The Role of Background Bacteria in Preventing the Isolation and Detection of *Shigella* and STEC in Foods and Novel MuSIC ddPCR Method to Improve STEC Detection in the Presence of Background Bacteria

by

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Abstract

Foodborne illness caused by Enterohemorrhagic *E. coli* (EHEC) and *Shigella* remain a public health issue in developed countries. The current microbiological methods for detecting EHEC and *Shigella* typically involve an enrichment step that aims to amplify the pathogenic organisms relative to the background bacteria flora present in foods. Following enrichment, there is a screening step to identify pathogens based on presence of virulence genes (ex. *stx* and *eae* for EHEC). False-positive detection of EHEC can occur if virulence targets are present within the population of bacteria found in foods, but not in any single organism. In this study, a Multiplexed Single Intact Cell Droplet Digital PCR (MuSIC ddPCR) was developed to reduce the false-positives in the EHEC method. During enrichment, EHEC and *Shigella* are often outcompeted by non-target bacteria. The role of microbial antagonism of non-target bacteria in preventing growth of EHEC and *Shigella* in enrichment culture was also investigated.

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Table of Contents

List of Tables

List of Illustrations

List of Appendices

List of Abbreviations

ρg: Picograms

µg: Micrograms

µL: Microliters

µM: Micromolar

BHI: Brain Heart Infusion

bp: Base Pairs

cfu: Colony Forming Units

ddPCR: Droplet Digital Polymerase Chain Reaction

DNA: Deoxyribonucleic Acid

EHEC: Enterohemorrhagic *E. coli*

EIEC: Enteroinvasive *E. coli*

gDNA: genomic Deoxyribonucleic Acid

HUS: Hemolytic-Uremic Syndrome

kDa: Kilodalton

LEE: Locus of Enterocyte Effacement

mL: Milliliters

mm: Millimeters

mTSB: modified Tryptone Soya Broth

MuSIC ddPCR: Multiplexed Single Intact Cell Droplet Digital Polymerase Chain Reaction

Ng: Nanogram

nL: Nanoliter

PBS: Phosphate-buffered saline

PCR: Polymerase Chain Reaction

PIC: Protease involved in colonization

qPCR: Real-time Quantitative Polymerase Chain Reaction

RNA: Ribonucleic Acid

rRNA: Ribosomal Ribonucleic Acid

ShET1: *Shigella* Enterotoxin 1

ShET2: *Shigella* Enterotoxin 2

spp.: Species

STEC: Shiga Toxin-Producing *E. coli*

stx: Shiga Toxin gene

Stx: Shiga toxin protein

TE: Tris-EDTA

VBNC: Viable but Nonculturable

1 Preface

The Multiplexed Single Intact Cell Droplet Digital PCR (MuSIC ddPCR) Method for Specific Detection of Enterohemorrhagic *E. coli* (EHEC) in Food Enrichment Cultures manuscript in Chapter 3 was published in March 2017 in Frontiers of Microbiology (McMahon, Blais, Wong, & Carrillo, 2017). It is reproduced in whole in my thesis. I helped design the experiments with Dr. Catherine Carrillo, Dr. Burton Blais and Dr. Alex Wong. I performed all of the experiments in the manuscript and analyzed most of the data. Dr. Carrillo helped with some analyses. I wrote most of the manuscript with the help of Dr. Carrillo, Dr. Blais and Dr. Wong.

The Inhibition of Shiga toxin-producing *E. coli* (STEC) and *Shigella* by Bacteria Present in Food Enrichment Cultures manuscript in Chapter 4 is a nearly completed final draft that will be submitted to the Journal of Food Protection. I helped design the experiments with Dr. Carrillo, Dr. Blais and Dr. Wong. I performed most of the experiments in the manuscript and analyzed most of the data. Hilda Hoo performed some experiments and Dr. Carrillo helped with some analyses. I wrote most of the manuscript with the help of Dr. Carrillo, Dr. Blais and Dr. Wong.

In Chapter 4, the term STEC is used instead of EHEC for simplicity. The rest of the document uses both the terms STEC and EHEC (subset of STEC with intimin (*eae*) gene).

2 Chapter: Introduction

2.1 Foodborne illnesses

Foodborne illnesses are caused by consuming foods that contain pathogenic bacteria, parasites, viruses, toxins, metals and prions (Thomas *et al.*, 2013). There is an estimated 4 million cases of foodborne illnesses in Canada each year (Thomas *et al.*, 2015). This is a significant amount as it accounts for about 11% of the population of Canada or 1 in 8 Canadians. The most common bacteria that cause foodborne illnesses are *Salmonella*, *Campylobacter*, Shiga toxin-producing *E.coli* (STEC) and *Shigella* (Public Health Agency of Canada, 2013). It is estimated that about 1,200 of the 4 million foodborne illnesses in Canada are caused by ingestion of *Shigella* and 33,000 are caused by STEC (Thomas *et al.*, 2013).

These cases cause a burden on society as they can lead to hospitalizations, complications, long-term health effects or death (Smith, Fratamico, & Gunther IV, 2014). For example, the STEC complication Hemolytic Uremic Syndrome (HUS) can cause patients lasting problems that require medical care and hospitalization after they have recovered from the STEC infection (Bláhová, Janda, Kreisinger, Mateˇjková, & Šedivá, 2002; Jenssen *et al.*, 2016). Sockett *et al.* (2014) estimated the annual cost of STEC infections in Canada (including hospitalizations, loss of productivity and premature deaths) is $403.9 million. The cost of individual illnesses varies from $43-$535 for mild and moderate cases to $5,780-$485,000 for severe cases and complications. These costs demonstrate the impact these foodborne illnesses have on society.

STEC and *Shigella* both have low infectious doses (10-100 for STEC and 10-500 for *Shigella*) which is a cause for concern as extremely low amounts of bacterial

contamination in foods can cause illness (Nguyen & Sperandio, 2012; Warren, Parish, & Schneider, 2006). The main source of STEC is ruminant animals, especially cattle (Bosilevac & Koohmaraie, 2011; Geraldine Duffy, Burgess, & Bolton, 2014; Krüger & Lucchesi, 2015). Cattle are a reservoir for STEC as they are asymptomatic but still shed the STEC in their faeces (Nguyen & Sperandio, 2012). The contaminated faeces can come into contact with meat during slaughter and vegetables through contaminated water. *Shigella* is most commonly transmitted through the fecal-oral route (The, Thanh, Holt, Thomson, & Baker, 2016). In developed countries, where sanitary practices may be more highly regulated, flies may be an important route of transmission of *Shigella* from fecal matter to food (Lindsay *et al.*, 2012).

2.2 Shiga toxin-producing *E. coli* (STEC) and Enterohemorrhagic *E. coli* (EHEC)

STEC are a pathogenic *E. coli* that contain at least one of two Shiga toxins (stx1 and stx2) and can cause foodborne illness (Public Health Agency of Canada, 2013; Thomas *et al.*, 2013). The Shiga toxins are encoded by a bacteriophage and have many different variants (Donnenberg & Whittam, 2001; Geraldine Duffy *et al.*, 2014; Krüger & Lucchesi, 2015; Smith *et al.*, 2014; Wasilenko *et al.*, 2012). There are three variants of Stx1 (a, c and d) and seven variants of stx2 (a, b, c, d, e, f and g). Certain variants of the Shiga toxins can cause more severe symptoms. For instance, an infection with a bacterium containing stx2c is more likely to cause hemolytic-uremic syndrome (HUS) (Bosilevac & Koohmaraie, 2011). Shiga toxin 1 is very similar to the Shiga toxin (Stx) in *Shigella dysenteriae* and *Shigella sonnei* (Beutin, Strauch, & Fischer, 1999).

