>	<p>215 Fecal samples</p>	<p>Not all fecal samples were amplified using the primers (geese and chickens); six major clusters arose - deer and cow, cow, pig, human and dog, horse. Human marker was not amplified in all human samples and did not amplify in non host-specific samples.</p>
<p>Identification of Nonpoint sources of fecal pollution in coastal waters by using host-specific 16s ribosomal dna genetic markers from fecal anaerobes. <b>Bernhard and Field, 2000</b></p>	<p>Fecal samples - cow, human; water samples - bay and river sites and DNA extracted using a variety of methods</p>	<p>Bac, Bif</p>	<p>To develop 16S rDNA markers based on fecal anaerobes and distinguished human fecal pollution from cow fecal pollution, and to show that they can measure and distinguish source.</p>		<p>Certain fragments were identified that were specific to human or cow, and potential host specific markers were identified - seven were identified, an additionally 5 more were identified by cutting with other restriction endonucleases</p>
<p>Host distributions of uncultivated fecal bacteroidales bacteria reveal genetic markers for fecal source identification. <b>Dick et al., 2005</b></p>	<p>Fecal samples from dogs, cats, elk, pigs, gulls humans, horses; DNA extracted using FastDNA Spin kit for Soi</p>	<p>Bac</p>	<p>To establish the extent to which host-sp distributions occur in Bacteroidales, introducing new host-sp markers to identify fecal pollution</p>	<p>90 fecal samples</p>	<p>Some of the cloned sequences from different hosts appear to be similar to each other as well as cultivated species; no ruminant specific sequences that were within <i>Bacteroides</i></p>

Table 6, Continued

<p>Persistence of PCR-detectable <i>Bacteroides disatoniis</i> from Human feces in river water. <b>Kreader, 1998</b></p>	<p>River water and Fresh human fecal matter; no extraction protocol</p>	<p>Feces were dispersed in river water. Collected water samples were centrifuged and the supernatant removed. Pellet was suspended in Tris-HCl - 0.1 mM EDTA, and used directly for PCR, using BSA to reduce inhibition; for evaluation of predation, water was filtered using 0.45 <math>\mu</math>m, and cyclohexamide was added to others</p>	<p>To determine the persistence of live <i>Bacteroides</i> in river water samples due to human fecal pollution</p>		<p>Temperature greatly affects the persistence of <i>B. disatoniis</i> - seasonality. Those samples which were filtered, persisted 14 days longer than those that were unfiltered and treated with cyclohexamide</p>
<p>Detection and quantification of the human-sp HF 183 <i>Bacteroides</i> 16S rRNA genetic marker with real-time PCR for assessment of human faecal pollution in freshwater. Seurinck et al., 2004</p>	<p>Animal feces and WW, freshwater, and sewage samples were collected; DNA extracted using QIAamp Stool Mini Kit</p>	<p>HF183</p>	<p>To develop a SYBR Green real-time PCR assay to quantify HF 183 marker in feces and environmental samples</p>	<p>7 Human, 5 dog, 5 horse, 4 cow, 3 chicken, 2 pig</p>	<p>HF 183 detected <math>10^5</math> to <math>10^9</math> markers per g human feces and <math>10^9</math> to <math>10^{10}</math> per liter of WW treatment water</p>

## SAMPLE PREPARATION

Considerations in sample processing include sample type and sample volume. In fact, these two variables are intimately related to one another. Understanding that the prevalence of *Bacteroidales* markers can be just as low as the prevalence of pathogens, a general rule of thumb is to use as large a sample as possible. However, the more “polluted” or “dirty” the sample, the smaller the volume necessary to process by virtue of its higher likelihood of contamination and the greater difficulty in processing samples having a high degree of filth. Sample sizes used in previous studies have ranged from a low of several milliliters or milligrams, to a high of several liters or grams. In general, water sample volumes are usually high (40 mL – 5 L); sewage and fecal sample sizes are lower (100 – 250 mg) (Layton et al., 2006, Ju-Yong et al., 2010; Dick et al., 2010).

The most common methods for water sample preparation are centrifugation and filtration. Centrifugation is usually done at forces up to 14,000 x g for 10 minutes, and filtration is done using 0.45 µm or 0.22 µm membranes with vacuum. Large volumes of water can be processed and concentrated using filtration and the genetic material can be extracted off of the filter (Layton et al., 2006; Seurinck et al., 2005; Savichtcheva and Okabe, 2006).

In processing fecal or sewage samples, many studies employ extraction of DNA directly from the fecal matter. These protocols are relatively standard, beginning with cell

extraction and/or lysis, followed by nucleic acid precipitation, and finally, purification. Arguments can be made for both direct lysis and cell extraction followed by lysis methods, although for host-specific discrimination assays, cell isolation and then lysis appears to be the more useful method. With direct cell lysis, non-microbial DNA can co-concentrate, potentially interfering with downstream molecular-based detection methods. Lysis can be done mechanically and/or chemically, with the latter being a gentler option. However, for Gram-positive organisms, such as some species of *Bacteroidales*, incorporation of mechanical methods (such as bead beating, freeze-thaw, or ultrasonication) may be necessary to achieve adequate DNA yields. Unfortunately, these methods can also result in shearing of the DNA. Chemical lysis generally involves a combination of detergents (e.g., sodium dodecyl sulfate or SDS) and enzymes. Other compounds such as polyvinylpolypyrrolidone (PVPP) can be added to aid in the removal of humic substances, which interfere with PCR, in the case of samples with high organic load. Some kits combine mechanical and chemical methods. DNA extraction itself is frequently done using commercial kits such as the FastDNA Spin kit for Soil (MP Bio), or the QIAamp DNA Stool kit (Qiagen), with the potential for additional DNA purification steps using ethanol or isopropanol. To overcome residual humic substances, some studies use bovine serum albumin in end-point PCR, or electrophorese the concentrated DNA to purify it (Roose-Amsaleg et al., 2001). Note that while the DNA extraction kits are specifically designed for use with “dirty” samples, they vary in their recovery efficiencies and ability to remove matrix-associated

inhibitors. Hence, choice of the appropriate kits is frequently matrix-specific. None of the studies evaluated in this review discussed the variability in extraction yields between kits; however some did mention residual compounds that may not be removed from environmental samples, even after rigorous sample clean-up.

#### THE IMPORTANCE OF MATRIX-ASSOCIATED INHIBITION

Despite rigorous DNA extraction and purification methods, residual matrix-associated compounds can persist and these can inhibit subsequent PCR amplification reactions. An important factor directly related to sample preparation and likewise impacting assay performance is the impact of residual matrix-associated inhibitory compounds on qPCR amplification efficiency. Many common inhibitory compounds such as tannic, humic, and fulvic acids, acidic plant polysaccharides, and phenolic compounds, can inhibit *Taq* polymerase binding affinity by direct hydrogen bonding, thereby reducing PCR efficiency.

Four strategies have been suggested to overcome co-extraction of compounds that can be inhibitory to PCR. First, the use of lysis buffers with increased salt content can increase the level of humic acid contamination in an extract (Krsek and Wellington, 1999); however higher levels of salt may be required for those samples with higher organic matter concentrations (Wikstrom et al., 1996). The optimum salt concentration for environmental samples must be evaluated. Based on these studies, the salt content can very significantly affect the co-precipitation (or lack thereof) of organic acids. A

second way of reducing contamination is by precipitating DNA with polyethylene glycol or isopropanol. There have been mixed results with the use of propylene glycol; however a few studies have shown its utility in the reduction of humates, in combination with sodium chloride (LaMontagne et al., 2002; Arbeli and Fuentes, 2007). Filtration of DNA (prior to PCR) using highly specific carbohydrate gels, made of agarose and dextrans, such as Sepharose, are useful in the separation of DNA from other compounds, such as humates and fulvics. These methods are based on size fractionation as well as ion-ion interactions (Miller et al., 1999). Finally, compounds such as bovine serum albumin (BSA), or phage T4 gene 32 protein can be added to a PCR mastermix to scavenge inhibitors (Shriewer et al., 2010; Park et al., 2010). BSA may work by a variety of means: binding up residual lipids; preventing *Taq* polymerase inhibition by residual proteases; acting as a scavenger of residual phenolic compounds; and/or generally aiding in maintaining the integrity of the *Taq* polymerase molecule (Park et al., 2010, Kreader, 1996). Aside from adding components to the mastermix of a PCR, extracted DNA can be serially diluted prior to PCR, effectively reducing the concentration of residual amplification inhibitors (Arbeli and Fuentes, 2007; Miller et al., 1999). Of course, this approach also results in dilution of the template, effectively resulting in poorer assay detection limits. Currently, the full implications of PCR inhibition are not fully understood, and each system (water, soil, food) brings its own issues and variety of inhibitors. Environmental samples are rarely inhibitor-free and

understanding those limitations are key to developing more sensitive and effective detection methods.

#### INCLUSION OF APPROPRIATE CONTROLS

It is also necessary to assure that the qPCR reaction is functioning properly, in other words, that matrix-associated inhibition (or user error, for that matter), is not compromising the assay results. Two approaches are generally used in this regard; one is sample dilution, and the other is inclusion of an internal amplification control (IAC). The former is discussed above. With respect to IACs, two types can be used, competitive (homologous) and noncompetitive (heterologous) (Hoorfar et al., 2004). In a competitive control, the target DNA and the control DNA are amplified by the same set of primers in the same reaction tube at the same time. A major advantage of this approach is that a standard reagent set and amplification protocol can be used for both the target and the IAC. Critical to the usefulness of this approach is to identify the lowest concentration of both the control and the template that can be simultaneously amplified. Without this understanding, there is an increased risk of false positive or false negative results, with the control either outcompeting the target or vice versa. In addition, the size of the control amplicon relative to the target amplicon must be considered. Theoretically, reaction kinetics of PCR would favor the amplification of the smaller amplicon. Therefore, it is advisable to have a target that is smaller than the control. Finally, manipulating reagent concentrations (i.e., primers, dNTPs, and

polymerase, usually used at suboptimal concentrations) can help push a reaction toward increased amplification efficiency for the template.

When a noncompetitive IAC is used, the primer sets used to amplify the target differ from those used to amplify the IAC, so the reaction functions as a multiplex PCR. The control is usually derived from a “universal” gene target (i.e., *aspC* for *E. coli* O157:H7 [Johnson et al., 2008]), but for foodborne pathogens, the universal gene target will vary from species to species (Tasara and Stephan, 2007; Johnson et al., 2008). Drawbacks to this method include selection of appropriate target sequences for the IAC; and non-specific amplification that can occur unless diligence has been taken regarding primer selection. Because the successful use of a noncompetitive IAC requires two optimized amplification assays, both must be optimally functional within the parameters of each other for the entire system to work appropriately.

Interpreting the results of a PCR relies on understanding the functionality of the IAC. If the IAC amplifies at the expected level with the prescribed dilution, it is safe to say that the PCR was successful, and the results garnered regarding the target are useful. However, if the IAC fails, and no amplification at the expected level occurs, the PCR has failed, and should be repeated.

A few studies have employed IACs as applied to *Bacteroidales* MST. For example, the universal primers Bac32F and Bac708R were used as an IAC by Dorai-Raj, et al. (2009) and Shanks et al. (2009), the idea being that if this primer pair failed to amplify, the

assay would be considered unsuccessful. Specifically, Shanks et al. (2009) developed a competitive IAC and used it to enumerate bovine-specific pollution wastewater and fecal samples, and to characterize the abundance of a variety of bovine-specific markers. The IAC DNA was designed to have a longer sequence (adding 9-36 bp), essentially giving the target DNA a competitive advantage because a longer target requires more reagent and more time to complete a full round of amplification. Effectively, this produced a higher probability of amplifying the target relative to the control, particularly in those crucial early amplification rounds. This IAC was also designed to incorporate multiple primer binding sites, allowing it to be used in multiple assays and also as a DNA standard for calibration purposes.

Non-competitive IACs have also been employed in *Bacteroidales* assays. In a study by Green et al. (2011), the purpose of which was to evaluate the decay rates of markers in marine and freshwater microcosms, a non-competitive IAC was introduced alongside a competitive control, with the former being a SYBR Green-based assay targeting the genome of a genetically engineered strain of *P. aeruginosa* (PAO-T7). The latter was a control developed for the Entero1 assay, referenced in Table 1.5 (Primers and Probes), the same assay that was developed in Shanks, et al., 2009. The study reported no significant impact from inhibitory compounds on DNA recovery or qPCR efficiency.

Interestingly, however, the vast majority of *Bacteroidales* studies have not used processing or extraction controls. A few studies have employed extraction controls, derived from a pristine water blank (Dick et al., 2010; Jenkins et al., 2009). If target

DNA were identified in these samples, this can be concluded as a presumptive positive. Alternatively, in some studies, samples are seeded with surrogate organisms in an effort to estimate extraction and processing losses. Such surrogates must (i) be routinely absent from the native sample; (ii) concentrate concurrently with the environmental target; (iii) behave similarly (i.e., in terms of lysis and recovery) relative to the target microorganism; and (iv) be recovered with the same efficiency as the target. In a study done by Stoeckel et al. (2009), two spike-and-recovery surrogates were used: *E. coli* with an inserted plasmid construct (pDsRed2 – 3.3 kb), and *Pantoea stewartii*, a plant pathogen carrying and exopolysaccharide synthesis gene, *cpsD*, which was used as a target for PCR amplification. The surrogates were added to all samples prior to DNA extraction, and their recovery monitored by PCR in conjunction with the target. Results indicated good correlation between recovery of both the markers and the target. In a study done by Silkie and Nelson (2009), *P. syringae* (a Gram-negative organism) was used as a surrogate for extraction efficiency, but in this case, recovery efficiency varied widely when applied directly to environmental samples.

In the absence of a formal IAC, other studies have used DNA extracted from pooled fecal samples as a control. Specifically, this DNA was inoculated into naturally contaminated samples, and then the samples were serially diluted and subjected to qPCR targeting the inoculated DNA. For example, Silkie and Nelson (2009) serially diluted (10-1000-fold) the DNA derived from fecal samples, and then amplified using a qPCR assay. If the concentration of DNA in the undiluted sample was lower than that of the diluted sample

(based on a qPCR standard curve), this was interpreted as evidence of inhibition. In that study, there was no improvement in amplification efficiency at serial dilutions greater than 1:10, so the investigators applied a 1:10 dilution to all experimental samples subjected to qPCR.

#### INTERPRETATION OF RESULTS

*Bacteroidales* assays can be designed to evaluate presence-absence of the target gene marker, or they can be at least semi-quantitative (determining the relative concentration of the gene marker if present). Many studies employ a standard curve using a known *Bacteroidales* 16S rDNA sequence that is cloned into a plasmid vector to serve as a quantification control. This sequence, when serially diluted and subjected to qPCR, is used to produce a standard curve that can be used to calculate gene copies in unknown samples. These values are used as proxies for the concentration of *Bacteroidales* and ultimately level of fecal contamination (Layton, et al., 2006; Stapleton, et al., 2009; Seurinck et al., 2004; Shanks et al., 2010; Jenkins et al., 2009; Kildaire et al., 2007). Others have developed plasmid standards, diluting these in sheared salmon sperm DNA to provide an artificially complex sample matrix (Silkie and Nelson, 2009). This approach was apparently effective in estimating *Bacteroidales* copy number and overall target concentration in the experimental samples to which it was applied, including fresh fecal samples from a variety of animals (dogs, geese, and cows), as well as raw sewage.

The complexity of interpretation of the results of experiments such as these cannot be underestimated, and must be carefully considered for an assay to be of value, and its results reliable. The use of real-time PCR assays in determining concentration can be done in two ways: based on relative changes, and absolute concentration. Absolute concentration involves the use of a standard curve based on known copy numbers, while relative concentration is based on changes in experimental DNA concentration relative to a known control. Determining absolute concentrations can be challenging. Theoretically, qPCR amplification efficiency assumes a doubling of product with every cycle. The concern here is that, in reality, amplification efficiency changes over the course of a series of amplifications, particularly towards the end, as competition increases for reagents and target. This reaction kinetic also assumes that amplification of the standard and experimental DNA occur with identical efficiency, which as stated above, is not always the case, especially when matrix associated inhibitors are present (Pierson et al., 2003).

For quantification using relative changes, the concentration of the target is measured against that of genes that are naturally occurring in the system, and amplify consistently and regularly. As the amplification of these “control” genes occurs, changes in the target gene can be measured, and that change can be quantified. The change in amplification of the control gene is then compared against the change in amplification of the target. This approach does not require the creation of a classic standard curve, so can be relatively simple. It can also be designed to measure relative changes in the

concentrations of a variety of genes, which may provide greater flexibility for quantification. However, the method requires an understanding of the types and relative concentrations of the control genes. The latter is usually done through characterization of standard curves based on amplification of the genomic DNA. To our knowledge, no *Bacteroidales* assays to date have employed this quantification approach.

One major issue in assay interpretation is determination of a cut-off value, or the  $C_T$  value that is used to delineate presumptively positive samples from those that are negative. This is complicated and dependent on a variety of characteristics of the system, including total number of amplification cycles, initial template (and IAC) concentration, and degree of matrix-associated inhibition. The cutoff point is intrinsically important, nonetheless, as low concentrations of experimental target amplified in a complex nucleic acid matrix can be subject to inhibition (also known as the Monte Carlo effect) (Bustin et al., 2005). Also, amplification that occurs at later cycles can produce primer dimer and result in the production of non-specific amplification products. Both of these phenomena can skew interpretation of quantitative results and may even create false positive interpretations. Shanks et al. (2009) used a cutoff  $C_t$  value of  $33.3 \pm 1.0$  for their Entero1 assay (to determine rRNA genes of fecal indicator bacteria) as a means by which to determine “no significant inhibition.” In other words, if the IAC was amplified at any cycle threshold below 33.3, the PCR was considered reliable and the resulting target  $C_T$  value was considered valid.

This value came from 50 repeated experiments measuring the single assay reaction (or simplex) mean  $C_T$  values containing 25 copies of the IAC.

#### THE VIABILITY DILEMMA

It is well known that molecular-based assays are limited in their ability to discriminate between viable and non-viable bacterial cells. This is extremely important for a strict anaerobe such as *Bacteroidales*, which cannot proliferate outside of its host but may persist in the environment. Hence, detection of the presence of 16S rDNA corresponding to this group of organisms does not necessarily mean that fecal contamination was recent, or even that viable cells were present at the time of sample collection. Bae and Wertz (2009) attempted to deal with this issue by using the DNA-intercalating agent propidium monoazide (PMA) to help discriminate between live and dead *Bacteroidales*. Propidium monoazide is able to enter dead cells (because of their compromised cellular envelope), but not live cells. When the compound does enter cells, upon exposure to ultraviolet light, it binds to DNA, rendering it resistant to amplification using PCR. Theoretically, after treatment with PMA, only the DNA associated with viable cells will be amplifiable by PCR. These investigators compared the persistence of *Bacteroidales* over time in wastewater influent and effluent, demonstrating that a two log reduction in titer occurred within 28 hours if qPCR was preceded by a PMA treatment, whereas without PMA pre-treatment, qPCR signals could

be detected around the limit of detection of the assay, without major changes for up to 24 days.

#### USEFULNESS OF *BACTEROIDALES* ASSAYS IN MST

All comprehensive MST studies to date have focused on using the *Bacteroidales* 16S rDNA typing method to examine sources of fecal contamination in water or sewage. In some instances, the goal has been to determine if the method can be used for fecal source tracking; in others, if there is a relationship between *Bacteroidales* and the presence of traditional microbiological indicators and/or pathogens in relevant environmental samples.

In terms of its usefulness for sourcing fecal contamination, Layton et al (2006) used *Bacteroides* 16S rDNA assays to quantify concentrations of fecal material associated with different host types (bovine, human, dog, horse, etc), and then applied markers associated with those feces to creek water samples. These data were compared against traditional indicators such as *E. coli*. The study found a linear correlation between the log<sub>10</sub> concentration of *Bacteroides* 16S rDNA and the log<sub>10</sub> *E. coli* concentration. This study also evaluated the geographic prevalence of *Bacteroides* 16S rRNA gene sequences, finding a high degree of similarity in gene sequences present in a single host species across different geographic locations.

In a study done by Schriewer et al (2010), co-occurrence of pathogens and indicators (alternative and traditional) was evaluated against *Bacteroidales* assays applied to riverine and estuarine waters in California. General (universal) *Bacteroidales* markers, total coliforms and fecal coliforms were detected in 99% of 143 riverine and estuarine samples; however, far fewer host-specific markers were found (37% for humans and 10% for dog or cow-specific markers). While these investigators observed a correlation between fecal coliforms and *Enterococcus*, a similar correlation between universal *Bacteroidales* markers and fecal coliforms was not observed. Combining their data with that from a previous study, these same investigators determined that the universal *Bacteroidales* assay sensitivity was virtually 100%, while the sensitivity of host-specific markers was 75%, 15%, 19% and 37% for fecal matter associated with humans, dogs, cows, and horses, respectively. A summary of the various studies described here can be found in Table 1.6.

## CONCLUSIONS

All of the *Bacteroidales* assays reviewed to date have been applied for MST in water-based systems, such as recreational or drinking waters, or to wastewaters. Assays have not yet been published for matrices other than waters and wastewaters, so very little information, if any, is available for systems such as fresh produce. However, assays are available for detection of general as well as host-specific fecal contamination. Theoretically, then, these assays should be applicable to food production systems, including fresh produce.

Of the indicators discussed above, *Bacteroidales*-associated markers appear to hold the most promise as molecular markers of fecal contamination. Specifically, they are more highly-associated with fecal contamination than are some of the alternative novel indicators; they provide information on host-specificity; and they appear to have environmental persistence similar to (or better than) some key pathogens. Many of the studies described here report a positive relationship between detection of the *Bacteroidales* general fecal marker and the presence of pathogens.

The information gathered through literature review provided background for how to approach the study at hand. In this thesis, we describe the results associated with development and evaluation of the use *Bacteroidales* MST for tracking fecal contamination along the production-packing continuum. The purpose of our study was to identify sources of fecal contamination in at-risk fresh produce items (tomatoes, hot peppers, cantaloupe melons) in Northern Mexico along the production-harvesting-packing continuum. In addition, various microbiological assays were done (for pathogens and indicators). We also investigated the utility of novel MST methods for identification of fecal contamination in general, and for MST, in particular.

## RESEARCH PAPER

## INTRODUCTION

Each year in the US there are roughly 9.4 million cases of foodborne illness associated with known pathogens, and another 38 million caused by unspecified agents (Scallan et al., 2011a, b). Although many different food commodities are associated with these illnesses, the incidence of fresh produce associated foodborne disease has been on the rise for the last two decades. This increase is due to an increase in fresh produce consumption (Sivapalasingam et al., 2004), as well as a relative increase in the proportion of outbreaks in which fresh produce was identified as a source of pathogen exposure. Fresh produce contamination with enteric pathogens is now estimated to be annually responsible for nearly 15% of food-related outbreaks (CSPI, 2009).

Certain groups of fresh produce appear to cause the vast majority of outbreaks. These include leafy greens, tomatoes, melons, and fresh herbs; key pathogens include *Salmonella* and pathogenic *E. coli* strains (Anderson et al., 2011). Most of the foodborne pathogens associated with fresh produce are enteric (fecal) in origin, and fecal matter can enter product anywhere along the farm-to-fork chain. In the fresh produce production phase, common points of entry include: 1) the use of improperly composted animal manures, 2) production waters (including surface waters contaminated by runoff from animal operations, 3) waters used for irrigation, pesticide, or fungicide application, and 4) wild or domestic animal encroachment (Greger, 2007; Kinsey et al.,

2006; Brackett, 1999; Tauxe, 1997). 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. Poor facility sanitation can also be a source of enteric pathogen contamination (Richards, 2001).

The produce industry and regulatory agencies have increased the frequency of microbial testing, both for pathogens and for microbiological indicators (Busta et al., 2003) to identify contamination, and potentially prevent unsafe product from entering the food supply. Testing for pathogens on produce or at points along the farm to table production chain is expensive and time consuming, and since pathogen contamination occurs infrequently, many samples may need to be screened before even a single positive sample is identified. A second option, sampling points in the production chain and produce for fecal indicators such as fecal coliforms (e.g. *E. coli*, fecal streptococci (*Enterococci*)) is more likely to identify fecal contamination (Busta et al., 2003). However, there is a relatively poor correlation between the presence of pathogens, fecal indicator loads (Horman et al., 2003; Winfield and Groisman, 2003; Wu et al., 2011) and source of contamination; human, livestock, or wildlife, all of which are critical information for the implementation of specific control measures (Field et al., 2003; Scott et al., 2002; Simpson et al., 2002).

Microbial source tracking (MST) can be used to quantify the dominant **source(s)** of fecal contamination, usually in waters (Layton et al., 2006; Bernard and Field., 2000).

Unique targets are identified that can be attributed to different host sources. Microbial source tracking is generally categorized as library-dependent or library-independent (Scott et al., 2002). The library-dependent methods require an extensive database (or library) upon which to make comparisons. In contrast, library independent methods can be more universally applied without extensive prior efforts in data collection or standardization. The major requirement for these assays is the identification of genetic “markers” that demonstrate host specificity and can easily be detected, and perhaps quantified, using molecular methods such as PCR (Simpson et al., 2002). Library-independent methods include those based on sequence diversity in the bacterial 16S rRNA gene (Layton et al., 2006). Some regions of the 16S gene are highly conserved, leading to the production of universal PCR primers targeting entire genera, families, or orders; other regions are quite variable, and many of these demonstrate host-specificity (Layton et al., 2006). One group of organisms in particular, the order *Bacteroidales*, has proven useful for MST. This order, which is within the phylum *Bacteroidetes*, contains at least four different families: *Bacteroidaceae*, *Prevotellaceae*, *Porphyromonaceae*, and *Bikenellaceae* (Coyne and Comstock, 2008). The genus *Bacteroides* makes up about 30% of bacterial isolates from the fecal matter of warm-blooded animals, and their concentrations in fecal material are usually higher than those of the fecal coliforms or enterococci (Layton et al., 2006). They also have a high degree of host specificity (Bernard and Field, 2000). Other factors supporting their utility as indicators of fecal contamination include a high degree of environmental persistence (Kreader, 1998) and

the inability to proliferate in the environment (Bernard and Field, 2000; Bernard and Field, 2000b; Layton et al., 2006; Kreader, 1995; Dick and Field, 2004).

A variety of studies have sought to characterize the *Bacteroidales* populations in specific host species, including humans, cows, dogs, horses, and various bird species (Ju-Yong, et al., 2010; Shanks, et al., 2010; Silkie et al., 2009; Mieszkin et al., 2009; Elmir et al., 2009; Lamendella et al., 2009; Walters, et al., 2009; Bernhard and Field, 2000; Lui et al., 2011; Reischer et al., 2006, Shanks, et al., 2007; Stapleton et al., 2009; Fogarty and Voytek, 2005; Bernhard and Field, 2000b; Dick et al., 2005; Seurinck et al., 2004). These have then been used to identify different fecal pollution sources in recreational water (Elmir et al., 2009; Stapleton et al., 2009; Green et al., 2010; Layton et al., 2006), drinking water (Sokolova et al., 2011; Layton et al., 2006), and estuarine, marine, and river waters (Walters and Field, 2008; Elmir et al., 2009; Dick et al., 2010; Green et al., 2011; Seurinck et al., 2005). However, to our knowledge, there have been no efforts to use this MST approach to detect the presence of generic fecal matter, and its source, in environmental samples collected from the fresh produce production environment.

A large, multi-institutional project was conducted to identify sources of fecal contamination in at-risk fresh produce items (tomatoes, hot peppers, and cantaloupe melons) in Northern Mexico along the production-harvest-packing continuum. Fresh produce production practices were documented, and the prevalence/concentration of

key pathogens and bacterial/viral indicators was determined. In addition, the utility of novel MST methods for identification of total fecal contamination, and associated source of that contamination was examined. In this particular work, we describe the results associated with development and evaluation of the use *Bacteroidales* MST for tracking fecal contamination along the fresh produce production-harvest-packing continuum.

## MATERIALS AND METHODS

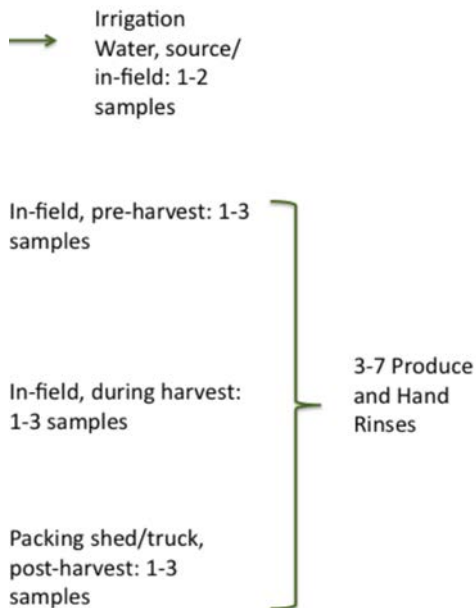
*Study area.* The study area was comprised of the contiguous northeastern states of Mexico, Nuevo Leon, Coahuila, and Tamaulipas (Figure A) on the U.S.-Mexico border. This region was chosen because it is a major agricultural area that regularly exports to the U.S. and has high production volumes of crops that are considered at elevated risk for contamination with enteric pathogens (i.e., cantaloupes, tomatoes, jalapeño peppers. Participation from farms was solicited. Those who agreed to participate were interviewed and enrolled. A total of nine different farms participated in this study. Four farms produced hot peppers, and of those four, two produced tomatoes, as well. A total of three farms produced tomatoes, including the two that produced hot peppers, and finally, four different farms produced cantaloupe melons. Institutional review board approval was received by Emory University, covering the duration of the study (approval number IRB00035460).



**Figure 1: Geographical Representation of Study Area between the Border of Mexico and the United States**

*Sample Collection.* The objective of the sampling plan was to identify points of entry for microbiological contamination of fresh produce, from production up to immediately prior to distribution. For each sampling event, a variety of sample types (water, fresh produce, and rinsates from hands) were collected at various locations on one single production site (farm and packing shed, as relevant). Each sampling event was referred to as a “chain;” each chain contained between three and nine samples. In an effort to understand the relationship between contamination of fresh produce and potential sources of such contamination, each chain consisted of a collection of rinses and waters representative of the external points of contact with source, irrigation and/or packing

waters; and contact of the produce with pickers/packers or collection bins. Each chain also contained rinsates obtained from the corresponding fresh produce items obtained before harvest, during harvest, and during packing or just prior to distribution. Each sample type had a slightly different sample collection protocol, each of which is described below (Figure 3). All samples were placed on ice immediately after collection, driven (by car) to the laboratory at La Universidad Autónoma de Nuevo León (UANL), and stored at 4°C until shipment to our laboratory at North Carolina State University. Samples were shipped on ice via DHL. Samples were received from at UNAL within 48 hours of harvest (for peppers and tomatoes), and within 72-96 hours for melons. The samples were held in refrigeration (4°C) until processed. Samples were processed within 24-72 hrs of receipt.



**Figure 2: Outline of Samples in a Given Chain**

**Product: Up to 4 samples per chain**  
(field preharvest, field at harvest, distribution, packing shed)

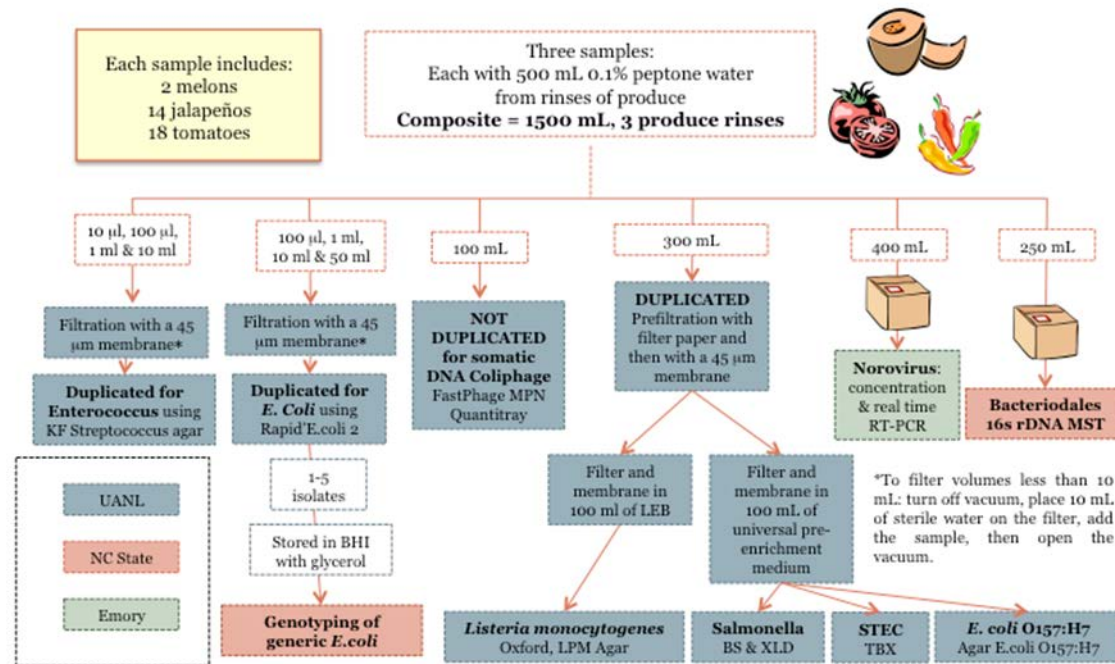


Figure 3: Chain Outline for Produce Samples (Combination of Produce Rinse, Hand Rinse, and Irrigation Waters)

## Handwash rinses: Up to 3 samples per chain (in the field at harvest, at distribution, in the packing shed)

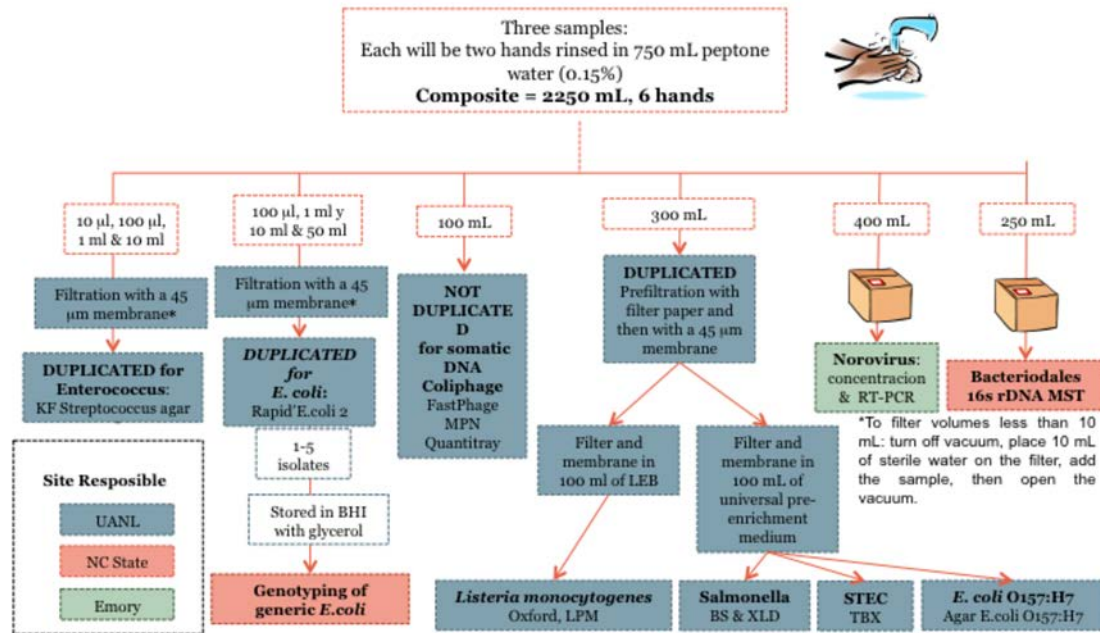


Figure 4: Chain Outline for Hand Rinse Samples (Combination of Produce Rinse, Hand Rinse, and Irrigation Waters)

## Water: Up to 2 samples per chain (at source and point of use for irrigation)

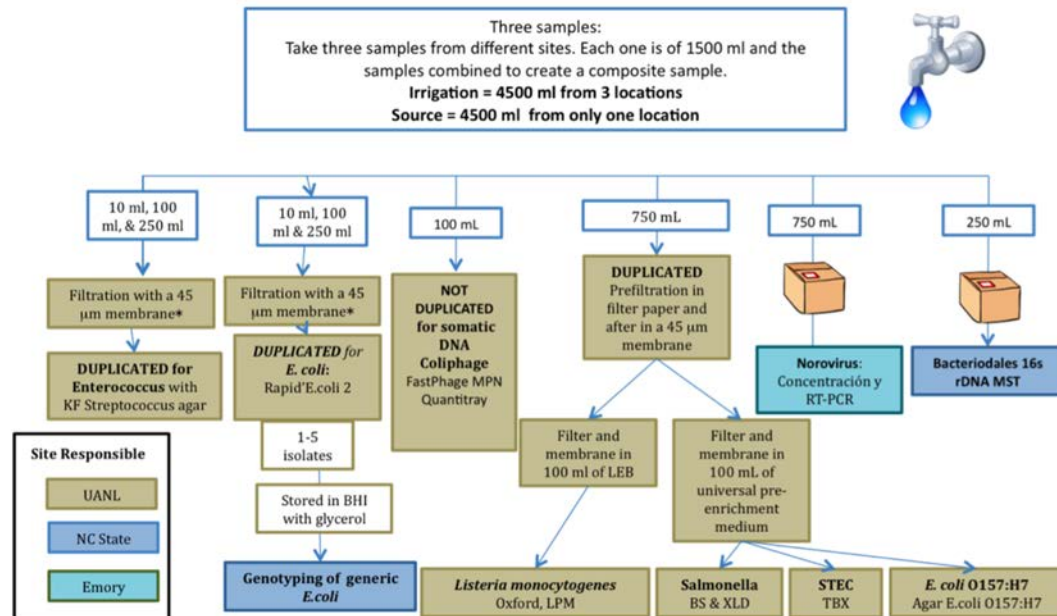


Figure 5: Chain Outline for Irrigation Water Samples (Combination of Produce Rinse, Hand Rinse, and Irrigation Waters)

*Irrigation water.* Samples of irrigation waters were collected from the source (well) and just prior to application on the field (irrigation). Samples from the well for source waters were collected by first disinfecting the pump or faucet with 200 ppm hypochlorite. The pump was allowed to run for 30 sec and then 3 water samples of 1.5 liters each were collected. For all farms, irrigation was done via a drip tape distribution system. When possible, irrigation waters were collected at the harvest row where the drip tape deposited irrigation water, or as close as possible to the harvest row. When this was not possible, samples were collected from the center of the distribution system, prior to division into drip tapes. For collection of samples from the irrigation system, the hose or the drip tape was rinsed quickly with 200 ppm hypochlorite, and the water was run for about 30 seconds prior to collection. Three samples of approximately 1.5 L each were collected in a Whirl-pak bag (Nasco, Ft. Atkinson, WI), without the hose or tape touching the inside of the bag. The three source water and irrigation water samples were composited to produce one large sample of about 4.5 L, each, in volume. This composited sample was divided into smaller subsamples for specific testing (microbiological indicators, bacterial pathogens, norovirus, or MST).