The Shiga toxins are formed of one A unit and a pentamer of identical B subunits (Donnenberg & Whittam, 2001; Smith *et al.*, 2014; Wasilenko *et al.*, 2012). The pentamer of B subunits binds to glycolipids on the membrane and the toxin is endocytosed into the host cell. Inside the cell, part of the A subunit is released into the cytosol where it acts on the 28S rRNA of the 60S ribosomal subunit. The toxins cause the host's endothelial cells to undergo apoptosis by depurinating a specific adenine on the 28S rRNA and inhibiting protein synthesis (Donnenberg & Whittam, 2001; Smith *et al.*, 2014). The production of the Shiga toxins depends on the induction of the *stx* phage (Krüger & Lucchesi, 2015; Melton-Celsa, Mohawk, Teel, & O'Brien, 2011). An important mechanism of phage induction is the SOS response occurring when bacterial stress results in extensive DNA damage. High levels of Shiga toxin increase the side effects and the chances of acquiring HUS. For this reason, antibiotics are avoided when treating STEC infections since certain antibiotics cause the induction of phages (Krüger & Lucchesi, 2015; Smith *et al.*, 2014).

The Shiga toxins can cause severe symptoms and complications such as hemorrhagic colitis, thrombotic thrombocytopenic purpura, hemolytic anemia and HUS (Donnenberg & Whittam, 2001; Perelle, Dilasser, Grout, & Fach, 2004; Smith *et al.*, 2014). Between 5-15% of patients that have a STEC infection will develop HUS. This complication strongly affects the kidneys but can also affect intestines, central nervous system, lungs and other organs (Smith *et al.*, 2014). The damage done to the kidneys or other organs can be permanent and can result in long lasting effects such as kidney disease.

In addition to Shiga toxins, STEC typically require additional virulence genes to cause infection. For example, a mechanism for attachment to host cells encoded by genes such as *eae* and *aggR*, is often required. STEC that are capable of attaching to host cells are typically classified as Enterohemorrhagic *E. coli* (EHEC).The *eae* gene encoding the intimin protein is the most common mechanism of attachment in EHEC and is part of the locus of enterocyte effacement (LEE) (Bosilevac & Koohmaraie, 2011). The intimin protein enables the production of attachment and effacing lesions in the host The process of colonization begins as the intimin protein attaches to the host cell, causing the destruction of the microvilli and changing the structural components of the host's cell (Hartland & Leong, 2013; Melton-Celsa *et al.*, 2011). . The *aggR* gene is less common and it acts as a regulator for genes that encode the aggregative adherence fimbriae protein used for attaching and adhering to the host's cell (Harrington, Dudley, & Nataro, 2006; Morin, Santiago, Ernst, Guillot, & Nataro, 2013). Even though *aggR* is not common among STEC strains it was found in the O104:H4 outbreak strain in Europe (Boisen, Melton-Celsa, Scheutz, O'Brien, & Nataro, 2015). *E. coli* O157:H7 causes most EHEC outbreaks in North America (Melton-Celsa *et al.*, 2011). However, non-O157 EHEC are increasingly associated with EHEC outbreaks (Bosilevac & Koohmaraie, 2011; Geraldine Duffy *et al.*, 2014; Smith *et al.*, 2014).

2.3 *Shigella*

Shigella is closely related to *E. coli*, especially Enteroinvasive *E. coli* (EIEC) and has evolved from *E. coli* on multiple occasions (Schroeder & Hilbi, 2008; The et al., 2016). There are four species of *Shigella* which are *Shigella dysenteriae, Shigella flexneri, Shigella boydii* and *Shigella sonnei* (Adam & Pickings, 2016; Warren *et al.*,

2006). Each species is divided into multiple serotypes, except *S. sonnei* which has only one serotype. *Shigella dysenteriae* serotype 1 is the only *Shigella* that can cause HUS as it contains a Shiga toxin. Some strains of *S. sonnei* also have the *stx* virulence gene, but this species has not been associated with HUS (Beutin *et al.*, 1999).

Evolution of both *Shigella* and EIEC involved acquisition of a virulence plasmid with the Type III Secretion System, enterotoxins and invasion plasmid antigens (Adam & Pickings, 2016; Schroeder & Hilbi, 2008; Ud-Din & Wahid, 2015), followed by inactivation of multiple chromosomal genes that were incompatible with virulence factors encoded on plasmids (anti-virulence genes). For example, both EIEC and *Shigella* have lost their flagella and the ability to decarboxylate lysine (Bliven & Maurelli, 2016; Ud-Din & Wahid, 2015) . EIEC is more closely related to *E. coli* as it has lost fewer genes. Most organisms classified as *Shigella* spp. have also lost their ability to ferment salicin, hydrolyze esculine, ferment mucate and use acetate (Ud-Din & Wahid, 2015). These extensive phenotypic differences as well as clinical phenotypes of the disease caused by ingesting *Shigella* led to classification of *Shigella* as a separate genus.

Shigella spp. can produce a variety of toxins. *S. dysenteriae* serotype 1 and some *S. sonnei* encode a Shiga toxin very similar to the Shiga toxin 1 in STEC whereas most other *Shigella* spp. encode enterotoxins. These enterotoxins contribute to the watery diarrhea associated with shigellosis (Adam & Pickings, 2016). *Shigella* enterotoxin 1 (ShET1) is encoded on the chromosome and is only found in certain serotypes of *S. flexneri* (Adam & Pickings, 2016; Warren et al., 2006). *Shigella* enterotoxin 2 (ShET2) is encoded on the virulence plasmid and is found in most *Shigella* as well as EIEC (Adam & Pickings, 2016; Warren *et al.*, 2006). Protease involved in colonization (PIC) and

SepA are two other enterotoxins that have been recently described (Adam & Pickings, 2016; Faherty *et al.*, 2012). PIC is chromosomally encoded and SepA is encoded on the virulence plasmid.

2.4 Methods to detect EHEC in foods

Similar methods in Canada and the USA are commonly used to detect EHEC in food products. The first step of all of the methods involves an enrichment of the bacteria in the food product. The purpose of the enrichment is to increase the number of EHEC while minimizing the growth of other bacteria using broth culture media with or without antibiotics. The Canadian and USA methods for detection of EHEC in foods use modified Tryptone Soya Broth (mTSB) with or without antibiotics such as vancomycin and cefsulodin (B. Blais, Martinez, *et al.*, 2014; USDA-FSIS, 2014). The mTSB media contains bile salts which provide some selectivity as enteric bacteria have adapted to grow in the presence of bile, whereas most bacteria cannot grow in these conditions (Begley, Gahan, & Hill, 2005; Merritt & Donaldson, 2009). Vancomycin targets Gram-positive bacteria, whereas cefsulodin targets *Pseudomonas aeruginosa* (D. P. Levine, 2006; Robson & Baddiley, 1977; Suginaka *et al.*, 1979). The EHEC enrichment broth does reduce the non-enteric bacteria with mTSB as well as Gram-positive bacteria and/or *Pseudomonas aeruginosa* with added antibiotics. However, even with the antibiotics added to the enrichment broth, there are still many non-pathogenic bacteria that can survive. This is a problem as the EHEC can be present in low numbers compared to all of the other bacteria that can still grow in the enrichment broth (Catarame *et al.*, 2003; Delannoy *et al.*, 2016). False -negative results of food testing analyses can occur if the EHEC do not grow to levels detectable by downstream analyses (Vimont *et al.*, 2007).

After enrichment, there is a PCR screening for a number of virulence genes characteristic of EHEC. To be classified as EHEC both a toxin gene (*stx1* or *stx2*) and an intimin gene (*eae*) have to be present (B. W. Blais, Gauthier, Deschênes, & Huszczynski, 2012; USDA-FSIS, 2014). With a high population of *E. coli* in the enrichment culture, false-positive results during the PCR screening can occur when virulence targets are encoded within separate strains (i.e. one non-pathogenic *E. coli* with *stx* and a second non-pathogenic *E. coli* with *eae*) (Delannoy *et al.*, 2016; Livezey, Groschel, & Becker, 2015). False positives are a problem as they can result in unnecessary work to find non-existent EHEC bacteria.

There have been testing methods that could reduce false positives such as using new gene targets (Delannoy, Beutin, & Fach, 2013). This has the potential to reduce false positives, but in the future, may not be effective if non-pathogenic bacteria acquire these genes or pathogenic bacteria lose these genes. As *E. coli* have the propensity to acquire genes easily, this may be a concern. Another approach for reducing false positive detection of EHEC has been the incorporation of an intercalating dye to prevent the amplification of DNA from dead cells (B. Li, Hu, & Elkins, 2014; Liu & Mustapha, 2014). This is useful for eliminating false positives caused by dead EHEC that are not pathogenic; however, it does not help with false positives caused by two different live bacteria (STEC *eae*-negative and *E. coli eae*-positive).

2.5 Methods to detect *Shigella* in foods

The *Shigella* method is very similar to the EHEC method. It involves an enrichment followed by PCR screening for the invasion plasmid antigen H (*ipaH*) gene

(Andrews & Jacobson, 1998; Kingombe, Cerqueira-Campos, Trottier, & Houle, 2006a). The methods for *Shigella* detection use *Shigella* broth with Novobiocin that is often enriched in a CO_2 environment (Andrews & Jacobson, 1998; Kingombe, Cerqueira-Campos, Trottier, & Houle, 2006b). The *Shigella* broth does not have bile salts to select for enteric bacteria, but the CO_2 environment does select for bacteria that can grow in an anaerobic environment. Also, as with the EHEC method, the *Shigella* method selects against most Gram-positive bacteria, in this case with novobiocin. This leads to the *Shigella* enrichment reducing the background bacteria to mostly Gram-negative bacteria that can grow in an anaerobic environment. Similar to the EHEC method, enrichment cultures can sustain growth of many species of Gram-negative bacteria.

Detection of *Shigella* in foods is unreliable using current methodology. There have been many cases where epidemiological investigations have connected a *Shigella* outbreak to a specific food product but no *Shigella* isolates were found in the implicated food (Kozak, MacDonald, Landry, & Farber, 2013). These false negative results are a serious concern as the contaminated foods may not be identified resulting in delayed recalls and prolonged public exposure to dangerous food products. For example, in May and June 2001, there was an outbreak in British Columbia that appeared to be linked to spinach. However, *Shigella* was not detected in the spinach or the ditch water which was believed to be the source of the contamination (Kozak *et al.*, 2013). In 2007, an outbreak of *Shigella* was linked to mini-carrots. No *Shigella* was isolated from the carrots (Kozak *et al.*, 2013). In addition, *Shigella* is rarely isolated in routine food testing programs, likely due to problems with methodology. Improved detection of *Shigella* in foods would

result in a better understanding of foodborne illness attributed to consumption of *Shigella* in foods.

Recent efforts to improve detection of *Shigella* in foods have been limited. There have been a few studies looking to improve the molecular methods for detection and characterization of *Shigella* such as microplate immunocapture, targeted sequencing and DNA microarray assays (Fakruddin, Hossain, & Ahmed, 2017; Ferrario *et al.*, 2017; Y. Li, 2016). However, there have been very few studies that aim to improve the problem that exists during the enrichment of *Shigella*. If the enrichment step is not improved, the molecular methods may not function properly, particularly if there is no or very few *Shigella* in the enrichment culture. It is known that *Shigella* is very difficult to detect (Pollock & Dahlgren, 1974; Uyttendaele, Bagamboula, De Smet, Van Wilder, & Debevere, 2001). The problem with detection is usually attributed to transition of *Shigella* cells into a viable but nonculturable (VBNC) state or inhibition of growth due to the presence of organic acids and unfavourable pH (Uyttendaele *et al.*, 2001; Zhang *et al.*, 2011).

2.6 Microbial antagonism

Microbial antagonism occurs when one bacterium prevents the growth of another bacterium. Microbial antagonism has long been known to be an issue affecting selective enrichment of foodborne pathogens (Nissle, 1916). In 1946, an early experiment exploring microbial antagonism showed the production of diffusible substances, designated colicins, was common among coliform bacteria (Gratia & Fredericq, 1946). A

study of 2458 coliform strains in 1948 found that 11.7 % of these coliforms inhibited the growth of *S. flexneri* and *S. sonnei* (Halbert, 1948). Later, Levine and Tanimoto (1954) found that 19% of 124 *E. coli* isolates from stool and water and 69% of *E. coli* from infant throat cultures exhibited antibiotic properties against Gram-negative bacteria.

Bacteria use a variety of mechanisms to prevent the growth of competing organisms. Mechanisms of microbial antagonism include production of bacteriophages, bacteriocins, and other antibiotics. Bacteriocins are peptides that have antimicrobial activity against closely related bacteria (Riley & Wertz, 2002; Yang, Lin, Sung, & Fang, 2014). Bacteriophages are viruses that can infect bacteria that are often encoded in bacterial genomes. When activated, bacteriophages can infect and kill other bacteria. A variety of other antimicrobial compounds can also be produced by bacteria. Classes of antibiotics that are produced by bacteria include tyrothricin, actinomycin, bacitracin, aminoglycosides, chloramphenicol, tetracyclines, macrolides, glycopeptides, streptogramins, lincosamide, ansamycins and aminocoumarins (Mohr, 2016). Many of these antibiotics are produced by Gram-positive bacteria of the Genera *Streptomyces*, *Bacillus* and *Amycolatopsis*.

2.7 Purpose

The goal of this work was to improve current microbiological methods for the detection of EHEC and *Shigella* in foods and ultimately to reduce the false positives and false negatives in methods for detection of these organisms. In the first part of this study, the new technology of Droplet Digital PCR (ddPCR) was used as the basis for the development of an improved EHEC screening method that has the potential to eliminate

false-positive detection of this pathogen in food enrichment culture. The hypothesis of this study is that ddPCR with live cultures can reduce the false positive detection caused by background bacteria by enabling detection of diagnostic targets in single-cell assays. In the second part of the study, the impact of microbial antagonism of background organisms during the enrichment broth culture of *Shigella* spp. and EHEC was investigated. The hypothesis of this study is that bacteriocins and bacteriophages produced by background bacteria prevent the growth of *Shigella* and EHEC in food enrichment cultures. The method development and information gained from these studies will enable the improvement of current methods for the detection and isolation of *Shigella* and EHEC.

3 Chapter: Multiplexed Single Intact Cell Droplet Digital PCR (MuSIC ddPCR) Method for Specific Detection of Enterohemorrhagic *E. coli* (EHEC) in Food Enrichment Cultures

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Keywords: STEC, intimin, EHEC, Shiga toxin, droplet digital PCR, quantitative PCR.