*Produce rinsates.* Multiple produce items at each sampling time (before and during harvest, at packing) were batched for rinsate collection. Each “batch” of produce was rinsed together to produce one single sample rinsate. Specifically, two whole melons were composited into one sample for cantaloupes; 18 individual whole tomatoes were composited into one sample for this commodity; and 14 Jalapeno or Serrano constituted

a single sample for the pepper commodity. Each batch of a produce item was subdivided in half, placed in a Whirl-pak bag, containing 500 ml 0.1% peptone water (PW), and shaken for 30 sec, massaged for another 30 sec, and shaken again for an additional 30 sec. The first half batch of fruit was removed, replaced with the second half and the process was repeated. For each produce item at each point (pre-harvest, harvest, distribution and packing), three batches of produce were sampled to collect one composited sample of 1500 ml. The composited sample was divided into smaller subsamples for specific testing, as shown in the flow diagrams (Figures 3-5).

*Hand rinses.* Researchers in the field asked permission to sample the hands of workers prior to rinsing. Workers gave oral consent about participating in the study, and were compensated with a soft drink and snack. Once verbal consent was given, the worker placed his or her hand in a Whirl-pak bag containing 750 ml peptone water (PW). The worker was asked to shake his hand for 30 sec, and then his hand was massaged for an additional 30 sec, paying particular attention to manipulate the fingers. The first hand was gently removed from the buffer, and the second hand was replaced in the same bag. The same process was repeated. Three pairs of hands (three individual pickers or packers) were composited as single sample, for a total of three pairs of hands, each of 750 ml, making a total sample size of 2250 ml. This composited sample was divided into smaller subsamples for specific microbiological testing.

*Escherichia coli screening.* Upon receipt at the UANL laboratory, each sample was aliquoted with subsamples destined for different testing protocols (for microbiological

indicators, bacterial pathogens, noroviruses, or MST) to different partner institutions (Figures 3-5). The UNAL laboratory conducted the microbiological indicator analyses (total coliforms, *E. coli*, and total *Enterococcus*), following a prescribed membrane filtration protocol. Briefly, a 47 mm diameter 0.45 µm pore size S-Pack filter (Millipore, Billerica, MA) was placed on a vacuum apparatus. Vacuum was applied to the sample, and once the entire sample passed through, the filter was removed, and directly placed onto solidified media for each assay. Each filtered sample ranged in volume from 1 µl to 250 ml. For total coliforms and *E. coli*, the Rapid'2 *E. coli* 2 agar was used (Bio-Rad, Hercules, CA), and for total *Enterococcus*, KF Streptococcus agar (Neogen, Lansing, MI) was used. Plates were incubated inverted and overnight at 44°C. After 24 h, plates were enumerated. *Escherichia coli* colonies appeared on the Rapid'2 *E. coli* 2 agar as blue-colored, and other coliforms appeared as red colonies. Calculation of total coliforms was done by summing the number of *E. coli* colonies and coliform colonies.

*Sample processing for Bacteroidales detection.* *Bacteroidales* MST testing was conducted at NCSU, (Jaykus Laboratory, Dept. Food, Bioprocessing and Nutrition Sciences). Three different methods, centrifugation, filtration and combined centrifugation and filtration (Figures 6-8) were evaluated for sample preparation, each including the sequential steps of sample concentration followed by DNA extraction.

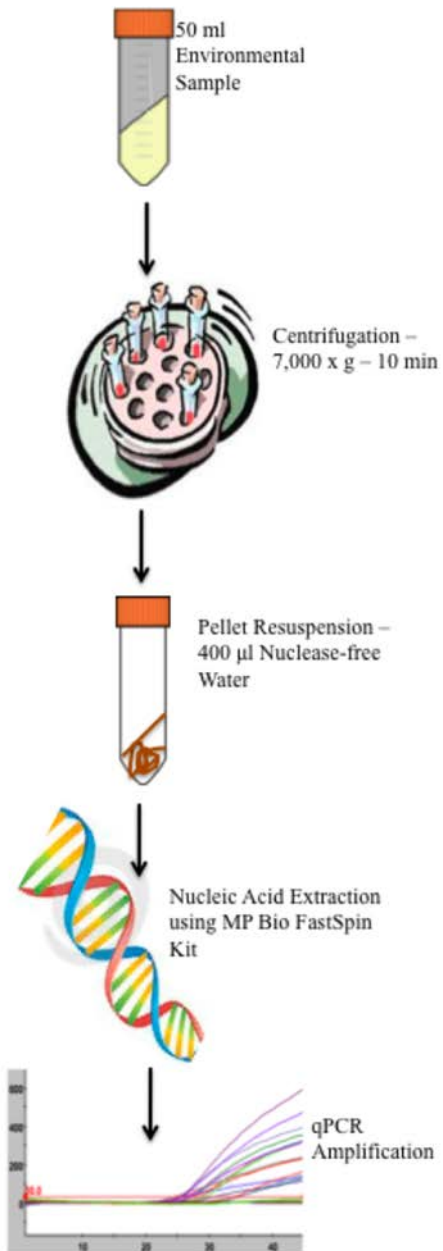
*Centrifugation.* In a small pilot study, the first method was applied to local lake water collected from Lake Johnson, Raleigh, NC. The purpose of these studies was to determine the limit of detection of the *Bacteroidales* assay. Specifically, 50 mL water

sample volumes were centrifuged at  $7,000 \times g$  for 10 min. The supernatant was discarded and the pellet resuspended in 400  $\mu\text{L}$  of DNase-free water. The suspended pellet was stored at  $-20^\circ\text{C}$  until DNA extraction.

*Filtration.* Filtration methods were developed using experimental samples during the first harvest season. Samples were filtered 50 mL at a time, until the entire sample volume, roughly 250 mL, was processed, using 47 mm 0.45 $\mu\text{m}$  pore, Millipore S filters (Millipore, Billerica, MA), pulled by vacuum at 25 kPa. The filtrate was discarded and the filter was either processed immediately for DNA isolation or else was frozen at  $-20^\circ\text{C}$  until extraction.

*Combined centrifugation/filtration.* This method was also developed using experimental samples from the first harvest season. The protocol combined the centrifugation and filtration methods described above, with some sample-specific modifications. For irrigation water samples, a sole filtration method was used. Hand rinse and produce rinse samples were processed by centrifuging at  $8,000 \times g$  for 10 minutes two different 50 mL aliquots (100 mL total processed), then sequentially (one aliquot at a time) filtering the resulting supernatant in a 47 mm 0.45  $\mu\text{m}$  pore, Millipore S filter, maintaining one filter per sample as long as possible. The sediment-containing pellet, which was resuspended in 400  $\mu\text{L}$  of DNase-free water, and the filter were then stored in the same 50 mL tube, and placed in the freezer at  $-20^\circ\text{C}$  until DNA extraction. In cases where the sample was too filthy, multiple filters were used. In this situation, the sample was filtered using a single filter as long as possible and when further

filtration could not proceed, the filter was exchanged, and filtration continued. All filters and corresponding resuspended pellets were stored at  $-20^{\circ}\text{C}$  until extraction. For a visual of how these methods were completed, see Figure 6.



**Figure 6: Schematic outlining Centrifugation Protocol for Sample Concentration**

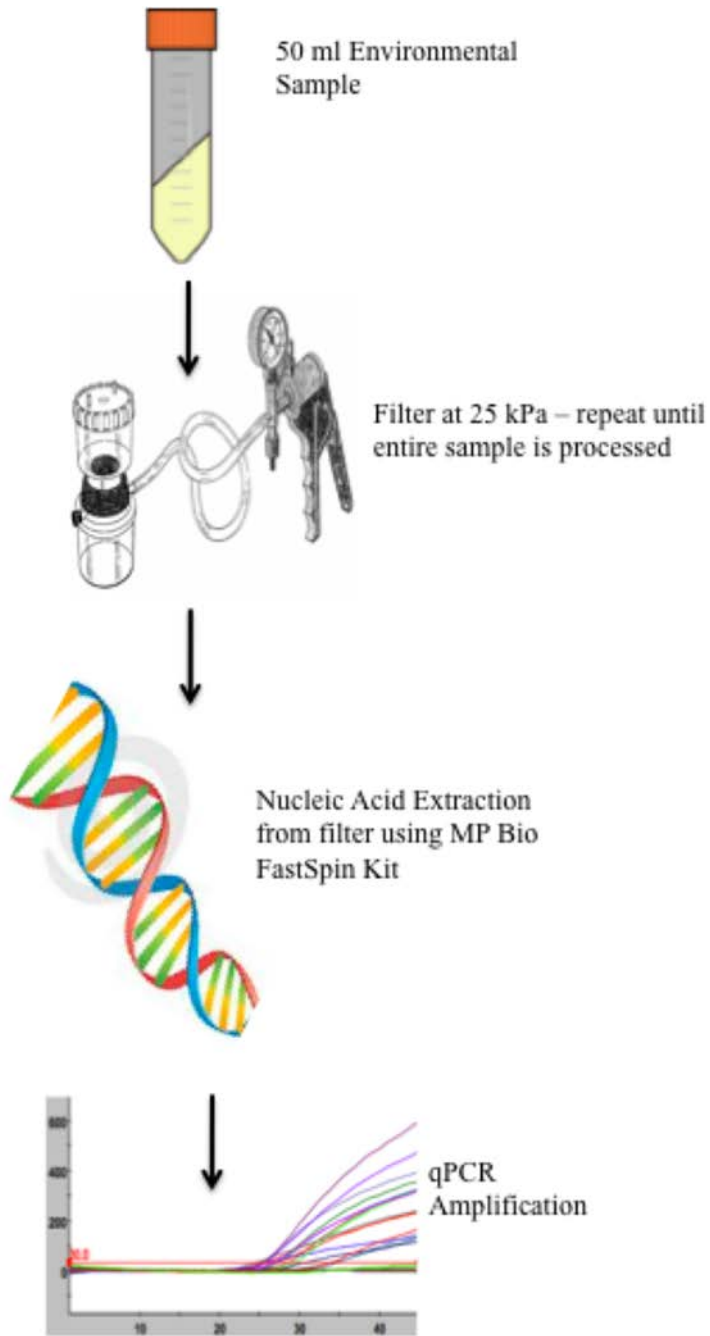
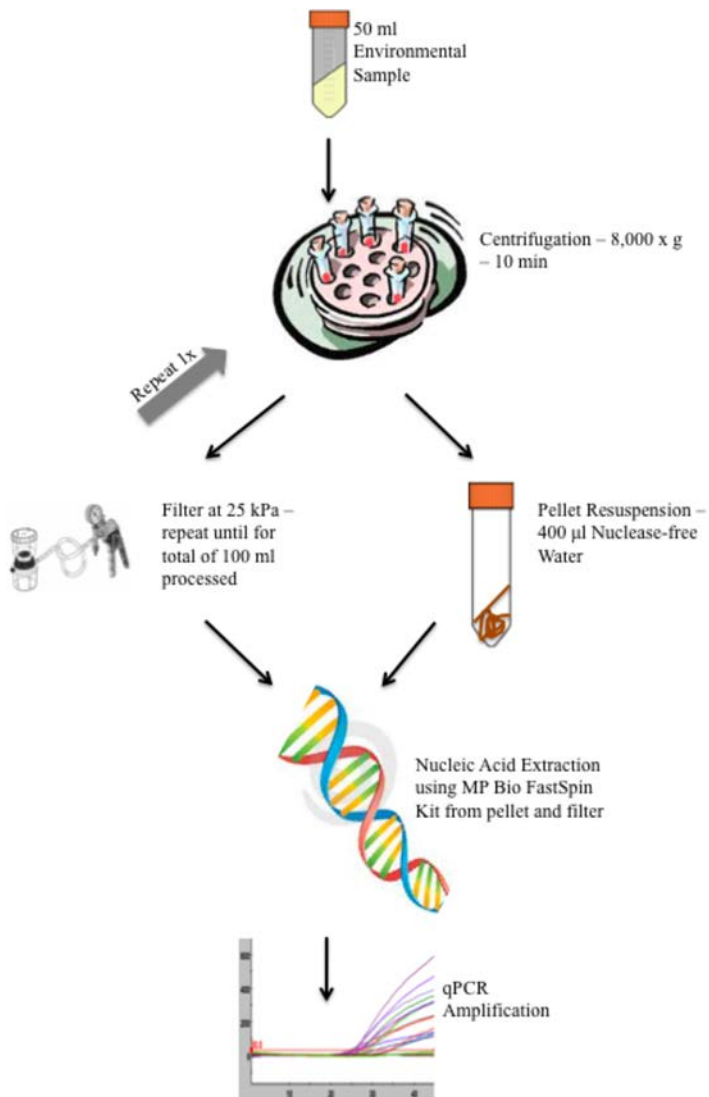


Figure 7: Schematic Outlining Filtration Method for Sample Concentration



**Figure 8: Schematic Outlining Combination Method of Centrifugation and Filtration for Sample Concentration**

*DNA Extraction.* Three DNA extraction kits were evaluated, as well as a bead beater-based method. The commercial methods evaluated were the PowerSoil kit (MoBio, Carlsbad, CA), the DNEasy Blood and Tissue kit (Qiagen, Valencia, CA), and the FastSpin for Soil kit (MP Bio, Solon, OH). The extraction methods corresponding to each kit were performed according to the manufacturers' instructions. The bead beater method was tested on 20% human stool specimens (human feces suspended 20% in DNase-free water). Specifically, 350  $\mu$ L of the suspension was combined with 250  $\mu$ L of acid-washed glass beads (Sigma-Aldrich, St. Louis, MO) in a microcentrifuge tube. This tube was placed in a bead beater (Mini BeadBeater, BioSpec Products, Bartlesville, OK) and processed for 7 sequential cycles, each consisting of 30 sec beating followed by 30 sec cooling on ice. The tube was then centrifuged at 18,600  $\times g$  (or 14,000 rpm for bench top microcentrifuge) for two min and the supernatant recovered. DNA was precipitated by the addition of one-tenth volume of 3M sodium acetate and one volume of ice-cold isopropanol followed by freezing at -20°C for 15 min. The tube was centrifuged for 2 min at 18,600  $\times g$ . After pouring off the supernatant, the pellet was washed with 300  $\mu$ L of 70% ethanol, centrifuged to remove remaining ethanol, and resuspended in 10  $\mu$ L DNase-free water.

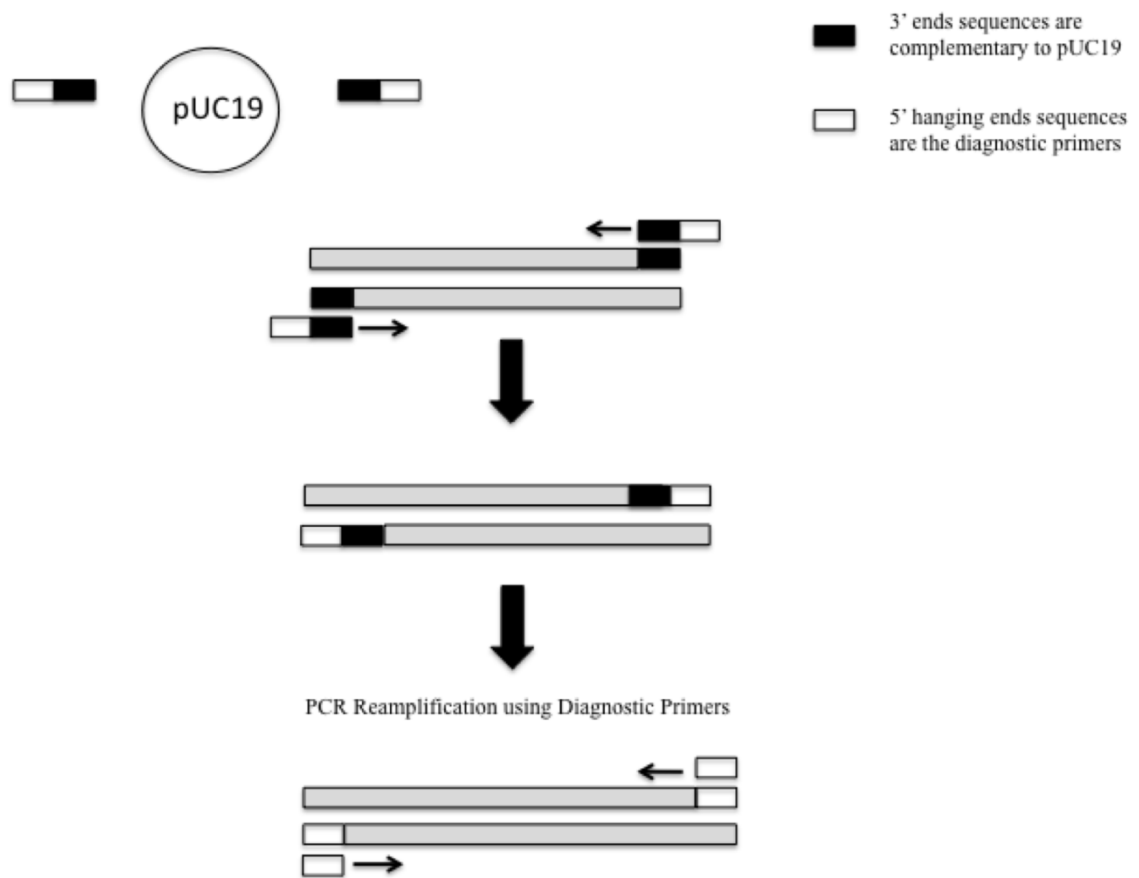
*Quantitative real-time PCR (qPCR).* Two different qPCR assays were used in this study, amplifying 16S rDNA genes within *Bacteroidales*. Those assays were AllBac (for universal *Bacteroidales* identification) and BoBac (for identification of bovine-specific *Bacteroidales*). The bovine-specific assay was chosen prior to understanding the wildlife

population surrounding the farms. Later it was determined that domesticated cattle were not present nearby. For each, an internal amplification control (IAC) was produced. Each of these assays is described below.

*Construction of an Internal Amplification Control (IAC).* An IAC is a non-target nucleotide sequence that is co-amplified simultaneously with the target sequence and can serve as a means by which to identify reaction failure (false negative results occurring because of improper amplification conditions, errors in reagent composition, or inhibition of polymerase activity as might be the case for the presence of matrix-associated inhibitory substances) (Hoorfar et al., 2004). A competitive IAC, constructed to be amplified by the same primers as the target (Figure 9), was produced using the composite primer technique for each of the two primer sets (Table 7) used in this study (Siebert et al, 1992). This IAC could be amplified using the assay diagnostic primers, but had an internal sequence corresponding to a region of the pUC19 vector (New England Biolabs, Ipswich, MA). Hence, the target DNA could be co-amplified using the *Bacteroidales* diagnostic primers with a FAM-labeled probe for the diagnostic assay, and a TET reporter dye for the IAC.

Briefly, the IAC was synthesized by overlap extension PCR, after which the 230 bp PCR product was separated by gel electrophoresis, purified using the QIAquick PCR/Gel purification kit (Qiagen, Valencia, CA (Figure 9). The final IAC concentration was determined using a Nanophotometer Pearl (Implen, Munchen, Germany) and its

concentration for use in qPCR was optimized by applying PCR (described below) to 10-fold serial dilutions of the concentrated IAC stock. The dilution displaying a consistent Ct value of about 30 was used to screen for qPCR inhibition in all environmental sample extracts tested.



**Figure 9: Construction of IAC - Diagnostic overhanging primers complementary to plasmid pUC19, and amplified by PCR. Amplicons obtained are able to be reamplified using the diagnostic primers**

*AllBac qPCR Assay.* Detection of a universal, conserved 16S rDNA marker for *Bacteroidales* (producing an amplicon of 106 bp) was used in this TaqMan™ assay. Primers used (Forward 5' – GAG AGG AAG GTC CCC CAC – 3'; Reverse 5' – CGC TAC TTG

GCT GGT TCA G – 3’; and TaqMan probe 5’ – (FAM) – TGA AGG ATG AAG GTT CTA TGG ATT GTA AAC TT - (BHQ-1) – 3’) were reported in Layton et al. (2006). The assay was carried out using the SmartCycler PCR system (Cepheid, Sunnyvale, CA). A 25 µL PCR reaction, containing 1X PCR buffer (Life Technologies, Grand Island, NY), 200 nM dNTP mix (Applied Biosystems, Warrington, UK), 1.5 mM MgCl<sub>2</sub> (Life Technologies), 400 nM forward primer, 400 nM reverse primer, 200 nM TaqMan probe, 200 nM TaqMan IAC probe (Integrated DNA Technologies, Coralville, IA), 1.0 U Taq polymerase (Life Technologies), 2.5µL experimental DNA, and DNase-free water to bring to volume. Amplification parameters were as follows: initial denaturation at 95°C for 2 minutes, followed by 45 cycles of denaturation at 95°C for 15 sec, annealing at 53°C for 30 sec, extension at 72°C for 30 sec.

BoBAC qPCR ASSAY. Detection of a bovine-specific 16S rDNA *Bacteroidales* marker (an amplicon of 100 bp) was done using a TaqMan™ qPCR protocol. Primers used (Forward 5’ – GAA G(G/A) CTG AAC CAG CCA AGT A – 3’; Reverse 5’ – GCT TAT TCA TAC GGT ACA TAC AAG – 3’; and TaqMan probe 5’ – (FAM) – TGA AGG ATG AAG GTT CTA TGG ATT GTA AAC TT (BHQ-1) – 3’) were reported in Layton et al. (2006). The assay was carried out in the SmartCycler PCR system. Reactions consisted of the qPCR mastermix, which consisted of 1X PCR Buffer (Life Technologies), 200 nM dNTP mix (Applied Biosystems), 1.5 mM MgCl<sub>2</sub> (Life Technologies), 400 nM forward primer, 400 nM reverse primer, 200 nM TaqMan probe, 200 nM TaqMan IAC probe (Integrated DNA Technologies), 1.0 U Taq polymerase (Life Technologies), 2.5µL experimental DNA, and

DNase-free water to bring to volume. Amplification parameters were as follows: initial denaturation at 95°C for 2 minutes, followed by 45 cycles of denaturation at 95°C for 15 sec, annealing at 57°C for 30 sec, extension at 72°C for 30 sec.

**Table 7: Primers and Probes used in this Study; AllBac Assay Designates General Fecal Material, and BoBac Designates Bovine-specific Fecal Material**

Assay	Primer/Probe Sequence 5'-3'	Annealing Temp	Limit of Detection
AllBac	AllBac296f, 5'-GAG AGG AAG GTC CCC CAC-3'	53	10 <sup>-2</sup> - 10 <sup>-3</sup>
	AllBac412r, 5'-CGC TAC TTG GCT GGT TCA G-3'		
	AllBac375Bhqr, 5'- (FAM)CCATTGACCAATATTCCTCACTGCTGCCT(BHQ-1)-3'		
AllBac IAC	AllBac Forward (GAG AGG AAG GTC CCC CAC) – modified – TTC TCA TAG CTC ACG CTG TAG – 3'		
	– 5' – AllBac Reverse (CGC TAC TTG CGT GGT TCA) - modified -TCG CTC TGC TAA TCC TGT TAC – 3'		
BoBac	BoBac367f, 5'-GAA G(G/A)C TGA ACC AGC CAA GTA-3'	57	10 <sup>-2</sup>
	BoBac467r, 5'-GCT TAT TCA TAC GGT ACA TAC AAG-3'		
	BoBac402Bhqf, 5'- (FAM)TGAAGGATGAAGGTTCTATGGATTGTAACTT(BHQ-1)-3'		
BoBac IAC	BoBac367f, 5'- (GAA G(G/A)C TGA ACC AGC CAA GTA) - modified- TTC TCA TAG CTC ACG CTG TAG - 3'		
	BoBac467r, 5' – (GCT TAT TCA TAC GGT ACA TAC AAG) - modified TCG CTC TGC TAA TCC TGT TAC - 3'		
IAC Probe	5'-(TET)-ATC TCAGTT CGG TGT AGG TCG TTC GCT CC- 3BHQ_1-3'		

*Construction of standard curves for quantification of total Bacteroidales.* A key difference between traditional PCR and real-time PCR is the ability to semi-quantitatively determine concentration of amplified regions. Standard curves were constructed using both a plasmid standard as well as a genomic DNA standard. For the latter, an ATCC genomic DNA preparation of *Bacteroides thetaiotaomicron* 29148D-5

was procured from American Type Culture Collection (ATCC, Manassas, VA). Serial dilutions of the genomic DNA were made, and amplified using the AllBac primer set and the conditions described above. For the plasmid DNA quantitative standard, the ATCC strain *Bacteroides thetaiotaomicron* (ATCC 29148) was grown anaerobically, at 37°C for 42 hours. Genomic RNA was extracted from the pure culture as described above. Genomic RNA was amplified using the BthetaFor (CAACCCATAGGGCAGTCATCC) and BthetaRev (GGTAACGGCTCACCAAACCT) primers designed using the basic local alignment tool (BLAST, National Center for Biotechnology Information [Bethesda, MD]) with cycling conditions as described above. Amplified DNA was cloned into the pCR™ 2.1-TOPO® vector using the TOPO TA Cloning kit (Life Technologies, Grand Island, NY). Plasmid DNA was isolated from *E. coli* using the PureLink Quick Plasmid Miniprep (Life Technologies) and quantified using a Nanodrop Spectrophotometer (Thermo Scientific, Wilmington, DE). A serial dilution of the plasmid DNA was made and amplified using the AllBac primer set using the conditions described above.

*Interpretation of qPCR amplification results.* Based on results of the genomic DNA standard curve, the highest cycle threshold (Ct) value at which target amplification consistently occurred was roughly 36-38 (corresponding to about 0.5 genomic equivalent copies), presumptively positive samples were defined as any amplification yielding a Ct  $\leq$ 35. Samples with amplification at Ct values greater than 35 were determined as potential false positives. Optimized IAC Ct values ranged from 29-31, and hence when amplified in the presence of the target, Ct values of 29-31 for the IAC

were considered supportive of adequate PCR amplification reagents and conditions, and the absence of matrix-associated inhibitory compounds. Because the IAC can out-complete the target, particularly when template levels are low, each qPCR amplification reaction was done both with and without inclusion of the IAC. If the IAC failed to be amplified, or was amplified at Ct values exceeding 31, the sample was further 10-fold diluted, up through a maximum 1,000-fold dilution, and re-amplified. If the IAC failed to amplify at the highest sample dilution (1,000 X), the sample was designated as “uninterpretable.”

STATISTICAL ANALYSIS. The Ct values for each dilution of DNA were averaged and plotted against genome equivalent copies to develop a standard curve using least squares linear regression analysis. Residual values were plotted to assess normality of the distribution. This curve and the associated equation were used for quantification of approximate genome equivalent copies in unknown samples, expressed as GEC per 100 ml sample. This value took into account sample dilution (when necessary) due to qPCR failure associated with residual matrix-associated inhibition.

The limit of detection for *E. coli* assays was determined on a sample-by-sample basis. In some instances, growth was too numerous to count, however in other instances no growth appeared. For samples where growth was too numerous to count, 9999 was entered as the growth value, and was divided by the smallest volume plated. This value

was used as the limit of detection for the assay. For example, if a 5 ml sample that was plated yielded too numerous to count values, 9999 cfu was divided by 5 ml to give a limit of detection of 1999.8 cfu per ml. In situations where no visible growth was noted, less than one cfu per greatest volume plated was used as the limit of detection. For example, if growth was not noted on a sample where 50 ml were plated, then the limit of detection for that sample would be  $< 2.0 \times 10^{-2}$  cfu per ml. In cases where no reportable number was available, the limit of detection served as the concentration of *E. coli* colonies. These values, alongside the reportable numbers, were log-transformed to normalize, and compared against log-transformed GEC values for correlation values. Spearman's correlations were performed to determine whether a relationship between the presence of *E. coli* predicted the presence of *Bacteroidales*. The log-transformed values of both *E. coli* and *Bacteroidales* were plotted against each other, and a linear regression analysis was done to determine possible correlations between the two assays.

Predictive analysis for the presence of *Bacteroidales* using *E. coli* presence was done using JMP software package (JMP, Version 10. SAS Institute, Inc., Cary, NC, 1989-2013). Presence and absence, and log concentration of *Bacteroidales* and *E. coli* was recorded and compared using Spearman's Rho to determine whether there was a statistically significant relationship present.

#### SAMPLE PROCESSING.

For processing and concentration of the environmental samples, a combination centrifugation and filtration method was chosen. Filtration alone, as applied to the entire sample volume, took virtually hours for samples having high organic load (i.e., hand and produce rinsates). Much of the larger debris in such samples was readily precipitated by centrifugation, but there was concern that fine debris might remain in the discarded supernatant. Using a combined centrifugation-filtration method, we were able to capture Bacteroidales associated with both the solids and liquid portions of the sample, and effectively process half the sample volume in approximately one hour for each sample, compared to potentially hours using the filtration method alone.

DNA EXTRACTION. The Bead Beater method yielded high concentrations of DNA (on average, upwards of 1,700 ng/ $\mu$ L), however the purity of the DNA (OD260/OD280) was poor (around 1.2). When applied to qPCR, DNA extracted from fecal specimens using the bead beater method failed to be amplified. Further data on this method can be viewed in Appendix A. The PowerSoil kit, by MoBio, provided the lowest yields of DNA, averaging roughly 5 ng/ $\mu$ L, with OD260/OD280 ratios of around 1.5. The other two kits, DNEasy and FastSpin for Soil, yielded higher concentrations, about 30 ng/ $\mu$ L for DNEasy and 35 ng/ $\mu$ L for FastSpin, with purities of 1.3 and 1.7, respectively. These data are provided in Appendix A. Since the concentrations were similar, and purities were so significantly different, the FastSpin Kit for Soil (MP Bio) was chosen for use on the experimental sample set. Minor modifications to the FastSpin kit protocol were done to

accommodate the environmental samples tested in this study. Specifically, extraction was done from both the pellet and the filter by cutting the filter in half, rolling it up and adding it to the extraction tube, alongside the resuspended pellet, prior to sample lysis. Secondly, lysis time was extended to one hour. The final modification was made at the elution step, where the spin filter was eluted with 60  $\mu$ L of DNase-free water, and placed in a heat block at 55°C for 5 minutes prior to final centrifugation.

qPCR OPTIMIZATION. Optimal annealing temperatures for the AllBac and BoBac assays were done using temperature gradient PCR. In this case, lake water was spiked with a 20% human fecal suspension and processed using the filtration protocol as described above. DNA was extracted from the pellet as well, using the MP Bio FastSpin for Soil DNA extraction protocol, following the manufacturer's protocol. Assays were set up as described above, with variation in annealing temperature. Temperatures ranged from 50°C to 60°C. Temperatures were evaluated for optimal performance based on the fluorescence profile of the output graph, and the Ct value. Optimized temperatures for the assays were as follows: AllBac, 53°C and BoBac, 57°C. An example of optimized PCR for both AllBac and BoBac assays can be found in Appendices B and C. Determination of the detection limits for the AllBac and BoBac assays was done by spiking 50 ml samples of lake waters with a 20% human fecal suspension, and creating 10-fold serial dilutions ranging from  $10^{-1}$ -  $10^{-5}$ . The samples were processed as described above, using centrifugation. DNA extraction was carried out according to the

MP Bio FastSpin for Soil protocol. Using the optimized annealing temperature for each assay, consistent amplification occurred for each assay at a sample dilution of  $10^{-2}$ . A schematic of this approach can be found in Appendix D. By back calculation, the lowest mass of fecal matter that could be detected in these assays, constituting the detection limit of the qPCR, was roughly 2 mg of feces per 100 ml water sample.

#### RESULTS FROM SAMPLES COLLECTED ACROSS THE FARM-TO-DISTRIBUTION CHAIN.

A total of 174 samples were processed, consisting of a variety of sample types [i.e., produce rinses (peppers, tomatoes, and cantaloupe), hand rinsates and irrigation waters]. The samples came from nine different farms that were sampled throughout the harvest season, beginning in May, and ending in October, 2011. Each sample type was not necessarily represented in each chain, but each chain did have a variety of sample types, and those sample types spanned the production through packing continuum.

DESCRIPTIVE ALLBAC RESULTS. The universal AllBac marker was identified in 36% (64/174) of the samples screened. The marker was not identified in 55% (95/174) of the samples screened, and finally, 8% (14/174) results were uninterpretable. Based on sample type, 30% (15/50) of hand rinse samples were positive for the AllBac marker, 45% (38/85) of produce rinsates were positive, and 28% (11/39) of irrigation water samples were positive for *Bacteroidales*. When grouping the samples in accordance

with the type of fresh produce item for which they were associated, positivity rates were 59% (33/56) for cantaloupes chains, 31% (24/78) for tomato chains, and 17% (7/40) for pepper chains. A table of these results can be found in Appendix E.

More specifically, for produce rinsates in cantaloupe chains, 66% (18/27) tested positive, 26% (7/27) tested negative, and 7% (2/27) were uninterpretable for the AllBac marker. In hand rinses associated with cantaloupes, 47% (8/17) were positive, 47% (8/17) were negative, and 8% (1/17) were uninterpretable. Finally, in irrigation waters, 75% (9/12) tested positive, 25% (3/12) tested negative; none were reported as uninterpretable. For pepper chains, 24% (4/17) produce rinsates tested positive and 76% (13/17) tested negative. For hand rinse samples, 14% (2/14) tested positive and 86% (12/14) were negative; 11% (1/9) irrigation waters tested positive, with the remaining 89% (8/9) samples testing negative. For tomato chains, 39% (16/41) of produce rinsates tested positive, 51% (21/41) tested negative and 10% (4/41) were uninterpretable. For hand rinse samples, 37% (7/19) tested positive, 37% (7/19) tested negative, and 26% (5/19) were uninterpretable. Finally, for irrigation water samples associated with tomatoes, 6% (1/18) tested positive, 89% (16/18) tested negative and 5% (1/18) was left uninterpretable. A visual representation of these results can be seen in Figures 10-15). Exact numbers of positives and negatives can be seen in Appendix E. Environmental samples that are broken down by produce type can be seen in Appendix F.

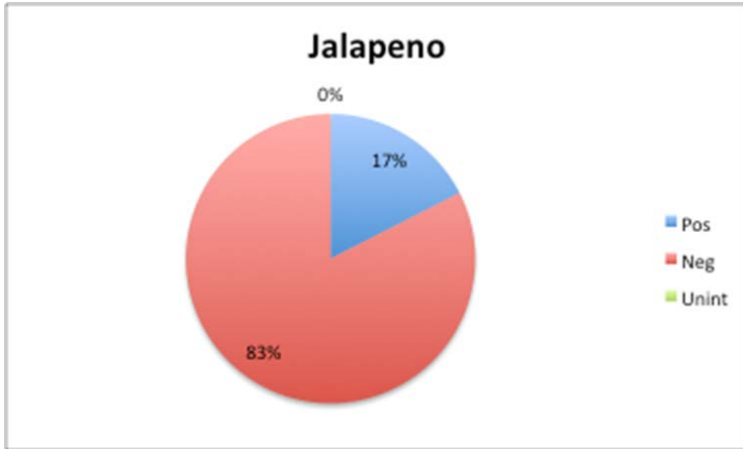


Figure 10: Jalapeno Results (Percent Positive, negative and uninterpretable)

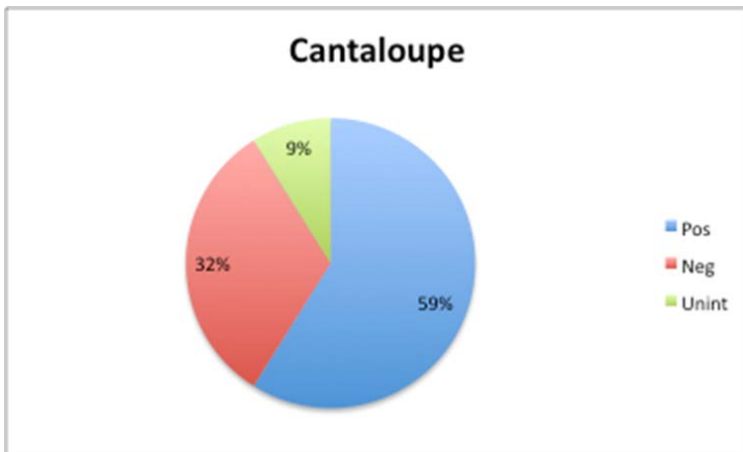


Figure 11: Cantaloupe Results (Percent Positive, negative and uninterpretable)

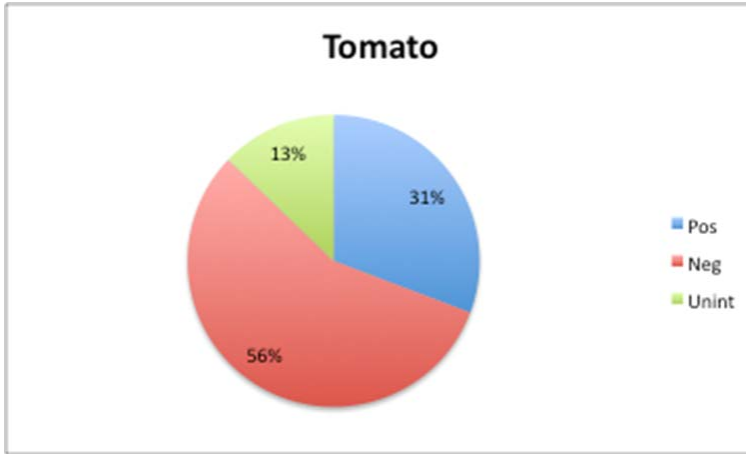


Figure 12: Tomato Results (Percent Positive, negative and uninterpretable)

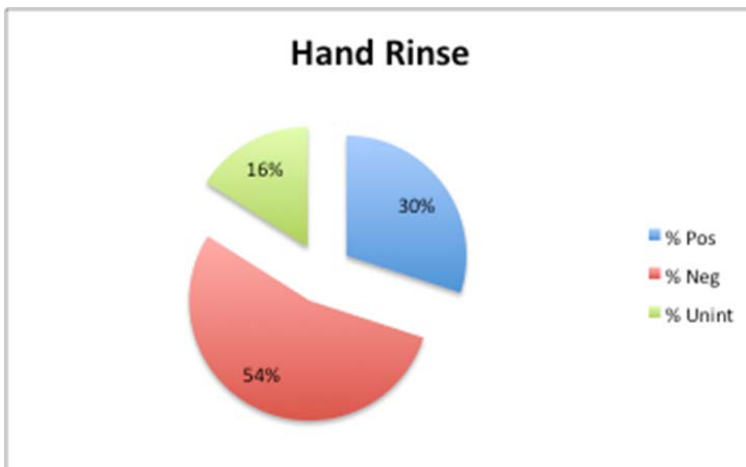


Figure 13: Hand Rinse Results (Percent Positive, negative and uninterpretable)

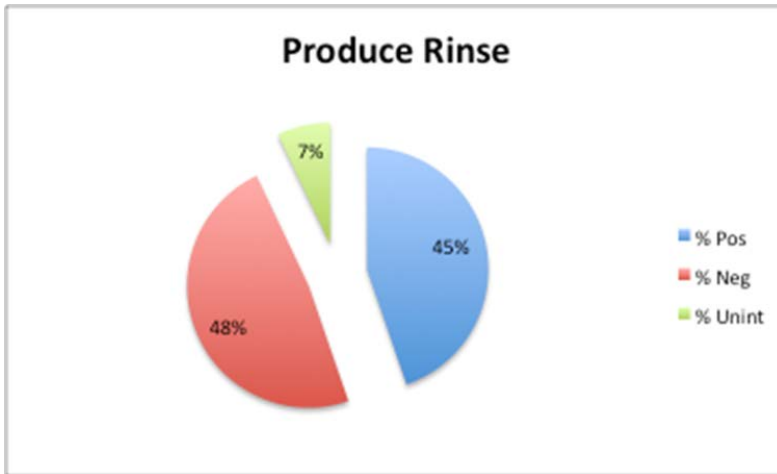


Figure 14: Produce Rinse Results (Percent Positive, negative and uninterpretable)

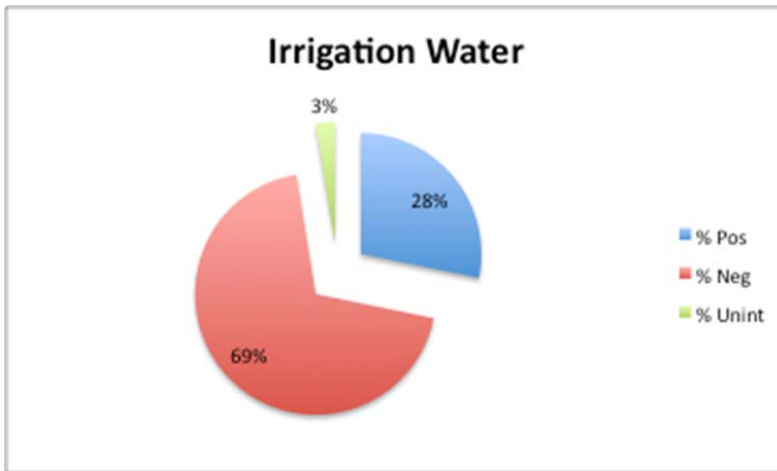


Figure 15: Irrigation Water Results (Percent Positive, negative and uninterpretable)

qPCR INHIBITION. A total of 37% (65/174) of the samples evaluated required further dilution of DNA extracts to obtain qPCR results that were interpretable. Of the 65, 77% (50/65) required dilution up to 1,000-fold and successfully amplified. This left 23% (15/65) of the samples “uninterpretable,” requiring dilution in excess of 1,000-fold. Of the 50 samples that were diluted and subsequently successfully amplified, 44% (22/50) were diluted 10-fold, 30% (15/50) were diluted 100-fold, and finally 26% (13/50) required one-thousand fold dilution. Of note, four samples were diluted 10,000-fold and amplified yielding a positive result; these were not included in the final calculations, as the cut-off for dilution of experimental samples was 1,000-fold, however this will be discussed further in the discussion.