3.1 Abstract

Foodborne illness attributed to enterohemorrhagic *E. coli* (EHEC), a highly pathogenic subset of Shiga toxin-producing *E. coli* (STEC), is increasingly recognized as a significant public health issue. Current microbiological methods for the identification of EHEC in foods often use PCR-based approaches to screen enrichment broth cultures for characteristic gene markers (i.e., Shiga toxin (*stx*) and intimin (*eae*)). However, false positives arise when complex food matrices, such as beef, contain mixtures of *eae*-negative STEC and *eae*-positive *E. coli*, but no EHEC with both markers in a single cell. To reduce false-positive detection of EHEC in food enrichment samples, a Multiplexed, Single Intact Cell droplet digital PCR (MuSIC ddPCR) assay capable of detecting the co-occurrence of the *stx* and *eae* genes in a single bacterial cell was developed. This method requires: (1) dispersal of intact bacteria into droplets; (2) release of genomic DNA (gDNA) by heat lysis; and (3) amplification and detection of genetic targets (*stx* and *eae*) using standard TaqMan chemistries with ddPCR. Performance of the method was tested with panels of EHEC and non-target *E. coli*. By determining the linkage (i.e., the proportion of droplets in which *stx* and *eae* targets were both amplified), samples

containing EHEC (typically greater than 20% linkage) could be distinguished from samples containing mixtures of *eae*-negative STEC and *eae*-positive *E. coli* (0-2% linkage). The use of intact cells was necessary as this linkage was not observed with gDNA extracts. EHEC could be accurately identified in enrichment broth cultures containing excess amounts of background *E. coli* and in enrichment cultures derived from ground beef/pork and leafy-green produce samples. To our knowledge, this is the first report of dual-target detection in single bacterial cells using ddPCR. The application of MuSIC ddPCR to enrichment-culture screening would reduce false-positives, thereby improving the cost, speed and accuracy of current methods for EHEC detection in foods.

3.2 Materials and methods

3.3 Introduction

Foodborne illness due to Shiga toxin-producing *E. coli* (STEC) continues to be an important public health concern in Canada and around the world (EFSA, 2013; Gould *et al.*, 2013; Thomas *et al.*, 2015). A sub-group of STEC, the enterohemorrhagic *E. coli* (EHEC), causes infections that can result in serious medical conditions including bloody diarrhoea, hemolytic-uremic syndrome (HUS), kidney failure and microangiopathic hemolytic anemia, and can occasionally be fatal (Karmali, Gannon, & Sargeant, 2010; Thomas *et al.*, 2015). Consumption of foods contaminated with EHEC is an important cause of illnesses associated with this pathogen. High-risk foods, such as ground beef and produce, are thought to become contaminated through exposure to animal fecal matter, particularly from ruminant animals in which STEC bacteria are prevalent (Geraldine Duffy *et al.*, 2014; Gill & Gill, 2010; Mathusa, Chen, Enache, & Hontz, 2010).

Surveillance and recall of EHEC-contaminated foods reduces the risk to the consumer, and improved methods will enable more extensive testing and further reduce the human-health risk attributed to this organism (Catford *et al.*, 2014; Geraldine Duffy *et al.*, 2014; EFSA, 2013; Seys, Sampedro, & Hedberg, 2015).

The most common EHEC is *E. coli* O157, which has been the focus of public health organizations. However, non-O157 EHEC foodborne illnesses have been increasingly identified (Catford *et al.*, 2014; Gould *et al.*, 2013; Johnson, Thorpe, & Sears, 2006; Luna-Gierke *et al.*, 2014). There are no biochemical features by which EHEC strains can be differentiated from commensal *E. coli* or other STEC that are not a public health concern. Nonetheless, it is universally recognized that foodborne EHEC can generally be defined on the basis of certain gene markers, including the Shiga toxin genes, *stx1* or *stx2*, the intimin-coding gene, *eae*, along with markers for specific serogroups of concern (e.g., O26, O45, O103, O104, O111, O121, O145 and O157) (B. W. Blais et al., 2012; Catford et al., 2014; EFSA, 2013). Note that while most EHEC strains have both *eae* and *stx* genes, priority serogroups vary among countries.

The method for detection and isolation of EHEC used in food-testing laboratories at the Canadian Food Inspection Agency (B. Blais, Martinez, *et al.*, 2014; B. Blais, Deschênes, Huszczynski, & Gauthier, 2014; Gill, Martinez-Perez, McIlwham, & Blais, 2012; Huszczynski, Gauthier, Mohajer, Gill, & Blais, 2013) as well as the ISO/CEN TS13136:2012 Technical specification (ISO, 2012) and the US MLG5B.05 (USDA-FSIS, 2014) methods, commonly used internationally, involve enrichment of samples in a selective broth and screening for the presumptive presence of EHEC using PCR assays targeting *stx1* and/or *stx2*, and *eae*. One of the challenges of screening enrichment broths

for EHEC is to distinguish samples with target EHEC carrying both *stx* and *eae* from samples containing mixed cultures in which these markers are present in different cells. Using current approaches, as much as 50% of samples identified as presumptive positives may be false positives, particularly in samples with high levels of non-target *E. coli* (Delannoy *et al.*, 2016; Livezey *et al.*, 2015). This high rate of false positives can negate the benefit of a screening procedure intended to identify presumptive EHEC (i.e., *E. coli* cells carrying both *stx* and *eae*) due to the need for unnecessary downstream processing of samples for the recovery and characterization of the target bacteria.

The aim of this work was to develop a screening method capable of distinguishing enrichments positive for EHEC from false-positive samples containing mixed cultures of *eae*-negative STEC and *eae*-positive *E. coli* using droplet digital PCR (ddPCR) technology (Figure 1). ddPCR is a quantitative PCR technique in which a standard PCR reaction mixture is distributed into thousands to millions of droplets prior to PCR amplification (Hindson *et al.*, 2011; Pinheiro *et al.*, 2012). The Bio-Rad QX200™ implementation of this technology involves conversion of 20 µL reaction volumes into approximately 20 thousand one-nanoliter droplets followed by PCR amplification within each droplet. The concentration of the input sample is adjusted to achieve a distribution of less than one template molecule per droplet with a Poisson distribution of the template molecules among droplets (Pinheiro *et al.*, 2012). TaqMan probe-based PCR assays are commonly used in this method, and amplification is determined by detection of fluorescence in each droplet by a droplet reader. The number of positive droplets indicates the number of template molecules in the sample and linkage between different targets (e.g., *eae*, *stx*) can be determined based on frequency of co-amplification of two

targets within a droplet. In samples containing EHEC, co-amplification of *eae*/*stx* targets would be expected to be high; whereas in false-positive samples (i.e., mixtures of *eae*-negative STEC and *eae*-positive *E. coli*) the two targets would generally be amplified in separate droplets, with only a small number of droplets in which both targets were amplified due to presence of two different bacterial cells.

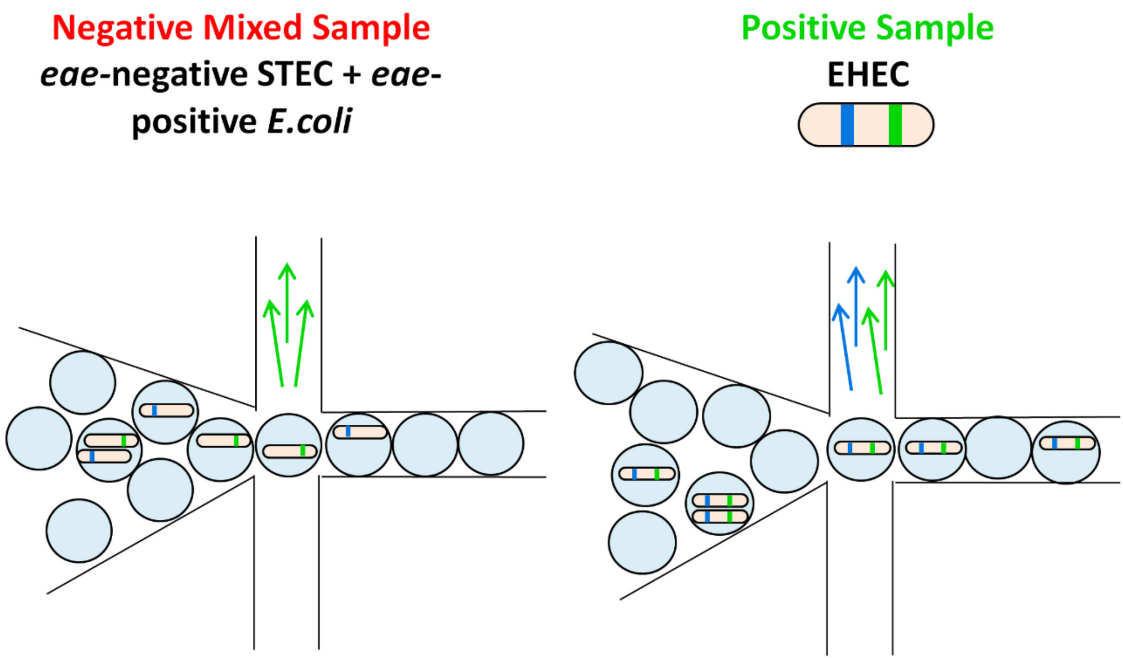

Figure 1. Droplet Digital PCR Method for specific detection of intact EHEC cells. Samples of food enrichment broths containing intact cells are added to the PCR reaction. A 20 µL PCR reaction is converted into 20,000 1 nL droplets prior to PCR-amplification. Amplification of *eae* (green) and *stx* (blue) is determined based on detection of fluorescent probes for each assay. In samples containing EHEC, both *eae* and *stx* targets will be detected in positive droplets. In samples containing mixtures of *eae*-negative STEC and *eae*-positive *E. coli*, most droplets will contain either *eae* or *stx*, unless both bacteria types are present within a single droplet.

Here we describe the development of a Multiplexed, Single Intact Cell ddPCR (MuSIC ddPCR) assay targeting detection of *stx* and *eae* genes and ensuring presence of the two targets within a single bacterial cell. In this assay, suspensions of bacterial cells

in enrichment broth are dispersed into droplets, followed by release of gDNA by heat lysis, and droplet digital PCR analysis. The method was evaluated to determine performance for specific identification of EHEC in enrichment-broth samples containing excess amounts of non-target bacteria.

3.3.1. Growth and maintenance of *E. coli* strains

A selection of *E. coli* strains of various serotype, *eae,* and *stx* gene profiles were used to evaluate the MuSIC ddPCR method (Table 1). An *E. coli* strain lacking the *stx* and *eae* genes was used as a negative control (OLC1543) and a strain containing a plasmid encoding fragments of the *stx1*, *stx2* and *eae* genes was used as a positive control (OLC2283, see description below). All strains were stored at -80°C in 25% glycerol and were plated on Brain-Heart Infusion agar (BHI) (OXOID, Ontario, Canada) overnight (14-16 hours) at 37°C prior to use. Samples were prepared for ddPCR experiments by transferring growth from a single colony into 10 mL of nutrient broth (OXOID) or modified Tryptone Soya Broth (mTSB)(OXOID) and growing overnight at 37°C. Prior to use, broths were diluted in nutrient broth (OXOID) to approximately 100 cells/µL. The strains used to generate mixed-culture samples were grown separately and mixed in equal amounts before diluting. Bacterial concentrations in overnight cultures (nutrient or mTSB broth) were initially determined by duplicate plating of serial dilutions on BHI agar. Subsequently, the A_{600} absorbance was used to estimate concentrations. In general, STEC strains reached a concentration of approximately 10^8 to 10^9 cells/mL after an overnight growth at 37°C. The plasmid control (OLC2283) and the generic *E. coli* (OLC1543) reached concentrations of approximately 10^9 to 10^{10} cells/mL.

Table 1. List of *E. coli* strains used in this study

Strain name	Serotype (toxin profile)	*stx*	*Eae*
OLC0455[1]	O111:H11 (*stx1a*)	+	+
OLC0456	O111:H8 (*stx1a*)	+	+
OLC0464[1]	O26:H11 (*stx1a*)	+	+
OLC0467[3]	O5:NM (*stx1a*)	+	+
OLC0639[2]	O26:H11 (*stx1a*)	+	+
OLC0669[2]	O76:H19 (*stx1*)	+	-
OLC0675[1]	O145:NM (*stx1a*)	+	+
OLC0679[1]	O103:H2 (*stx1a*)	+	+
OLC0684[1]	O145:NM	-	+
OLC0710[1]	O121:H19 (*stx2a*)	+	+
OLC0716[1]	O45:H2 (*stx1a*)	+	+
OLC0728[1]	O103:H11 (*stx1a*)	+	+
OLC0797[1]	O157:H7 (*stx1a*, *stx2a*)	+	+
OLC0986	O157:H7 (*stx2a, stx2c*)	+	+
OLC0997[2]	O118:H12 (*stx2b*)	+	-
OLC0998[2]	O73:H18 (*stx2d*)	+	-
OLC0999[2]	O2:H25 (*stx2g*)	+	-
OLC1001[2]	O128ac:H2 (*stx2f*)	+	+
OLC1002[2]	O174:H8 (*stx1c*, *stx2b*)	+	-
OLC1003[2]	O139:K12:H1 (*stx2e*)	+	-
OLC1059	O157:H7 (*stx2c*)	+	+
OLC1060	O166:H15 (*stx2d*)	+	-
OLC1069	O121:H19 (*stx2a*)	+	+
OLC1070	O157:H7 (*stx2a, stx2c*)	+	+
OLC1251	O91:H14 (*stx2b*)	+	-
OLC1254	O166:H15 (*stx2d*)	+	-
OLC1256	O55:H7 (*stx1a*)	+	+
OLC1258	O145:H34 (*stx2f*)	+	+
OLC1263	O26:H11 (*stx2a*)	+	+
OLC1267	O8:H10 (*stx2e*)	+	-
OLC1269	O2:H25 (*stx2g*)	+	-
OLC1335	O154:H31(*stx1d*)	+	-
OLC1535	O185:H7 (*stx2c*)	+	-
OLC1685	OUT:H23 (*stx2e*)	+	-
OLC2238	O159:H19 (*stx2a*)	+	-
OLC2250	O91:H14 (*stx1a*)	+	-
OLC2284	O157:H7 (*stx2c*)	+	+
OLC2285	O157:H7 (*stx1a*, *stx2a*)	+	+
OLC1543 (negative control)	O87:H7	-	-
OLC2283 (positive control)	-	fragment	fragment

[1] Strains were previously described in (Lambert, Carrillo, Koziol, Manninger, & Blais, 2015).
[2] Strains were previously described in (B. Blais, Deschênes, *et al.*, 2014).
[3] Strains were previously described in (Knowles, Lambert, Huszczynski, Gauthier, & Blais, 2015).
[4] OUT indicates O-untypeable

3.3.2. Preparation of positive-control plasmid and strain

A plasmid control was constructed by Integrated DNA Technologies (IDT, Iowa, USA) using an artificial sequence designed to incorporate the sequences corresponding to the amplicons of the *stx1*, *stx2* and *eae* genes used in the ddPCR assay (Appendix 1). Sequences of the gene fragments integrated into the plasmid were based on the *E. coli* O157:H7 Sakai strain (Accession number: BA000007)(Hayashi *et al.*, 2001). Fragments of other genes (*gyrB* and 16s rDNA) were inserted in between the *stx1*, *stx2* and *eae* genes for other applications. The sequence containing the gene fragments was inserted into the "Best-Fit" pIDTSMART-KAN Vector with a kanamycin marker (IDT), with an EcoRI restriction site at the 5' end and BamHI restriction site target at the 3' end of the artificial sequence.