GENOME EQUIVALENCE COPIES. Genome equivalence copies were calculated for each of the samples based on the Ct values and plasmid standard curve, correcting for sample dilution as appropriate. Overall log average genome equivalence (Appendix C) for samples that tested positive was 5.5, and ranged from 2.9 to 10.1. By environmental sample type, average log GEC for hand rinses was 5.3, ranging from 4.0 to 8.6. Average log GEC for irrigation waters was 6.0, and ranged from 3.6 to 7.7. Average log GEC for produce rinses was 6.1, ranging from 2.9 to 10.1. Alternatively, log GEC averages by produce type were slightly lower, with log average GEC for peppers at 4.6, and ranging from 3.6 to 6.8. For melons, average log GEC was 6.4, and ranged from 4.1 to 9.1. Finally, for tomatoes, average log GEC was 5.4 and ranged from 2.9 to 10.1. When

identifying log GECs for produce rinsates alone, it can be broken down by produce type, with average log GEC for tomatoes at 5.8, and ranging from 2.9 to 10.1. For peppers, the average produce rinsate log GEC concentration was 4.3, and ranged from 3.6 to 5.1, and finally, for melons, the average log GEC was 6.6, and ranged from 4.1 to 9.1.

The overall average Ct value for all positive samples was 30.4, and ranged from 20.0 to 35.0. The average for hand rinse samples was 31.8, and ranged from 20 to 35. In irrigation waters, average Ct value was 28.9, and ranged from 24.3 to 33.7. For produce rinses, average Ct value was 30.3, and ranged from 21.5 to 34.8. By produce type, average Ct value for peppers was 31.9, and ranged from 29.6 to 33.3. For melons, the average Ct value was 29.2, and ranged from 20.0 to 35.0. Finally, for tomatoes, the average Ct value was 31.5, and ranged from 21.8 to 34.8. A table of these values can be seen in Appendix G.

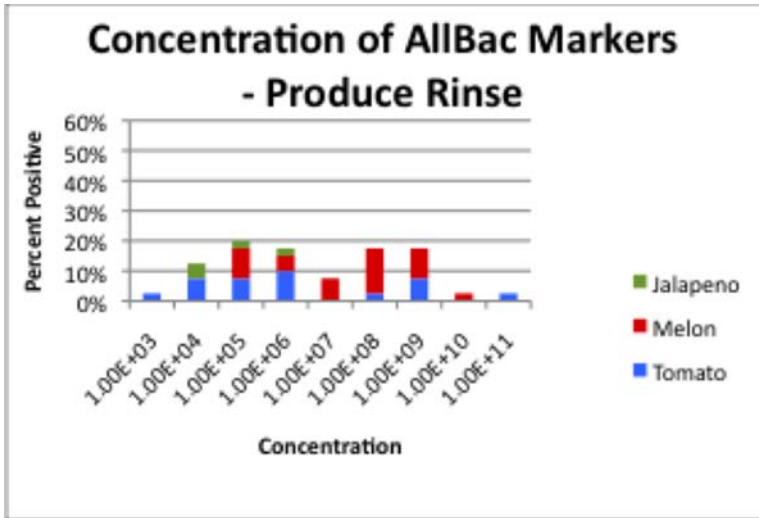


Figure 16: Concentration of AllBac Markers in All Types of Produce Rinses



Figure 17: Concentration of AllBac Markers in Hand Rinse Samples

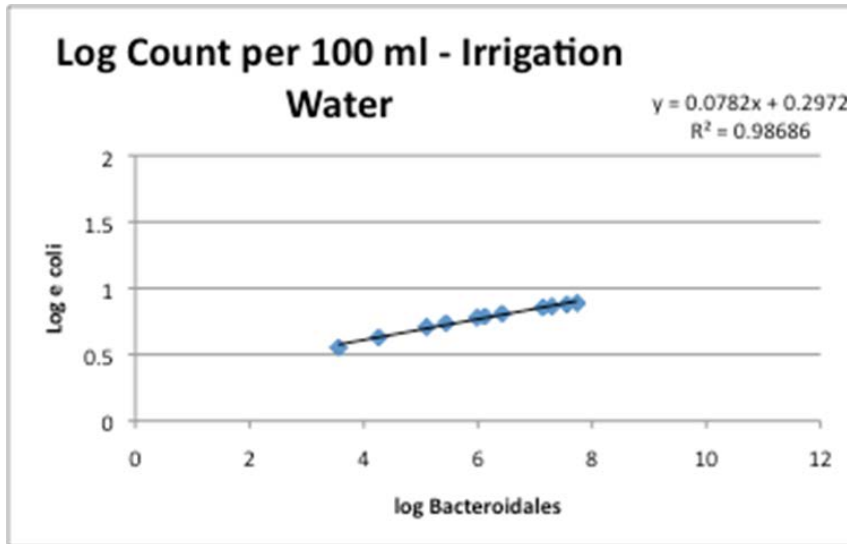


Figure 18: Log Count per 100 ml Bacteroidales to *E. coli*

#### STATISTICAL ANALYSIS OF *E. COLI* VERSUS *BACTEROIDALES*.

Regression of log *E. coli* concentrations for positive samples to those samples against those also positive for the AllBac marker showed a linear trend for the irrigation waters, with a corresponding  $R^2$  value of 0.99, indicating a strong relationship (Figure 18).

In determining whether there is a statistically significant relationship between *E. coli* and Bacteroidales, a Spearman's Rho correlation was used, and the overall relationship between *E. coli* and Bacteroidales was determined (Table 8). This result did not indicate a significant relationship overall between *E. coli* and Bacteroidales; however, when individual produce types were compared, a statistically significant relationship between *E. coli* and Bacteroidales was present in hand rinses. This relationship is an inverse correlation, meaning that when *E. coli* is present Bacteroidales is not.

Additionally, another statistically significant relationship was present between *E. coli* and Bacteroidales in produce rinses of tomatoes. When only samples that were positive for both *E. coli* and Bacteroidales were compared, a significant, positive relationship was found, as seen in Table 8.

**Table 8: Spearman's Rho Correlation by Relationship**

Groups Compared	Sample Type	Produce Type	Result		Sample Size
			Rho	Probability	
Log GEC <i>Bacteroidales</i> Log cfu <i>E. coli</i> (per 100 ml)	Hand Rinse	Jalapeno	0.3191	0.2661	14
		Melon	-0.7465	0.0014	15
		Tomato	-0.0514	0.8157	23
	Irrigation Water	Jalapeno	-0.5443	0.4557	4
		Melon	0.3262	0.3576	10
		Tomato	-0.5774	0.1340	8
	Produce Rinse	Jalapeno	-0.1880	0.4698	17
		Melon	-0.0022	0.9914	26
		Tomato	0.3555	0.0391	34
	Source Water	Jalapeno	No Data	No Data	
		Melon	0.866	0.3333	3
		Tomato	No Data	No Data	
	All Samples	All Produce Types	0.0296	0.7034	168
	Only Positive	All Produce Types	0.4316	0.0003	67

**Table 9: Spearman's Rho for Only Positive *E. coli* and *Bacteroidales* in Irrigation Water and Source Water**

Groups Compared	Sample Type	Data Set	Result		Sample Size
			Rho	Probability	
Log GEC <i>Bacteroidales</i>	Irrigation Water	Only Positives	0.7301	0.0165	10
Log cfu <i>E. coli</i> (per 100 ml)	Source Water	Only Positives	No Data	No Data	1

As seen in Table 9, samples that were positive for both *E. coli* and *Bacteroidales* were compared in irrigation water samples. The irrigation water samples were broken down into both source water and in-field irrigation waters. A significant relationship was found in irrigation waters, but not enough information was available for source waters.

**BoBAC RESULTS.** All samples that tested positive for the AllBac marker were evaluated for the presence of the BoBac marker, however, no sample returned a positive result, indicating no bovine fecal contamination within the samples processed.

**DISCUSSION**

As part of a broader project, the purpose of which was to identify sources of fecal contamination in at-risk fresh produce items (tomatoes, hot peppers, cantaloupe melons) in Northern Mexico, we investigated the utility of bacterial source identifiers, specifically members of the order *Bacteroidales*, as a novel MST method for identification of total fecal contamination, and the associated source of that contamination. Clearly, many of the samples did show evidence of fecal contamination,

since the universal AllBac marker was identified, at some level, in roughly 40% of the samples screened. Relatively speaking, 47% of produce samples, 34% of hand rinsates, and 28% of irrigation waters screened showed evidence of *Bacteroidales* contamination.

There are many ways that fecal contamination can be introduced into the fresh produce production environment. The most prominent among these are (i) the use of improperly treated compost or manure for fertilization; (ii) fecally-impacted irrigation waters; (iii) poor hygiene practices of pickers and packers; and (iv) wild or domestic animal encroachment in growing fields. Other potential but less likely sources of fecal contamination are poorly sanitized harvest/packing equipment, disease-carrying vermin (insects or rodents), or contaminated rinse waters or ice. Unfortunately, we do not know definitively which of these routes might have been associated with *Bacteroidales* contamination in the samples screened in this study. However, the following paragraphs discuss some hypotheses.

Workers hands were frequently positive for *Bacteroidales*. Hands can be a source of contamination during both harvest and packing. According to the farmers participating in this study, most of the workers employed were temporary, itinerant, Mexican natives. They were usually illiterate, speaking only native-tongues (not Spanish), with one “ringleader” who acted as an intermediary between the farmers and workers.

Workers, on the whole, never communicated directly with the farm managers, and vice versa. Migrant workers tend to have poorer sanitation habits (Whalley et al., 2009), and their practices could have impacted the high prevalence of *Bacteroidales* contamination on hand rinsates.

Relatively speaking, irrigation water samples were more often negative for *Bacteroidales* relative to the other sample types. This suggests that these waters were relatively free of fecal contamination, and also suggests that fresh produce contamination is not likely to occur via irrigation waters. This can be juxtaposed to previous work in which the *Bacteroidales*-based universal molecular markers were found in all freshwater samples screened (Mieszkin et al., 2009; Shriewer et al., 2010; Ju-Yong et al., 2009; Silkie and Nelson, 2009; Stapleton et al., 2009). These data cannot be directly compared to ours, however, as our irrigation waters were collected either directly from the water source (as in from the well) or from the irrigation system. The irrigation waters were likely to be less prone to fecal contamination relative to surface waters because the former are less accessible to wild or domestic animals. The one exception to this observation was the irrigation waters associated with cantaloupe production, which had a relatively high degree (75% of samples) of *Bacteroidales* contamination. Unlike tomatoes and peppers, cantaloupes do come in direct contact with irrigation water, and as such, water could have been a source of contamination for this commodity.

When comparing the presence of fecal coliforms (*E. coli*) against the presence of *Bacteroidales* AllBac markers in irrigation water, there is a very obvious trend. The strong relationship indicates that, in the case of irrigation water, the presence of the AllBac marker could signify the presence of fecal contamination, and can strengthen the argument for the use of this marker in evaluating the potential for fecal contamination in irrigation waters.

By produce item, AllBac markers were most frequently found in cantaloupe samples, followed by tomato samples, and lastly by peppers. In fact, the high positivity rate for cantaloupes had a dramatic influence on the overall positivity rate for fresh produce in general. The produce samples originated across the farm to packing continuum, and fecal contamination could occur during production, harvest, and/or packing. In the case of cantaloupe melons, contamination likely had to do with growing conditions for this product, i.e., the fact that the melons are in direct contact with the soil. As such, they may also come in direct contact with fertilizers, fecal material (such as that deposited due to animal encroachment or human defecation of fields), or by contact with contaminated irrigation waters.

Another important factor responsible for the higher prevalence of *Bacteroidales* markers (and perhaps fecal contamination) in cantaloupes is the relatively larger surface area of this product. In addition, and perhaps more importantly, are the characteristics of the surface of cantaloupes. The surface of the cantaloupe melon has ridges and crevices, referred to as netting, creating a degree of roughness that provides

areas for filth and microbes to easily lodge and proliferate, if given the right conditions. The netting also makes it more difficult to remove filth and associated microbes from cantaloupes during washing steps (Ukuku and Fett, 2002). Additionally, the leaves of the melon plant can provide some shelter for microorganisms present on the surface of the fruit, preventing temperature swings, and blocking sunlight (Stine et al., 2005). There is also concern that microbes can colonize the surface of cantaloupe, produce substances that can prevent disinfection, and create biofilms (Annous et al., 2008; Solomon and Sharma, 2009). Indeed, melons have been associated with outbreaks of a number of diverse foodborne disease agents over recent years (Mohle-Bohetani et al., 1997; Harris et al., 2006; Lynch et al., 2006).

Interestingly, the sample pool did not show any indication of bovine-related fecal contamination. It is possible that there might not be a presence of bovine fecal pollution, and as such, none was detected. However, common practice in fresh produce farming is the application of bovine manure as a fertilizer. In the US, 120 days must lapse between time of application of untreated manure and time of harvest, for the produce to be considered safe (Natvig et al., 2002). Time of year can also be considered. The climate in the Mexican regions considered tends to be very hot during the summer months. It is possible that any fecal pollution that may have been present was killed by the heat or sunlight. The study done by Natvig et al. (2002), determined that fecal material survived less during the spring to summer months. This study was also

conducted in Wisconsin, and so evaluation of geographical location would seem that the spring to summer months would be even more intense as farms neared the equator. However, in a study done by Mukherjee et al. (2003), found no presence of the fecal pathogen *E. coli* O157:7 in a variety of samples. It is possible that cow manure may not have been used on these farms, and other types of fertilizers were used.

A consistent problem with the assays used in this study was the presence of matrix-associated inhibitory compounds that frequently cause false negative PCR results, despite rigorous DNA extraction and purification methods. Many, many compounds have been demonstrated to be inhibitory of PCR, and environmental samples (waters, soils, produce) are notorious for PCR inhibition (Yeats et al., 1998; LaMontagne et al., 2001). Such inhibitors include tannic, humic, and fulvic acids and acidic plant polysaccharides (Arbeli and Fuentes, 2007); humic acids, common to environmental samples, are particularly troublesome (Yeats et al., 1998; LaMontagne et al., 2001). These can also interfere with DNA quantification using ultraviolet absorbance (Yeats et al., 1998). A variety of compounds can be added to the DNA extraction method, as well as to the PCR to prevent co-extraction, and later interference of the PCR. There are a variety of methods that can be used to reduce the effects of matrix associated inhibition, by focusing on the DNA extraction and purification step (LaMontagne, et al. 2001), pre-treatment of DNA prior to amplification by filtration (Miller et al., 1999), and/or the addition of PCR additive such as bovine serum albumin (BSA), or phage T4 gene 32

protein can be added to a PCR mastermix to scavenge inhibitors (Shriewer et al., 2010; Park et al., 2010).

Because our study was pilot-level and proof-of-concept, we did not investigate these options. Instead, we relied on sample dilution to “dilute out” potential inhibitors. About 60% of the positive samples required dilution of 10-fold or 100-fold before achieving interpretable qPCR results. Roughly 7% of the sample pool required up to 1,000-fold; another 8% of the sample pool was uninterpretable, based on limiting the level of dilution to 1,000-fold. However, four samples were evaluated past the 1,000-fold dilution cut-off. These samples were diluted 10,000-fold, and yielded a weakly positive result, with Ct values greater than 33. These samples could be positive, with very high levels of inhibitors (all four were melon samples), however, dilution of the template in a large volume of diluent may not be reliable in identifying weakly positive samples. Interestingly, the greatest number of uninterpretable results was associated with tomato samples, for reasons unexplained. As perhaps expected, cantaloupes demonstrated the highest degree of matrix-associated inhibition of the three commodity groups, and seems probably a function of the high degree of filth on this product, as demonstrated by the previously described samples that were diluted 10,000-fold.

The dilution approach has been used by others (Mieszkin et al., 2009; Stapleton et al., 2009; Bae and Wertz, 2009; Mieszkin et al., 2009b; Silkie and Nelson, 2009; Dick et al., 2010) to account for matrix-associated inhibition. Few of these studies specifically discuss effects of inhibition or even prevalence of inhibition, with one study stating that no inhibition was observed (Dick et al., 2010), however, one study did specifically discuss the potential of inhibition due to internal controls, or inhibition preventing the amplification of the internal controls. Shanks et al. (2007), developed two homologous controls and measured adequate amplification to determine the most effective cycle threshold for prevention of inhibition due to multiplex reactions. The IACs developed in this study did not appear to inhibit the amplification of the target, however, further discussion about the potential for inhibition was not discussed. Other studies have sought to deal with the inhibition problem using PCR enhancement agents like bovine serum albumin (BSA) (Schriewer et al., 2010, Bernard and Field, 2000). In the study done by Schriewer et al. (2010), low levels of inhibition were noted, indicating that the use of BSA as an inhibitor suppressant may be effective. Overall, the fact that some inhibited samples required dilution up to 1,000-fold does not necessarily bode well for the efficiency of AllBac assay, since template can easily be diluted out along with inhibitors. In short, a negative *Bacteroidales* assay result after sample dilution must be interpreted with caution, and perhaps should be termed as “presumptively negative.” Roughly 80% (50/64) tested presumptively positive, and of the diluted samples, roughly 30% (54/174) tested positive, and roughly 75% (82/174). Clearly, residual

qPCR inhibition had a major impact on the interpretation of our results, and perhaps on the usefulness of the assay in general.