The plasmid construct was transformed into *E. coli* DH5α cells using the Subcloning Efficiency™ DH5α™ Competent Cells (ThermoFisher Scientific Inc., Ontario, Canada), according to manufacturer's instructions. Transformed cells were cultured on nutrient agar (OXOID) containing 50 µg/mL of kanamycin (Sigma, Ontario, Canada). Plasmid DNA was extracted using the Midi Plasmid Kit (Qiagen, Ontario, Canada) and diluted to 5 fg/µL. Prior to use, the control strain was grown at 37°C overnight (14-16 hours) on nutrient agar containing 50 µg/mL of kanamycin. Broth

cultures were generated by transferring growth from a single colony into nutrient broth (OXOID) containing 50 µg/mL of kanamycin followed by overnight growth at 37°C.

3.3.3. Primers and probes

Primer and probe sequences for the *stx1*, *stx2* and *eae* genes were derived from the US Department of Agriculture's (USDA) non-O157 STEC Real-Time PCR (qPCR) Assay (Nielsen & Andersen, 2003; Perelle *et al.*, 2004; USDA-FSIS, 2014; Wasilenko *et al.*, 2012) (Table 2). The original USDA *stx* primers contain degenerate bases and the *stx1* and *stx2* primers, derived from the *stx* primers, have no degenerate bases. Primers and Probes (IDT) were rehydrated to stock concentrations of 100 µM using 1X Tris-EDTA (TE) and stored at -20°C.

Table 2. Primers and probes used in this study

Oligos	Sequence (5' → 3')	Amplicon size (bp)	Reference
Primers			
stx-F	TTT GTY ACT GTS ACA GCW GAA GCY TTA CG	129/133	(Perelle *et al.*, 2004)
stx-R	CCC CAG TTC ARW GTR AGR TCM ACD TC		(Perelle *et al.*, 2004; Wasilenko *et al.*, 2012)
eae-F	CAT TGA TCA GGA TTT TTC TGG TGA TA	102	(Nielsen and Andersen, 2003)
eae-R	CTC ATG CGG AAA TAG CCG TTM		(Nielsen and Andersen, 2003; Wasilenko *et al.*, 2012)
stx1-F	TTT GTT ACT GTG ACA GCT GAA GCT TTA CG	133	This paper
stx1-R	CCC CAG TTC AAT GTA AGA TCA ACA TC		This paper
stx2-F	TTT GTC ACT GTC ACA GCA GAA GCC TTA CG	129	This paper
stx2-R	CCC CAG TTC AGA GTG AGG TCC ACG TC		This paper
Probes			
stx1	56-FAM-CTG GAT GAT/zen/CTC AGT GGG CGT TCT TAT GTA A-3IABkFQ	-	(Perelle *et al.*, 2004; Wasilenko *et al.*, 2012)
stx2	56-FAM-TCG TCA GGC/zeN/ACT GTC TGA AAC TGC TCC-3IABkFQ	-	(Perelle *et al.*, 2004; Wasilenko *et al.*, 2012)
eae	5MAXN-ATA GTC TCG CCA GTA TTC GCC ACC AAT ACC-3IABkFQ	-	(Nielsen and Andersen, 2003; Wasilenko *et al.*, 2012)

Mixed bases: Y (C,T), W (A,T), R (A,G), M (A,C), D (A,G,T), S (C,G).

3.3.4. Genomic DNA extraction

Genomic DNA (gDNA) was extracted from 400 µL of culture grown in BHI broth for 3-4 hours (OLC1543 was grown in nutrient broth) using the Maxwell® 16 Cell DNA purification kit (Promega, Madison, WI) according to manufacturer's recommendations. The resulting gDNA was quantified using the Quant-iT™ High-Sensitivity DNA assay kit (ThermoFisher Scientific Inc.) according to manufacturer's recommendations and diluted to 5 pg/µL.

3.3.5. Detection of EHEC in samples containing high levels of non-target *E. coli*

To simulate the impact of high backgrounds of non-target commensal bacteria, EHEC cells were added in different ratios (1:10, 1:100, 1:1000, 1:10,000) relative to the generic *E. coli* to a final concentration of approximately 10^9 cells/mL. The STEC-*E. coli* samples were then diluted in nutrient broth as needed to reach a concentration of approximately 100 cells/µL of EHEC (10 cells/µL for the 1:10,000 dilution). For mixed cultures, overnight cultures of *eae*-negative STEC were mixed at a ratio of 1:1 with *eae*-positive *E. coli* prior to dilution as indicated above.

3.3.6. Detection of EHEC in food enrichment broths

To simulate EHEC enrichment in natural background flora found in raw meat and produce, 25 g of a mixture of ground beef and pork, or of samples of leafy-green produce (iceberg lettuce, kale, or spinach) were added to 225 mL of mTSB. Enrichment broths were incubated at 42°C for 16-18 hours. Aliquots of 50 mL of the enrichment broths

were centrifuged at 1500 x g for 1 minute to remove debris followed by transfer of supernatants to new tubes. EHEC bacteria were then added to the background flora at proportions of 1:100 and 1:1000 (v/v). For mixed cultures, overnight cultures of *eae*-negative STEC were mixed at a ratio of 1:1 (v/v) with *eae*-positive *E. coli* prior to diluting 1:10 (v/v) in enrichment broth.

3.3.7. Quantitative PCR (qPCR)

qPCR was carried out using a Lightcycler 96 instrument (Roche Diagnostics, Quebec, Canada) according to the manufacturer's recommendations. Each 25 µL PCR reaction contained 12.5 µL of FastStart Essential DNA Probes Master (Roche Diagnostics), 1.25 µM of *stx*1 and *stx*2 primers, 1.0 µM of *eae* primers, 0.25 µM of *stx1* and *stx*2 probes, 0.2 µM of *eae* probe and 5 µL of 1 ng/µL of gDNA. The thermocycler conditions were as follows: one cycle of 95°C for 10 min (initial denaturation); and 50 cycles of 94°C for 15 seconds (denaturation) and 55°C for 60 seconds (annealing). The data was analyzed using the Lightcycler 96 SW 1.1 Software.

3.3.8. Droplet digital PCR (ddPCR)

ddPCR was conducted using the QX200™ ddPCR system (Bio-Rad, Ontario, Canada) according to the manufacturer's recommendations. Each 25 µL PCR reaction mixture contained 1 X Supermix for Probes (Bio-Rad), 1.25 µM of *stx*, (or *stx*1 and *stx*2 primers), 1.0 µM of *eae* primers, 0.25 µM of *stx*1 and *stx*2 probes and 0.2 µM of *eae* probe. Approximately 500 intact cells or 25 pg gDNA was used in each assay (25 fg for plasmid DNA). An aliquot of 20 µL was taken from the 25 µL PCR reaction mix and

loaded into a DG8 cartridge (Bio-Rad) with a volume of 70 µL of Droplet Generation Oil for Probes (Bio-Rad). The cartridge was placed in the Droplet Generator (Bio-Rad) to form the nanoliter droplets. For the droplet generation step, the Droplet Generator was placed in a Biosafety Cabinet as a precaution since the safety of generating droplets with intact pathogenic bacterial cells has not been studied. Droplets were then transferred to a 96 well plate that was sealed with foil using a PX1 PCR Plate sealer (Bio-Rad) prior to amplification using the C1000 Touch Thermocycler (Bio-Rad). The thermocycler conditions were as follows: one cycle of 95°C for 5 min; 40 cycles of 95°C for 20 seconds and between 51°C and 61°C for 60 seconds; one cycle of 94°C for 10 minutes; followed by cooling to 4°C. The range of annealing temperatures 51°C-61°C was chosen based on the melting points of the primers and probes. The cooling to 4°C was to ensure the PCR product and droplets remained stable until further tests could be performed. For reactions with restriction enzyme, 2.5 µL of BamHI FastDigest (ThermoFisher Scientific Inc.) were added to each PCR reaction and the following steps were added to the beginning of the thermocycler protocol: 80°C for 2 min (heat lysis); 37°C for 45 min (restriction digestion); and 80°C for 5 min (enzyme inactivation).

Following PCR amplification, samples were analyzed on the QX200 Droplet Reader (Bio-Rad) using the QuantaSoft™ software (Bio-Rad). Samples with concentrations below 5 copies/µL and droplet counts below 10,000 droplets were discarded (Pinheiro *et al.*, 2012). The linkage value generated by the QuantaSoft™ software was used to assess association between the *eae* and *stx* targets; but, as this value is concentration-dependent, this linkage value was normalized by dividing by the concentration calculated for the *eae* assay and multiplying by 100 to generate a "percent

linkage" value to enable comparison among experiments. The concentration of the *stx* assay was not used in the linkage normalization as there were differences in the number of *stx* genes among the isolates used in this study. Averages and standard deviations were determined for replicates.

3.4 Results

3.4.1. Development of the Multiplexed Single Intact Cell (MuSIC) ddPCR Method

3.4.1.1. Selection of primers

Primers and probes for *stx* and *eae* genes were based on those described in the MLG 5B method developed by the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS, 2014) for detection of non-O157 STEC (MLG 5B, Appendix 1.01) (Nielsen & Andersen, 2003; Perelle *et al.*, 2004; USDA-FSIS, 2014; Wasilenko *et al.*, 2012). Due to the observation of lot-to-lot variations in the performance of these degenerate primers, described in an early implementation of the FSIS method, degenerate bases in the primers were removed and new primers specific to *stx1* or *stx2* were designed (Table 2). Performance of the specific primers relative to the degenerate primers was evaluated with a set of STEC with varying serological and toxin profiles using ddPCR (Figure 2A). Use of the specific primers resulted in higher numbers of positive droplets relative to results with degenerate primers, particularly for strains encoding *stx1* genes.

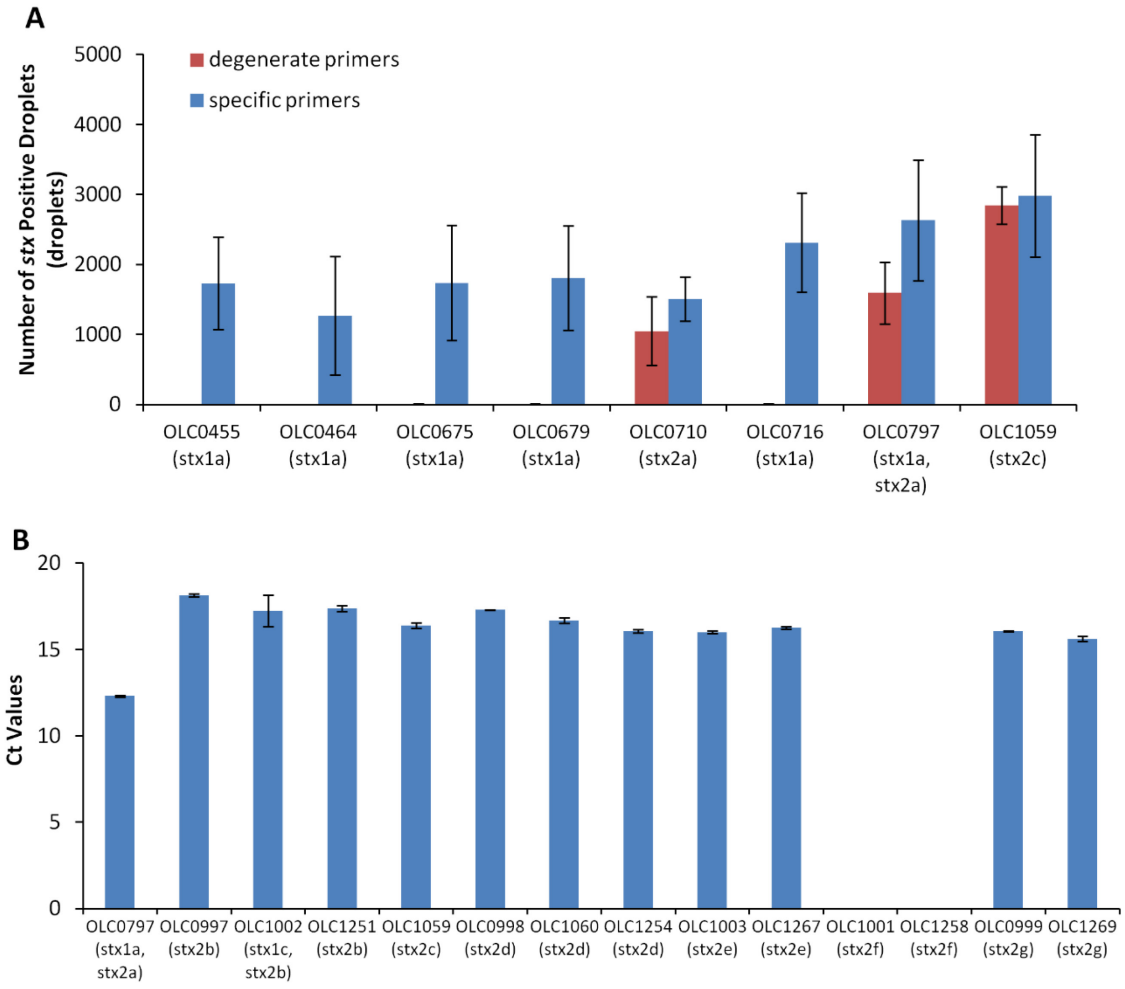

Figure 2. Development of primers for the detection of Shiga Toxin genes.
(A) Use of degenerate primers (red bars) in the ddPCR assay resulted in unreliable detection of *stx1* genes by ddPCR relative to the use of pools of specific primers (blue bars) in strains with varying Shiga toxin profiles. Error bars represent standard deviations of 4 replicates. **(**B) Evaluation of the specific primers using qPCR demonstrated reliability of the assay with *stx2* subtypes *stx2a*, *stx2b*, *stx2c*, *stx2d*, *stx2e* and *stx2g* but not *stx2f* (OLC1001 and OLC1258). Ct values were obtained using default parameters of the Lightcycler Software. Error bars represent the standard deviation of 2 technical replicates.

The specific primers were designed based on sequences for subtypes stx1a and stx2a of the *stx1* and *stx2* genes; however, there are 3 subtypes of *stx1* (*stx1a*, *stx1c*,

stx1d) and 7 subtypes of *stx2* (*stx2a*, *stx2b*, *stx2c*, *stx2d*, *stx2e*, *stx2f* and *stx2g*). To evaluate the performance of the specific primers for detection of all of seven of the variants of the *stx2* genes, real-time PCR amplification of targets from STEC with various *stx2* subtypes was conducted (Figure 2B). The *stx2* genes were detected within all isolates with the exception of isolates encoding the *stx2f* subtype. By comparison of the primer sequences to gene sequences within a database of full-length Shiga toxin genes (vtx.fsa, updated 16 March 2016) from the Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/data.php) (Joensen *et al.*, 2014), up to 3 mismatches to the specific *stx* primers were observed within the primer binding regions for most of the *stx2* genes. For the *stx2f* variants, up to 14 variable positions were observed for both the specific and degenerate primers. Conversely, the specific primers for *stx1* were generally 100% identical to sequences within *stx1a* subtypes, with only1-2 mismatches relative to sequences for *stx1c* and *stx1d* toxin subtypes.

An annealing temperature gradient was performed to determine optimal temperatures for the new primers. Optimal distinction between the fluorescence amplitude intensity of positive and negative droplets was observed at temperatures between 54 and 57°C for the *stx* assay and between 56 and 59°C for the *eae* assay (data not shown). An annealing temperature of 56°C was selected for subsequent experiments.