To address the potential for false negative results due to residual qPCR inhibition, we incorporated an internal amplification control (IAC). Regarding IACs, two types can be used, competitive (homologous) and noncompetitive (heterologous). In a competitive control, the target DNA and the control DNA are amplified simultaneously using the same set of primers and two differently labeled fluorescent probes. Even with the advantages of this approach (i.e., the ability to optimize annealing temperature, the relative increase in amplification efficiency that comes from circumventing duplex amplification) (Hoorfar et al., 2004), when template levels are low, the IAC can still out-compete the target. Consequently, we ran our qPCR amplifications both with and without inclusion of the IAC, for preliminary studies, to understand adequate cut-off points for determining appropriate concentration. Roughly 20% of the samples had an IAC Ct value that was outside of the acceptable range of 29-31. For those samples that were questionable, such as those that had coamplification of the target and control within roughly three cycle thresholds, subjective interpretations were made. Other studies in the literature have used both heterologous and homologous internal amplification controls that were either developed by the investigators (Shanks et al., 2009; Shanks et al., 2007; Green et al., 2011) or purchased from a supplier (Mieszkin et al., 2009; Mieszkin et al., 2009b). These controls appear to be effective in confirming the functionality of the PCR, and whether samples must be diluted to account for

potential inhibitors. Many of the studies discussing the use of controls do not go into great detail about the approach, or really the need for mechanisms of control. There were also a number of studies that did not use a control, or discuss the potential for inhibition (Stapleton et al., 2009; Jeong, et al., 2009; Silkie and Nelson, 2009; Reischer et al., 2006). Ultimately, our decision to use dilution as a means of inhibition alleviation was based on the results of a study done by Cao et al., (2012), where the researchers found that inhibition was most effectively reduced by diluting the template prior to use in PCR.

Standard curves were used to estimate *Bacteroidales* load based on genome equivalent copies (GEC) for the AllBac marker. The standard curve was constructed based on amplification of two different targets, the first being the *Bacteroides thetaiotaomicron* rDNA gene, which was inserted into a cloning vector, and the second being a preparation of genomic DNA derived directly from *B. thetaiotaomicron* and amplified by the AllBac primers. A key difference between using the plasmid standard versus the genomic DNA standard is that the AllBac marker appears within the genome of *B. thetaiotaomicron* five times. This difference is clearly reflected in the detection limits of the standard curves (Figure F). Because the first method corresponds to amplification of a single gene copy (rather than multiple gene copies), it was chosen for quantification of GEC in our study. It should be noted that, because *Bacteroidales* have multiple copies of the rDNA gene of interest, there is not a direct relationship between GEC and *Bacteroidales* cell numbers (BLAST Search). Further, since DNA can remain stable long

after cell death, there is no real relationship between *Bacteroidales* GEC and the presence of viable cells (Bae and Wuertz, 2009).

This approach to quantification of *Bacteroidales* as applied to various water systems (ground and surface waters) using the AllBac marker has been used by others. Marker concentrations vary from study to study, based on the volume of water processed, however, genome equivalence copies (GEC) range from  $10^2$  to  $10^8$  for the universal markers (Dick et al., 2010; Mieszkin et al., 2009; Shriewer et al., 2010; Jeong et al., 2009; Stapleton et al., 2009; Jeong et al., 2009;). For the host-specific markers, GEC also vary by study, but usually range from  $10^3$  to  $10^7$  over a variety of sample volumes, ranging from 1 ml to 1 liter (Mieszkin et al., 2009b; Reischer et al., 2006; Stapleton et al., 2009); human marker concentrations range from  $10^4$  to  $10^6$  GEC per 100 ml (Stapleton et al., 2009; Jeong et al., 2009).

One interesting remaining question is the relationship between alternative indicator concentrations, such as *Bacteroidales*, and the levels of traditional indicators of fecal contamination, such as *E. coli*. Overall, these are very poor correlations, suggesting that the presence of *Bacteroidales* markers has little relationship to the presence of *E. coli*. Overall, others have reported similarly poor correlations between the presence of *E. coli* and *Bacteroidales*. Specifically, Dorai-Raj et al. (2009) found that rural water samples from pasture-lands containing high numbers of *E. coli* (77 and 178 cfu/100 ml) were negative for ruminant-specific markers. Another study done by Shanks et al. (2006) found poor correlation between *Bacteroidales* ruminant-specific markers and *E. coli*

counts. Finally, Layton et al. (2006) found only a loose correlation between *E. coli* presence and presence of *Bacteroidales* markers. This observation could be a function of a number of factors. First, the relatively high level of inhibitors, results in the need for extensive sample dilution and in some instances, yields uninterpretable data. *Bacteroidales* may simply be more prevalent, or present at higher levels, compared to *E. coli*, making them easier to detect (Dick et al., 2010).

One issue in our study was the time between sample collection and *Bacteroidales* detection. In classic food microbiology studies, microbiological assays should be initiated within 24 h of sample collection when attempting to enumerate viable organisms. The literature clearly states that, while *Bacteroidales* persistence varies by the type of assay, these markers can be quite stable. For example, markers in *Bacteroides distasonis* were detected up to 14 days at 4°C, however, only 1-2 days at 24°C (Kreader 1998). Dick et al. (2010) found that the human-specific markers (HF 183, BacHum) decayed at a much faster rate did the AllBac marker or *E. coli* under high sunlight, salinity and temperature. On the other hand, others have reported that *Bacteroidales* marker levels declined much faster than did *E. coli* levels (Walters and Field, 2009; Dick and Walters, 2009). Our samples did arrive at refrigeration temperature, but frequently over 24 h after collection. They were, however, packaged in double-lined boxes, so marker degradation due to light was unlikely. It is possible that native bacterial populations (such as from the surface of the fruits or the hands of workers) may have introduced predator organisms that might have degraded host-

specific markers. Based on the body of literature, it is possible that the host-specific markers (HF 183 – human-specific; BoBac – bovine-specific) degraded rather quickly, but the universal marker (AllBac) may have persisted longer. This could explain, at least in part, our inability to detect ruminant-specific fecal contamination. However, when all is said and done, molecular-based assays such as the one reported here may be more amenable to lengthy gaps between sample collection and assay initiation, as may be necessary when collecting samples from great distances or in rural/developing areas with limited access to overnight shipping.

This study sought to provide proof-of-concept that *Bacteroidales* markers could be alternative indicators of fecal contamination in the fresh produce production through packing continuum. Overall, we were able to detect the general *Bacteroidales* marker, AllBac, in many different sample types with a high prevalence. There was little correlation between the presence of AllBac markers and *E. coli*, the traditional fecal indicator. Although the *Bacteroidales* assays offer promise as an indicator of fecal contamination, its use poses problems. These problems include: 1) the complexity of the analytic procedure, including sample preparation and DNA extraction; 2) the propensity for matrix-associated qPCR inhibition; and 3) a poor understanding of their environmental persistence. Further work clearly needs to be done to validate that the presence of species-specific *Bacteroidales* markers actually do correlate with the presence of species-specific fecal contamination. If so, then these methods may be

promising alternatives for evaluation of fecal contamination sources from farm-to-fork, including temporal associations. Despite the need for additional work, this study does demonstrate that the general *Bacteroidales* markers are quite prevalent in the fresh produce production environment, and that their detection may ultimately facilitate MST within this complex farm-to-fork chain.

**REFERENCES**

- Anderson, Kimberly L, John E Whitlock, and Valerie J Harwood. "Persistence and Differential Survival of Fecal Indicator Bacteria in Subtropical Waters and Sediments." *Applied and Environmental Microbiology* 71, no. 6 (June 2005): 3041–8. doi:10.1128/AEM.71.6.3041-3048.2005.
- Anderson, Maren, Lee-Ann Jaykus, Steve Beaulieu, and Sherri Dennis. "Pathogen-produce Pair Attribution Risk Ranking Tool to Prioritize Fresh Produce Commodity and Pathogen Combinations for Further Evaluation (P3ARRT)." *Food Control* 22, no. 12 (December 2011): 1865–1872. doi:10.1016/j.foodcont.2011.04.028.
- Annous, Bassam A., Ethan B. Solomon, Peter H. Cooke, and Angela Burke. "Biofilm Formation by Salmonella Spp. on Cantaloupe Melons\*\*." *Journal of Food Safety* 25, no. 4 (2005): 276–287. doi:10.1111/j.1745-4565.2005.00024.x.
- Annous, BassamA, PinaM Fratamico, and James L. Smith. "Quorum Sensing in Biofilms: Why Bacteria Behave the Way They Do." (2009) (n.d.).
- Arbeli, Ziv, and Cilia L Fuentes. "Improved Purification and PCR Amplification of DNA from Environmental Samples." *FEMS Microbiology Letters* 272, no. 2 (July 2007): 269–75. doi:10.1111/j.1574-6968.2007.00764.x.
- Bae, Sungwoo, and Stefan Wuertz. "Discrimination of Viable and Dead Fecal Bacteroidales Bacteria by Quantitative PCR with Propidium Monoazide." *Applied and Environmental Microbiology* 75, no. 9 (May 1, 2009): 2940–2944. doi:10.1128/AEM.01333-08.
- Bae, Sungwoo and Stefan Wuertz. "Rapid Decay of Host-specific Fecal Bacteroidales Cells in Seawater as Measured by Quantitative PCR with Propidium Monoazide." *Water Research* 43, no. 19 (November 2009): 4850–4859. doi:10.1016/j.watres.2009.06.053.
- Behravesh, Casey, Rajal K. Mody, Jessica Jungk, Linda Gaul, John T. Redd, Sanny Chen, Shaun Cosgrove, et al. "2008 Outbreak of Salmonella Saintpaul Infections Associated with Raw Produce." *New England Journal of Medicine* 364, no. 10 (2011): 918–927. doi:10.1056/NEJMoa1005741.

Berger, Cedric N, Samir V Sodha, Robert K Shaw, Patricia M Griffin, David Pink, Paul Hand, and Gad Frankel. "Fresh Fruit and Vegetables as Vehicles for the Transmission of Human Pathogens." *Environmental Microbiology* 12, no. 9 (September 2010): 2385–97. doi:10.1111/j.1462-2920.2010.02297.x.

Bhagwat, Arvind A, Robert A Saftner, and Judith A Abbott. "Evaluation of Wash Treatments for Survival of Foodborne Pathogens and Maintenance of Quality Characteristics of Fresh-cut Apple Slices." *Food Microbiology* 21, no. 3 (June 2004): 319–326. doi:10.1016/j.fm.2003.08.001.

Brion, G M, J S Meschke, and M D Sobsey. "F-specific RNA Coliphages: Occurrence, Types, and Survival in Natural Waters." *Water Research* 36, no. 9 (May 2002): 2419–25.

Bustin, S. A., V. Benes, T. Nolan, and M. W. Pfaffl. "Quantitative Real-time RT-PCR – a Perspective." *Journal of Molecular Endocrinology* 34, no. 3 (June 1, 2005): 597–601. doi:10.1677/jme.1.01755.

Office of the Commissioner, 2007 - FDA Finalizes Report on 2006 Spinach Outbreak. Office of the Commissioner, n.d.. <http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/2007/ucm108873.htm>

Costerton, J. W., Philip S. Stewart, and E. P. Greenberg. "Bacterial Biofilms: A Common Cause of Persistent Infections." *Science* 284, no. 5418 (May 21, 1999): 1318–1322. doi:10.1126/science.284.5418.1318.

Coyne, Michael J, and Laurie E Comstock. "Niche-specific Features of the Intestinal Bacteroidales." *Journal of Bacteriology* 190, no. 2 (January 2008): 736–42. doi:10.1128/JB.01559-07.

Davies, CM, JA Long, M Donald, and NJ Ashbolt. "Survival of Fecal Microorganisms in Marine and Freshwater Sediments." *Appl. Envir. Microbiol.* 61, no. 5 (May 1995): 1888–1896.

Desmarais, T. R., H. M. Solo-Gabriele, and C. J. Palmer. "Influence of Soil on Fecal Indicator Organisms in a Tidally Influenced Subtropical Environment." *Applied and Environmental Microbiology* 68, no. 3 (March 2002): 1165–1172. doi:10.1128/AEM.68.3.1165-1172.2002.

Dick, Linda K, Anne E Bernhard, Timothy J Brodeur, Jorge W Santo Domingo, Joyce M Simpson, Sarah P Walters, and Katharine G Field. "Host Distributions of Uncultivated Fecal Bacteroidales Bacteria Reveal Genetic Markers for Fecal Source Identification." *Applied and Environmental Microbiology* 71, no. 6 (June 2005): 3184–91. doi:10.1128/AEM.71.6.3184-3191.2005.

Dick, Linda K, and Katharine G Field. "Rapid Estimation of Numbers of Fecal Bacteroidetes by Use of a Quantitative PCR Assay for 16S rRNA Genes." *Applied and Environmental Microbiology* 70, no. 9 (September 2004): 5695–7. doi:10.1128/AEM.70.9.5695-5697.2004.

Elmir, Samir M, Tomoyuki Shibata, Helena M Solo-Gabriele, Christopher D Sinigalliano, Maribeth L Gidley, Gary Miller, Lisa R W Plano, Jonathan Kish, Kelly Withum, and Lora E Fleming. "Quantitative Evaluation of Enterococci and Bacteroidales Released by Adults and Toddlers in Marine Water." *Water Research* 43, no. 18 (October 2009): 4610–6. doi:10.1016/j.watres.2009.07.006.

Endley S., Lu L., Vega E., Hume M.E., and Pillai S.D. "Male-Specific Coliphages as an Additional Fecal Contamination Indicator for Screening Fresh Carrots." *Journal of Food Protection* 66, no. 1 (2003): 88–93.

Espinosa, Isabel Y., and Suresh D. Pillai. "Impaction-Based Sampler for Detecting Male-Specific Coliphages in Bioaerosols." *Journal of Rapid Methods & Automation in Microbiology* 10, no. 2 (2002): 117–127. doi:10.1111/j.1745-4581.2002.tb00017.x.

Field, Katharine G, and Mansour Samadpour. "Fecal Source Tracking, the Indicator Paradigm, and Managing Water Quality." *Water Research* 41, no. 16 (August 2007): 3517–38. doi:10.1016/j.watres.2007.06.056.

Fiksdal, L, J S Maki, S J LaCroix, and J T Staley. "Survival and Detection of Bacteroides Spp., Prospective Indicator Bacteria." *Appl. Envir. Microbiol.* 49, no. 1 (January 1985): 148–150.

Frank, Christina, Dirk Werber, Jakob P. Cramer, Mona Askar, Mirko Faber, Matthias an der Heiden, Helen Bernard, et al. "Epidemic Profile of Shiga-Toxin–Producing Escherichia Coli O104:H4 Outbreak in Germany." *New England Journal of Medicine* 365, no. 19 (2011): 1771–1780. doi:10.1056/NEJMoa1106483.

GRIFFIN, DALE W., ERIN K. LIPP, MOLLY R. McLAUGHLIN, and JOAN B. ROSE. "Marine Recreation and Public Health Microbiology: Quest for the Ideal Indicator." *BioScience* 51, no. 10 (March 2001): 817. doi:10.1641/0006-3568(2001)051[0817:MRAPHM]2.0.CO;2.

Griffin, DW, R Stokes, JB Rose, and JH Paul. "Bacterial Indicator Occurrence and the Use of an F(+) Specific RNA Coliphage Assay to Identify Fecal Sources in Homosassa Springs, Florida." *Microbial Ecology* 39, no. 1 (January 2000): 56–64.

Harris, L.j., J.n. Farber, L.r. Beuchat, M.e. Parish, T.v. Suslow, E.h. Garrett, and F.f. Busta. "Outbreaks Associated with Fresh Produce: Incidence, Growth, and Survival of Pathogens in Fresh and Fresh-Cut Produce." *Comprehensive Reviews in Food Science and Food Safety* 2 (2003): 78–141. doi:10.1111/j.1541-4337.2003.tb00031.x.

Harwood, Valerie J, Miriam Brownell, Shiao Wang, Joe Lepo, R D Ellender, Abidemi Ajidahun, Kristen N Hellein, Elizabeth Kennedy, Xunyan Ye, and Christopher Flood. "Validation and Field Testing of Library-independent Microbial Source Tracking Methods in the Gulf of Mexico." *Water Research* 43, no. 19 (November 2009): 4812–9. doi:10.1016/j.watres.2009.06.029.

Holdeman, L V, I J Good, and W E Moore. "Human Fecal Flora: Variation in Bacterial Composition Within Individuals and a Possible Effect of Emotional Stress." *Appl. Envir. Microbiol.* 31, no. 3 (March 1976): 359–375.

Hoorfar, J., B. Malorny, A. Abdulmawjood, N. Cook, M. Wagner, and P. Fach. "Practical Considerations in Design of Internal Amplification Controls for Diagnostic PCR Assays."

*Journal of Clinical Microbiology* 42, no. 5 (May 2004): 1863–1868.  
doi:10.1128/JCM.42.5.1863-1868.2004.

Iturriaga, Montserrat H., Mark L. Tamplin, and Eduardo F. Escartín. “Colonization of Tomatoes by Salmonella Montevideo Is Affected by Relative Humidity and Storage Temperature.” *Journal of Food Protection* 70, no. 1 (2007): 30–34.

Jay, Michele T, Michael Cooley, Diana Carychao, Gerald W Wiscomb, Richard A Sweitzer, Leta Crawford-Miksza, Jeff A Farrar, et al. “Escherichia Coli O157:H7 in Feral Swine Near Spinach Fields and Cattle, Central California Coast.” *Emerging Infectious Diseases* 13, no. 12 (December 2007): 1908–11.

Johnson, Timothy J., Yvonne M. Wannemuehler, and Lisa K. Nolan. “Evolution of the Iss Gene in Escherichia Coli.” *Applied and Environmental Microbiology* 74, no. 8 (April 15, 2008): 2360–2369. doi:10.1128/AEM.02634-07.

Johnston, Lynette M., Lee-Ann Jaykus, Deborah Moll, Juan Anciso, Brenda Mora, and Christine L. Moe. “A Field Study of the Microbiological Quality of Fresh Produce of Domestic and Mexican Origin.” *International Journal of Food Microbiology* 112, no. 2 (November 1, 2006): 83–95. doi:10.1016/j.ijfoodmicro.2006.05.002.

Kreader, Carol A. “Persistence of PCR-Detectable Bacteroides Distasonis from Human Feces in River Water.” *Appl. Envir. Microbiol.* 64, no. 10 (October 1998): 4103–4105.

Krsek, M., and E.M.H. Wellington. “Comparison of Different Methods for the Isolation and Purification of Total Community DNA from Soil.” *Journal of Microbiological Methods* 39, no. 1 (December 1999): 1–16. doi:10.1016/S0167-7012(99)00093-7.

Laksanalamai, Pongpan, Lavin A Joseph, Benjamin J Silk, Laurel S Burall, Cheryl L Tarr, Peter Gerner-Smidt, and Atin R Datta. “Genomic Characterization of Listeria Monocytogenes Strains Involved in a Multistate Listeriosis Outbreak Associated with Cantaloupe in US.” Edited by Nancy E. Freitag. *PloS One* 7, no. 7 (January 2012): e42448. doi:10.1371/journal.pone.0042448.

LaMontagne, M.G., F.C. Michel Jr., P.A. Holden, and C.A. Reddy. "Evaluation of Extraction and Purification Methods for Obtaining PCR-amplifiable DNA from Compost for Microbial Community Analysis." *Journal of Microbiological Methods* 49, no. 3 (May 2002): 255–264. doi:10.1016/S0167-7012(01)00377-3.

Leclerc, H, D A Mossel, S C Edberg, and C B Struijk. "Advances in the Bacteriology of the Coliform Group: Their Suitability as Markers of Microbial Water Safety." *Annual Review of Microbiology* 55 (January 2001): 201–34. doi:10.1146/annurev.micro.55.1.201.

Lemann, Jacob, Nancy D. Adams, and Richard W. Gray. "Urinary Calcium Excretion in Human Beings." *New England Journal of Medicine* 301, no. 10 (1979): 535–541. doi:10.1056/NEJM197909063011008.

Long, Sharon C., Samar S. El-Khoury, Sjon J.G. Oudejans, Mark D. Sobsey, and Jan Vinjé. "Assessment of Sources and Diversity of Male-Specific Coliphages for Source Tracking." *Environmental Engineering Science* 22, no. 3 (May 2005): 367–377.

Lopez-Torres, Arleen J., Terry C. Hazen, and Gary A. Toranzos. "Distribution and in Situ Survival and Activity of *Klebsiella Pneumoniae* and *Escherichia Coli* in a Tropical Rain Forest Watershed." *Current Microbiology* 15, no. 4 (July 1987): 213–218. doi:10.1007/BF01577533.

Lu, Jingrang, Jorge W Santo Domingo, Regina Lamendella, Thomas Edge, and Stephen Hill. "Phylogenetic Diversity and Molecular Detection of Bacteria in Gull Feces." *Applied and Environmental Microbiology* 74, no. 13 (July 2008): 3969–76.

Luo, Yaguang, Xiangwu Nou, Yang Yang, Isabel Alegre, Ellen Turner, Hao Feng, Maribel Abadias, and William Conway. "Determination of Free Chlorine Concentrations Needed To Prevent *Escherichia Coli* O157:H7 Cross-Contamination During Fresh-Cut Produce Wash." *Journal of Food Protection* 74, no. 3 (2011): 7. doi:<a href="http://dx.doi.org/10.4315/0362-028X.JFP-10-429">http://dx.doi.org/10.4315/0362-028X.JFP-10-429</a>.

LYNCH, M. F., R. V. TAUXE, and C. W. HEDBERG. "The growing burden of foodborne outbreaks due to contaminated fresh produce: risks and opportunities." *Epidemiology and Infection* 137, no. 03 (March 2009): 307–315.

Mead, P S, L Slutsker, V Dietz, L F McCaig, J S Bresee, C Shapiro, P M Griffin, and R V Tauxe. "Food-related Illness and Death in the United States." *Emerging Infectious Diseases* 5, no. 5 (n.d.): 607–25.

Meays, Cynthia L, Klaas Broersma, Rick Nordin, and Asit Mazumder. "Source Tracking Fecal Bacteria in Water: a Critical Review of Current Methods." *Journal of Environmental Management* 73, no. 1 (October 2004): 71–9.

Mieszkin, S, J-F Yala, R Joubrel, and M Gourmelon. "Phylogenetic Analysis of Bacteroidales 16S rRNA Gene Sequences from Human and Animal Effluents and Assessment of Ruminant Faecal Pollution by Real-time PCR." *Journal of Applied Microbiology* 108, no. 3 (March 2010): 974–84. doi:10.1111/j.1365-2672.2009.04499.x.

Mieszkin, Sophie, Jean-Pierre Furet, Gérard Corthier, and Michèle Gourmelon. "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, no. 10 (May 15, 2009): 3045–3054. doi:10.1128/AEM.02343-08.

Mohle-Boetani, Janet C., Roshan Reporter, S. Benson Werner, Sharon Abbott, Jeff Farrar, Stephen H. Waterman, and Duc J. Vugia. "An Outbreak of Salmonella Serogroup Saphra Due to Cantaloupes from Mexico." *Journal of Infectious Diseases* 180, no. 4 (October 1, 1999): 1361–1364. doi:10.1086/314995.

Mossel, D A, and C B Struijk. "[Escherichia Coli, Other Enterobacteriaceae and Additional Indicators as Markers of Microbiologic Quality of Food: Advantages and Limitations]." *Microbiología (Madrid, Spain)* 11, no. 1 (March 1995): 75–90.

Nutrition, Center for Food Safety, and Applied. *Bacteriological Analytical Manual (BAM) - Archived BAM Method: Rapid Methods for Detecting Foodborne Pathogens*. Center for Food Safety and Applied Nutrition, n.d.  
<http://www.fda.gov/food/scienceresearch/laboratorymethods/bacteriologicalanalyticmanualbam/ucm109652.htm>.

Osawa, S, K Furuse, and I Watanabe. "Distribution of Ribonucleic Acid Coliphages in Animals." *Appl. Envir. Microbiol.* 41, no. 1 (January 1981): 164–168.

Pachepsky, Y. A., and D. R. Shelton. "Escherichia Coli and Fecal Coliforms in Freshwater and Estuarine Sediments." *Critical Reviews in Environmental Science and Technology* 41, no. 12 (2011): 1067–1110. doi:10.1080/10643380903392718.

Park, Si Hong, Irene Hanning, Robin Jarquin, Philip Moore, Dan J Donoghue, Ann M Donoghue, and Steven C Ricke. "Multiplex PCR Assay for the Detection and Quantification of Campylobacter Spp., Escherichia Coli O157:H7, and Salmonella Serotypes in Water Samples." *FEMS Microbiology Letters* 316, no. 1 (March 2011): 7–15. doi:10.1111/j.1574-6968.2010.02188.x.

Peirson, Stuart N., Jason N. Butler, and Russell G. Foster. "Experimental Validation of Novel and Conventional Approaches to Quantitative Real-time PCR Data Analysis." *Nucleic Acids Research* 31, no. 14 (July 15, 2003): e73–e73. doi:10.1093/nar/gng073.

Pillai, Suresh D. "Bacteriophages as Fecal Indicator Organisms." In *Viruses in Foods*, edited by Sagar M. Goyal, 205–222. Food Microbiology and Food Safety. Springer US, 2006. [http://link.springer.com/chapter/10.1007/0-387-29251-9\\_8](http://link.springer.com/chapter/10.1007/0-387-29251-9_8).

Rayner, Joanna, Richard Veeh, and Janine Flood. "Prevalence of Microbial Biofilms on Selected Fresh Produce and Household Surfaces." *International Journal of Food Microbiology* 95, no. 1 (August 15, 2004): 29–39. doi:10.1016/j.ijfoodmicro.2004.01.019.

Richards, G. P. "Enteric Virus Contamination of Foods Through Industrial Practices: a Primer on Intervention Strategies." *Journal of Industrial Microbiology and Biotechnology* 27, no. 2 (August 1, 2001): 117–125. doi:10.1038/sj.jim.7000095.

De Roever, C. "Microbiological Safety Evaluations and Recommendations on Fresh Produce." *Food Control* 9, no. 6 (December 1998): 321–347. doi:10.1016/S0956-7135(98)00022-X.

Roose-Amsaleg, C.L, E Garnier-Sillam, and M Harry. "Extraction and Purification of Microbial DNA from Soil and Sediment Samples." *Applied Soil Ecology* 18, no. 1 (September 2001): 47–60. doi:10.1016/S0929-1393(01)00149-4.

Sapers, Gerald M., Ethan B. Solomon, and Karl R. Matthews. *The Produce Contamination Problem: Causes and Solutions*. Academic Press, 2009.

Savichtcheva, Olga, and Satoshi Okabe. "Alternative Indicators of Fecal Pollution: Relations with Pathogens and Conventional Indicators, Current Methodologies for Direct Pathogen Monitoring and Future Application Perspectives." *Water Research* 40, no. 13 (July 2006): 2463–2476. doi:10.1016/j.watres.2006.04.040.

Scallan, Elaine, Patricia M Griffin, Frederick J Angulo, Robert V Tauxe, and Robert M Hoekstra. "Foodborne Illness Acquired in the United States—unspecified Agents." *Emerging Infectious Diseases* 17, no. 1 (January 2011): 16–22. doi:10.3201/eid1701.091101p2.

Scallan, Elaine, Robert M Hoekstra, Frederick J Angulo, Robert V Tauxe, Marc-Alain Widdowson, Sharon L Roy, Jeffery L Jones, and Patricia M Griffin. "Foodborne Illness Acquired in the United States—major Pathogens." *Emerging Infectious Diseases* 17, no. 1 (January 2011): 7–15. doi:10.3201/eid1701.091101p1.

Scharff, Robert L. "Economic Burden from Health Losses Due to Foodborne Illness in the United States." *Journal of Food Protection*® 75, no. 1 (2012): 123–131. doi:10.4315/0362-028X.JFP-11-058.

Scharff, Robert L. *Health-Related Costs from Foodborne Illness in the United States*, 2010.

Schneider, Salome, Jürg Enkerli, and Franco Widmer. "A Generally Applicable Assay for the Quantification of Inhibitory Effects on PCR." *Journal of Microbiological Methods* 78, no. 3 (September 2009): 351–3. doi:10.1016/j.mimet.2009.06.010.

Schriewer, Alexander, Woutrina A Miller, Barbara A Byrne, Melissa A Miller, Stori Oates, Patricia A Conrad, Dane Hardin, et al. "Presence of Bacteroidales as a Predictor of Pathogens in Surface Waters of the Central California Coast." *Applied and Environmental Microbiology* 76, no. 17 (September 2010): 5802–14. doi:10.1128/AEM.00635-10.

Scott, T. M., S. Parveen, K. M. Portier, J. B. Rose, M. L. Tamplin, S. R. Farrah, A. Koo, and J. Lukasik. "Geographical Variation in Ribotype Profiles of Escherichia Coli Isolates from Humans, Swine, Poultry, Beef, and Dairy Cattle in Florida." *Applied and Environmental Microbiology* 69, no. 2 (February 2003): 1089–1092.

Scott, T. M., J. B. Rose, T. M. Jenkins, S. R. Farrah, and J. Lukasik. "Microbial Source Tracking: Current Methodology and Future Directions." *Applied and Environmental Microbiology* 68, no. 12 (December 2002): 5796–5803. doi:10.1128/AEM.68.12.5796-5803.2002.

Service, Oregon State University Extension, C. K. (Cindy K. ) Bower, S. Stan, M. Daeschel, and Y. Zhao. "Promoting the safety of Northwest fresh and processed berries" Technical Report, October 2003. <http://ir.library.oregonstate.edu/xmlui/handle/1957/20310>.

Seurinck, Sylvie, Tom Defoirdt, Willy Verstraete, and Steven D Siciliano. "Detection and Quantification of the Human-specific HF183 Bacteroides 16S rRNA Genetic Marker with Real-time PCR for Assessment of Human Faecal Pollution in Freshwater." *Environmental Microbiology* 7, no. 2 (March 2005): 249–59. doi:10.1111/j.1462-2920.2004.00702.x.

Shanks, Orin C., Karen White, Catherine A. Kelty, Sam Hayes, Mano Sivaganesan, Michael Jenkins, Manju Varma, and Richard A. Haugland. "Performance Assessment PCR-Based Assays Targeting Bacteroidales Genetic Markers of Bovine Fecal Pollution." *Applied and Environmental Microbiology* 76, no. 5 (March 1, 2010): 1359–1366. doi:10.1128/AEM.02033-09.

Siefring, S., M. Varma, E. Atikovic, L. Wymer, and R. A. Haugland. "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* 06, no. 2 (June 2008): 225. doi:10.2166/wh.2008.022.

Silkie, Sarah S, and Kara L Nelson. "Concentrations of Host-specific and Generic Fecal Markers Measured by Quantitative PCR in Raw Sewage and Fresh Animal Feces." *Water Research* 43, no. 19 (November 2009): 4860–71. doi:10.1016/j.watres.2009.08.017.

Stoeckel, Donald M., Erin A. Stelzer, and Linda K. Dick. "Evaluation of Two Spike-and-recovery Controls for Assessment of Extraction Efficiency in Microbial Source Tracking Studies." *Water Research* 43, no. 19 (November 2009): 4820–4827. doi:10.1016/j.watres.2009.06.028.

Straub, Timothy M, and Darrell P Chandler. "Towards a Unified System for Detecting Waterborne Pathogens." *Journal of Microbiological Methods* 53, no. 2 (May 2003): 185–197. doi:10.1016/S0167-7012(03)00023-X.

Sumathi Sivapalasingam, Cindy R. Friedman, Linda Cohen, and Robert V. Tauxe. "Fresh Produce: A Growing Cause of Outbreaks of Foodborne Illness in the United States, 1973 Through 1997." *Journal of Food Protection* 67, no. 10 (2004): 2342–2353.

Swaminathan, B, T J Barrett, S B Hunter, and R V Tauxe. "PulseNet: The Molecular Subtyping Network for Foodborne Bacterial Disease Surveillance, United States." *Emerging Infectious Diseases* 7, no. 3 (n.d.): 382–9.

Tallon, Pam, Brenda Magajna, Cassandra Lofranco, and Kam Tin Leung. "Microbial Indicators of Faecal Contamination in Water: A Current Perspective." *Water, Air, and Soil Pollution* 166, no. 1–4 (September 2005): 139–166. doi:10.1007/s11270-005-7905-4.

Tasara, Taurai, and Roger Stephan. "Evaluation of Housekeeping Genes in *Listeria Monocytogenes* as Potential Internal Control References for Normalizing mRNA Expression Levels in Stress Adaptation Models Using Real-time PCR." *FEMS Microbiology Letters* 269, no. 2 (2007): 265–272. doi:10.1111/j.1574-6968.2007.00633.x.

Tucker, Katherine L., Marian T. Hannan, Honglei Chen, L. Adrienne Cupples, Peter WF Wilson, and Douglas P. Kiel. "Potassium, Magnesium, and Fruit and Vegetable Intakes Are Associated with Greater Bone Mineral Density in Elderly Men and Women." *The American Journal of Clinical Nutrition* 69, no. 4 (April 1, 1999): 727–736.

Walters, Sarah P, and Katharine G Field. "Survival and Persistence of Human and Ruminant-specific Faecal Bacteroidales in Freshwater Microcosms." *Environmental Microbiology* 11, no. 6 (June 2009): 1410–21. doi:10.1111/j.1462-2920.2009.01868.x.

Wendel, Arthur M, Diep Hoang Johnson, Umid Sharapov, Juliana Grant, John R Archer, Timothy Monson, Cindy Koschmann, and Jeffrey P Davis. "Multistate Outbreak of Escherichia Coli O157:H7 Infection Associated with Consumption of Packaged Spinach, August-September 2006: The Wisconsin Investigation." *Clinical Infectious Diseases : an Official Publication of the Infectious Diseases Society of America* 48, no. 8 (April 2009): 1079–86. doi:10.1086/597399.

Whalley, Lara E., Joseph G. Grzywacz, Sara A. Quandt, Quirina M. Vallejos, Michael Walkup, Haiying Chen, Leonardo Galván, and Thomas A. Arcury. "Migrant Farmworker Field and Camp Safety and Sanitation in Eastern North Carolina." *Journal of Agromedicine* 14, no. 4 (2009): 421–436. doi:10.1080/10599240903389508.

Wheeler, Andrea L., Peter G. Hartel, Dominique G. Godfrey, Jennifer L. Hill, and William I. Segars. "Potential of as a Human Fecal Indicator for Microbial Source Tracking." *Journal of Environment Quality* 31, no. 4 (2002): 1286. doi:10.2134/jeq2002.1286.

Wikström, Per, Anna Wiklund, Ann-Christin Andersson, and Mats Forsman. "DNA Recovery and PCR Quantification of Catechol 2,3-dioxygenase Genes from Different Soil Types." *Journal of Biotechnology* 52, no. 2 (December 10, 1996): 107–120. doi:10.1016/S0168-1656(96)01635-5.

"DNA Recovery and PCR Quantification of Catechol 2,3-dioxygenase Genes from Different Soil Types." *Journal of Biotechnology* 52, no. 2 (December 10, 1996): 107–120. doi:10.1016/S0168-1656(96)01635-

*2008 Outbreak of Salmonella Saintpaul Infections Associated with Raw Produce — NEJM*, n.d. <http://www.nejm.org/doi/full/10.1056/NEJMoa1005741>.

*Epidemic Profile of Shiga-Toxin–Producing Escherichia Coli O104:H4 Outbreak in Germany — NEJM*, n.d. <http://www.nejm.org/doi/full/10.1056/NEJMoa1106483>.

*Food Safety and Food Security*, n.d.

<http://www.nature.com.prox.lib.ncsu.edu/scitable/knowledge/library/food-safety-and-food-security-68168348>.

*Salmonella Braenderup and Mango*, n.d. <http://www.easybib.com/cite/view>.

*ScienceDirect.com - Applied Soil Ecology - Extraction and Purification of Microbial DNA from Soil and Sediment Samples*, n.d.

<http://www.sciencedirect.com.prox.lib.ncsu.edu/science/article/pii/S0929139301001494>.

*ScienceDirect.com - Food Control - Microbiological Safety Evaluations and Recommendations on Fresh Produce*, n.d.

<http://www.sciencedirect.com.prox.lib.ncsu.edu/science/article/pii/S095671359800022X>.

*ScienceDirect.com - Journal of Microbiological Methods - Evaluation of Extraction and Purification Methods for Obtaining PCR- Amplifiable DNA from Compost for Microbial Community Analysis*, n.d.

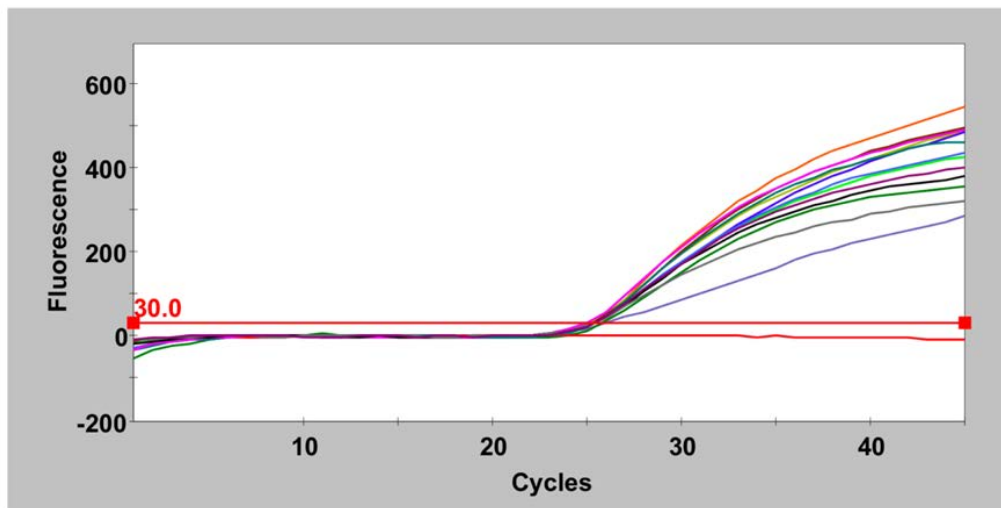
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**APPENDICES**

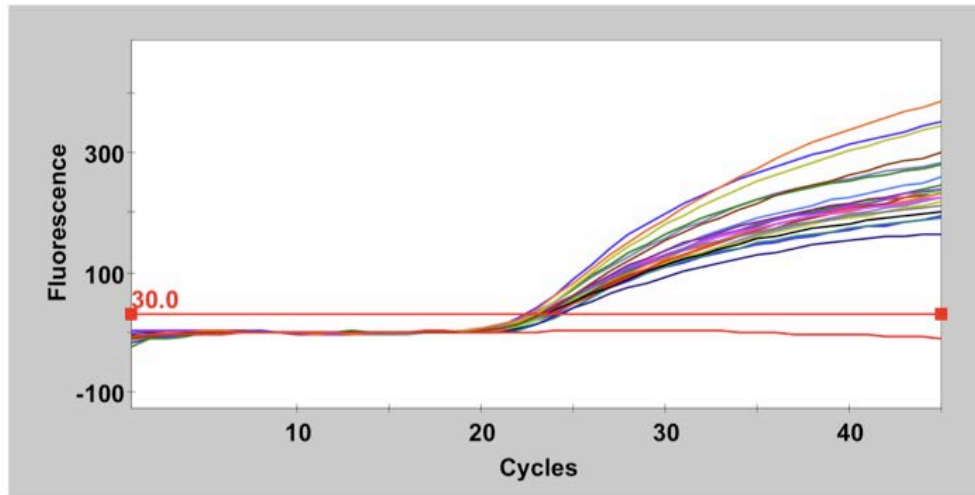
## Appendix A: Various methods used for DNA Extraction and their Characteristics

Method	Mean Conc	Mean Purity	Comments
Bead Beater	1870.15	1.175	Very low optical density - indicates contamination of DNA
PowerSoil	5.5	1.57	Low yield, low optical density
DNEasy	30.45	1.315	High yield, low optical density
FastSpin	36.45	1.675	High yield, closer to pure optical density

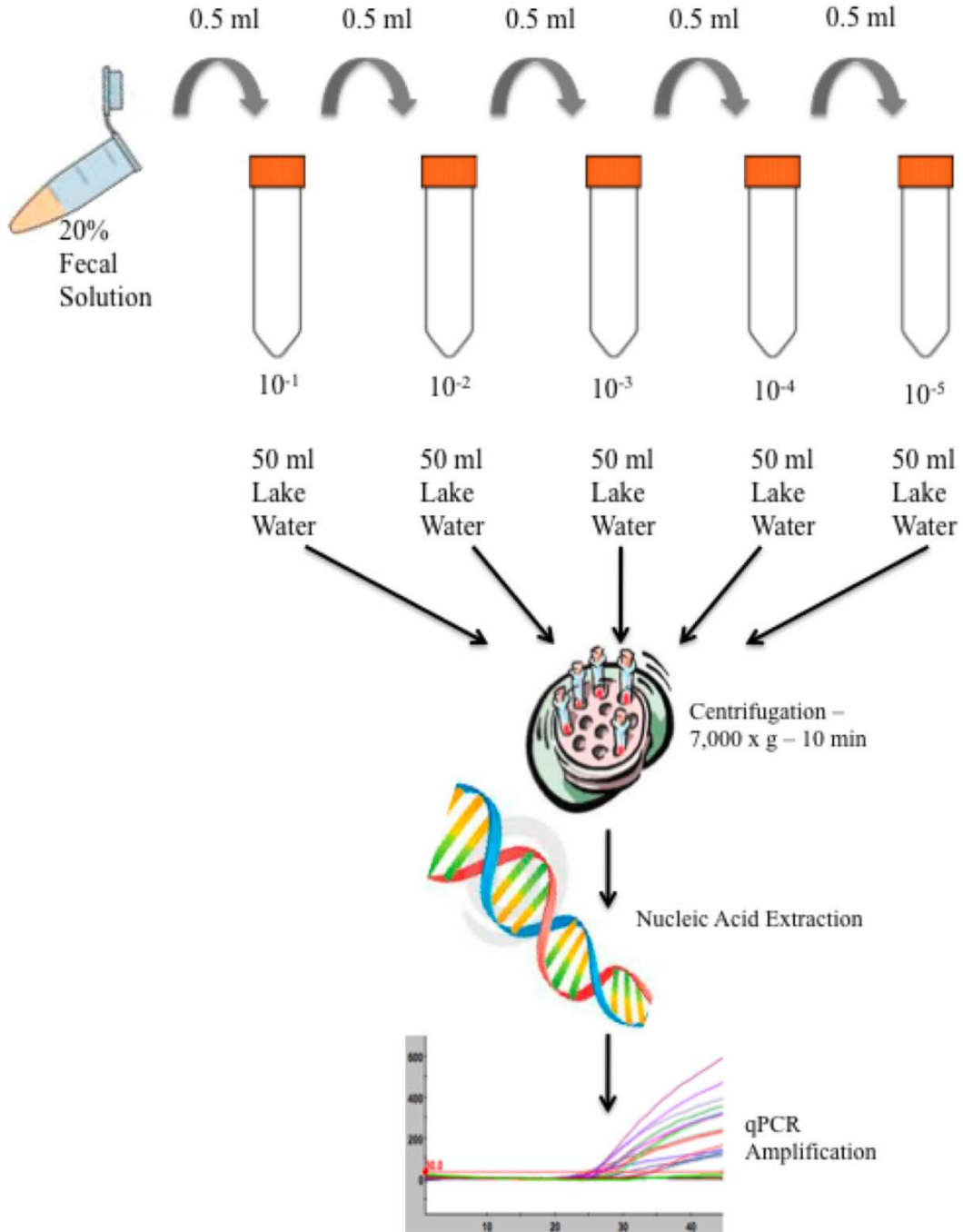
## Appendix B: AllBac Temperature Optimization



## Appendix C: BoBac Temperature Optimization



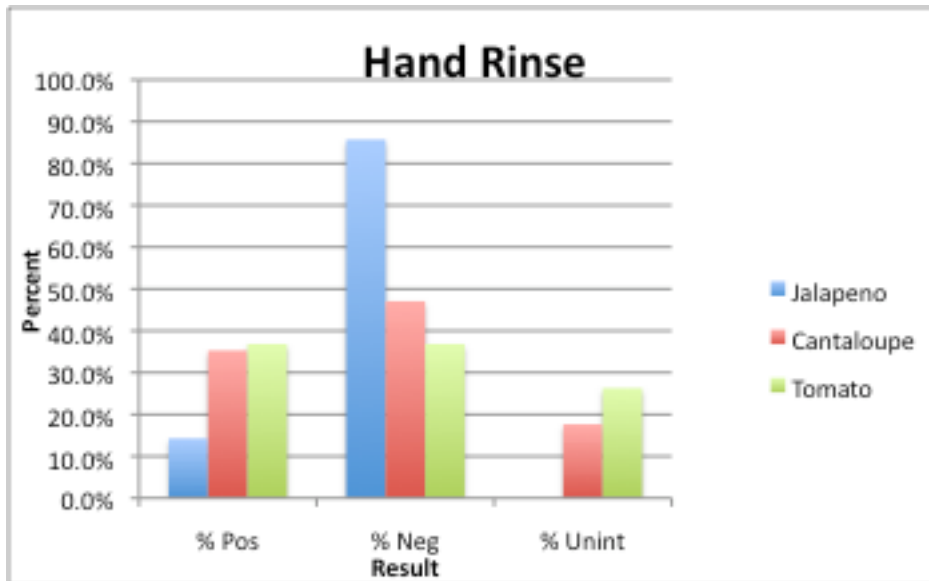
Appendix D: Spiking Experiments to Determine Limits of Detection for qPCR Assays



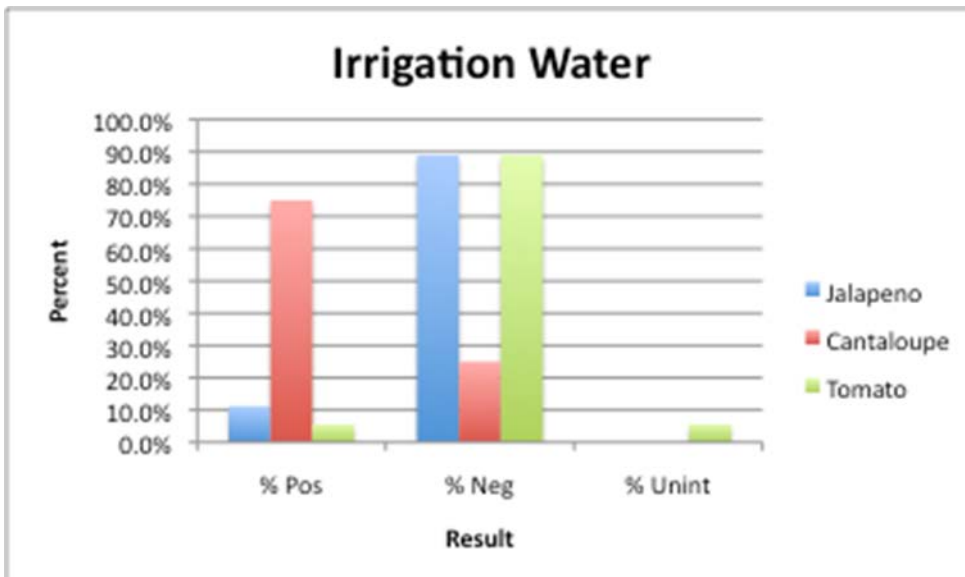
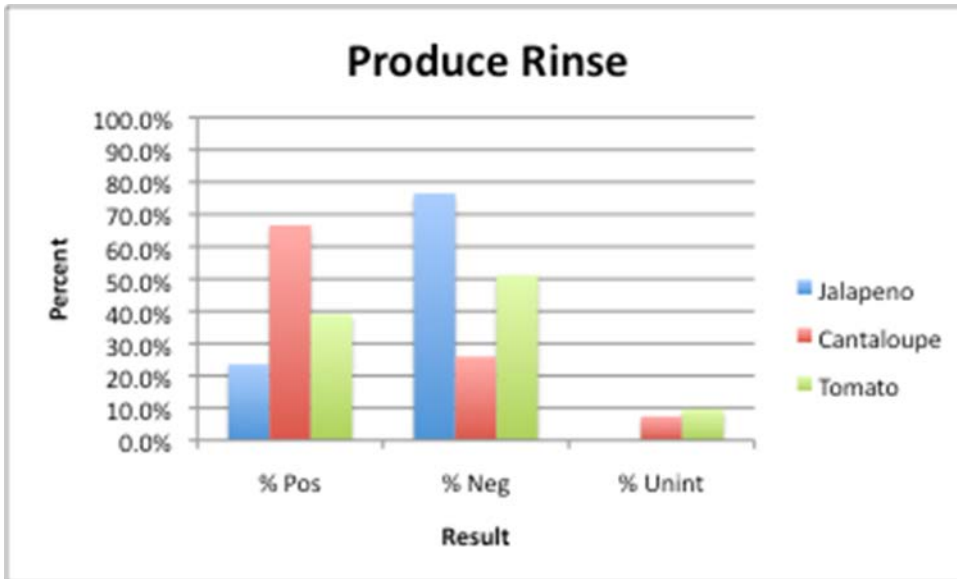
Appendix E: Presence/Absence of AllBac Marker in Environmental Sample Type

		Pos	Neg	Unint	Total
Hand Rinse	Jalapeno	2	12	0	14
	Cantaloupe	8	8	1	17
	Tomato	7	7	5	19
	Total	17	27	6	50
Irrigation Water	Jalapeno	1	8	0	9
	Cantaloupe	9	3	0	12
	Tomato	1	16	1	18
	Total	11	27	1	39
Produce Rinse	Jalapeno	4	13	0	17
	Cantaloupe	20	7	0	27
	Tomato	16	21	4	41
	Total	40	41	4	85
Produce Type	Jalapeno	7	33	0	40
	Cantaloupe	37	18	1	56
	Tomato	24	44	10	78
	Total	68	95	11	174

Appendix F: Results of Environmental Samples by Produce Type



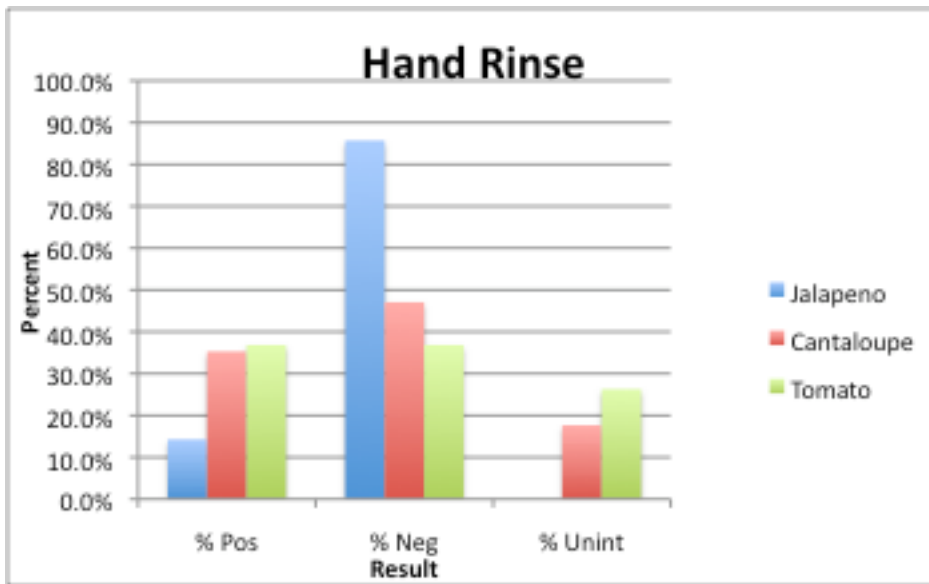
Appendix F, continued



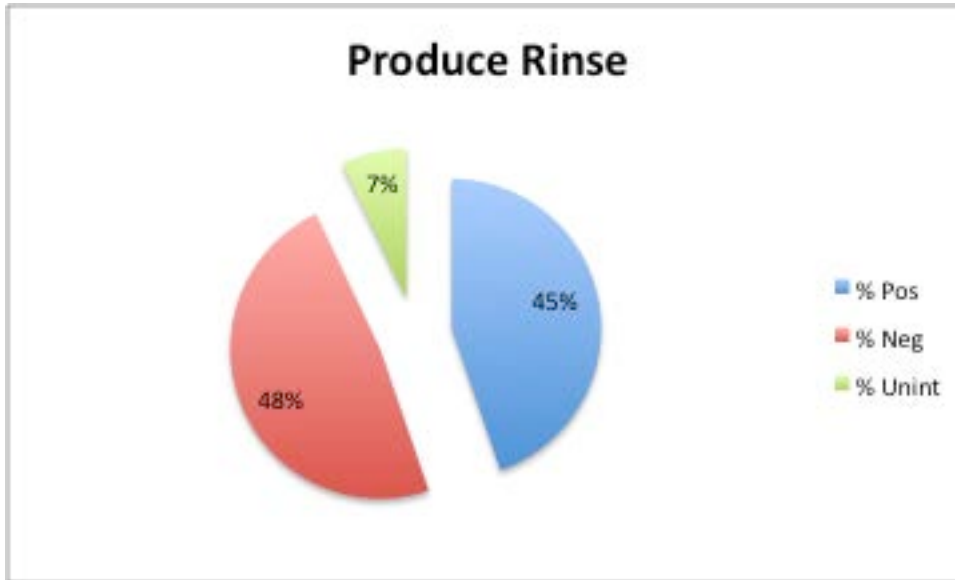
Appendix G: Average log Genome Equivalence Copies (GEC) for Environmental Samples, and by Produce Type with Corresponding Ct Values

Sample Type	Average log GEC	Range	Average Ct	Range
Hand Rinse	5.3	4.0-8.6	31.8	20.0-35.0
Irrigation	6.1	3.6-7.7	28.9	24.3-33.7
Produce Rinse	6.0	2.9-10.1	30.3	21.5-34.8
Pepper	4.6	3.6-6.8	31.9	29.6-33.3
produce rinses alone	4.3	3.6-5.1	31.7	29.5-33.2
Melon	6.4	4.1-9.1	29.2	20.0-35.0
produce rinses alone	6.6	4.1-9.1	29.2	21.5-34.6
Tomato	5.4	2.9-10.1	31.5	21.8-34.8
produce rinses alone	5.8	2.9-10.1	30.9	21.8-35.0
Overall	5.8	2.9-10.1	30.4	20.0-35.0

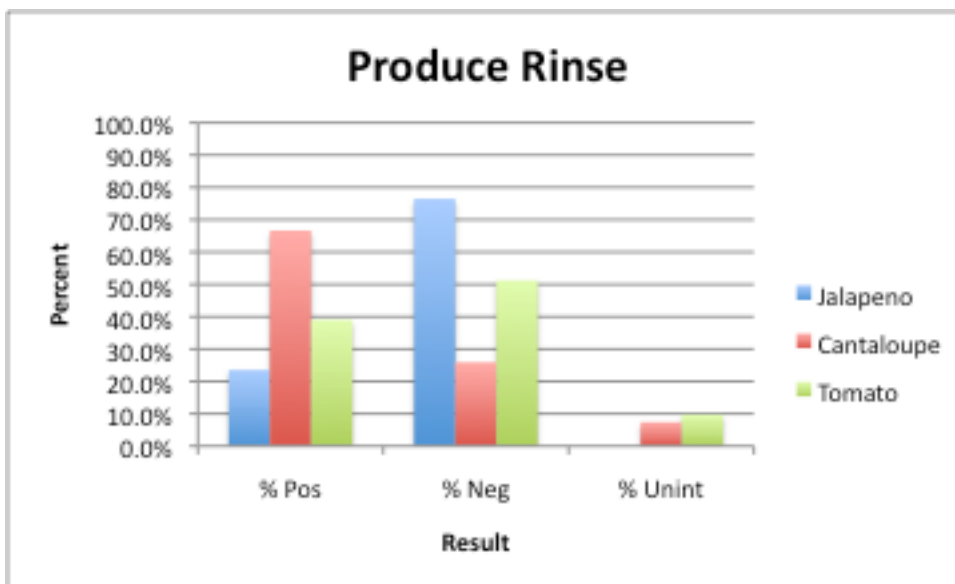
Appendix H: Percent Positive from Hand Rinse by Produce Type



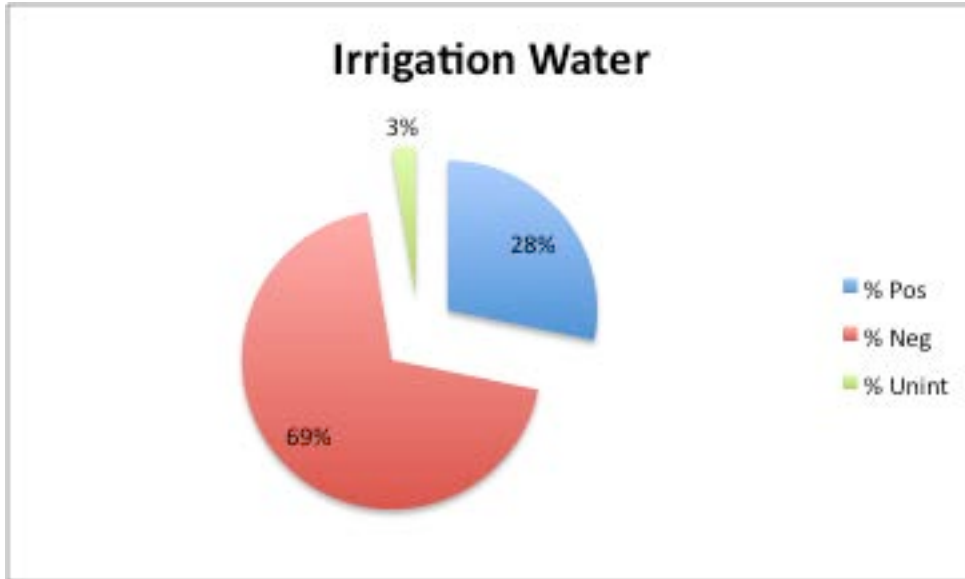
Appendix I: Percent Positive from Produce Rinse



Appendix J: Percent Positive from Produce Rinse by Produce Type



Appendix K: Percent Positive from Irrigation Water Samples



Appendix L: Percent Positive from Irrigation Water Samples by Produce Type