3.4.1.2. Comparison of the EHEC multiplex ddPCR using intact EHEC cells and genomic DNA extracts.

To demonstrate the feasibility of using ddPCR with intact *E. coli* cells, a multiplex ddPCR experiment was conducted on a panel of STEC with various genetic

profiles using both DNA extracts and intact cells (Figure 3). gDNA and overnight broth cultures were diluted to achieve appropriate concentrations for the ddPCR methods (~500 cells or 25 pg/reaction). While amplification of the *eae* and *stx* targets was observed with both whole cell and gDNA extract templates, co-amplification of the two targets (based on linkage values determined from the QuantaSoft™ program) was significantly higher (>31.8%) in reactions where intact cells were used as a PCR template compared to gDNA samples (<0.8%) (Figure 3A). In contrast, mean fluorescence amplitude of droplets in assays using gDNA template (Figure 3B) was higher, particularly for the *stx* target, than in assays where whole cells were used. Mean fluorescence amplitude was 6276 (cells) versus 11365 (gDNA) for the *stx* assay and 2820 (cells) vs 3748 (gDNA) for the *eae* assay. This difference in fluorescence amplitude indicates that amplification of targets may be impacted by the use of whole cells. Similarly, differences in fluorescence amplitude between positive and negative droplets were more distinct when gDNA was used as a template, relative to cells (Figure 3C). This was largely due to the increased mean fluorescence of the positive cells, as lower variability in the mean fluorescence intensity of the negative droplets was observed, with average mean fluorescence of 1685 (cells) and 1595 (gDNA) for the *stx* assay, and 520 (cells) and 532 (gDNA) for the *eae* assay.

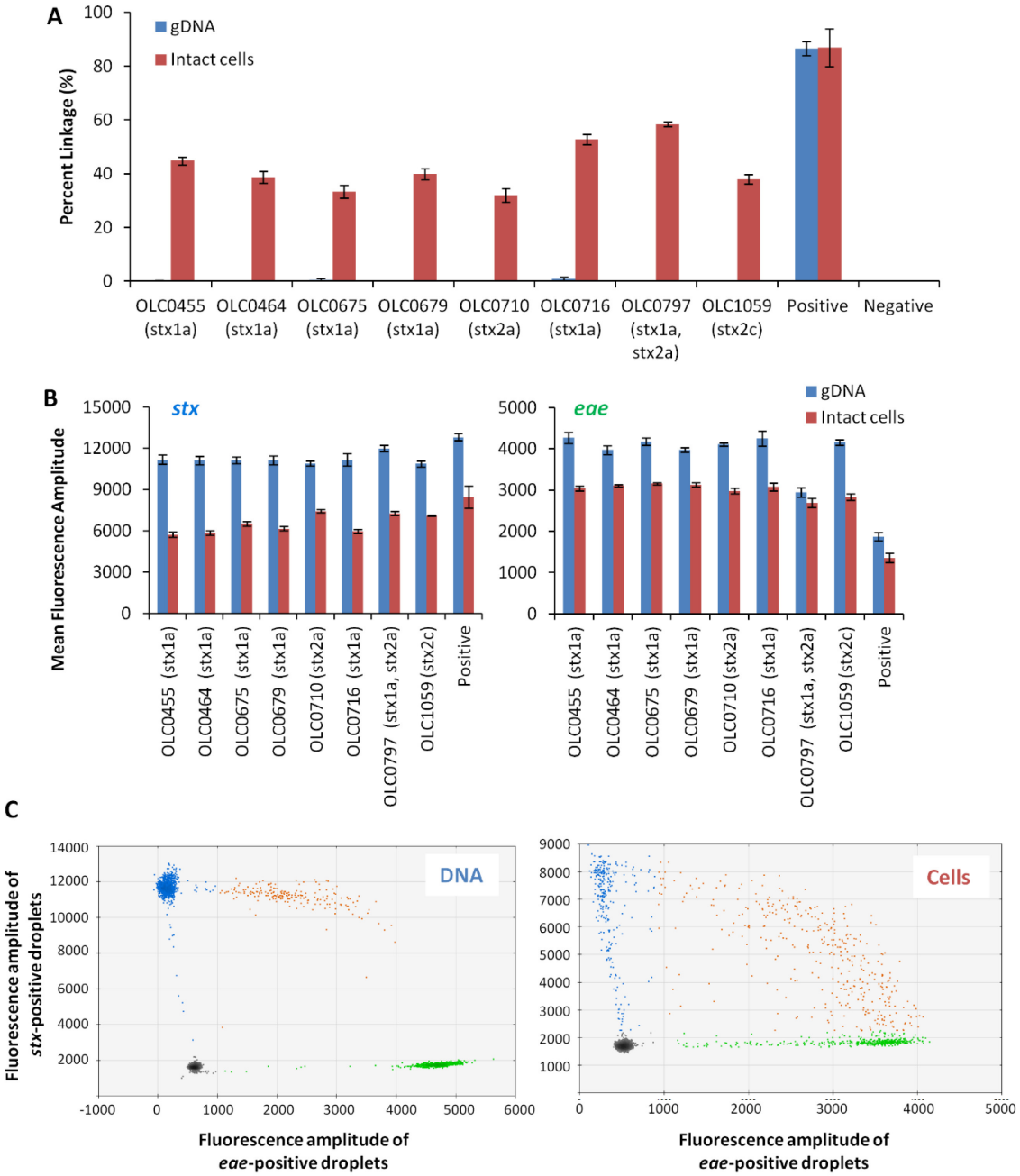

Figure 3. Linkage between *stx* and *eae* genes in intact STEC cells, but not in extracted gDNA using ddPCR.

(A) Co-amplification of *stx* and *eae* in ddPCR was evaluated with a panel of EHEC strains in assays using gDNA as template (blue bars) and assays using whole cells as template (red bars). Association of EHEC virulence targets was assessed by calculating a normalized value for linkage, based on the values provided by the QuantaSoft™ software (Percent linkage). A plasmid construct with both *stx* and *eae* genes or *E. coli* cells transformed with this plasmid (OLC2283), was used as a positive control. Error bars

represent the standard deviation of 4 technical replicates. (B) For both *stx* (right panel) and *eae* (left panel) assays, mean fluorescence of positive droplets was significantly higher when gDNA was used as a template relative to results using intact cells. (C) Representative 2D plots generated by the QuantaSoft™ analysis software for strain OLC0455 demonstrate more distinct partitions between positive and negative droplets when gDNA is used as a template (left panel) compared to intact cells (right panel). Droplets positive for *stx* amplification are blue, droplets positive for *eae* amplification are green, and droplets positive for both targets are orange.

Despite higher linkage of targets in whole-cell-based assays relative to assays using gDNA templates, the linkage of dual-amplification of targets within droplets was lower than was predicted (Figure 3C, Cells). To determine if this was due to reagent limitation in the droplets and/or problems with the use of intact cells in the ddPCR assay, a positive control plasmid containing segments of *stx1*, *stx2* and *eae* was created (Appendix 1) and transformed into *E. coli* cells. Greater than 86% linkage of the *stx*/*eae* targets was observed when either the plasmid DNA or intact cells containing the control plasmid (OLC2283) was used as template in the ddPCR reaction (Figure 3A), indicating that the limitation in dual-target amplification within a single droplet was not due to the use of intact cells, or exhaustion of reagents.

3.4.1.3. Incorporation of restriction enzyme digestion to improve droplet separation

To assess the possibility that the tertiary structure of the chromosomal DNA within the cells was preventing access to the target genes, the impact of digestion of the DNA through the integration of the restriction enzyme BamHI in the ddPCR mixture was evaluated (Figure 4). While ideal reaction conditions for the restriction enzyme digestion could not be achieved, modifications to the PCR conditions were incorporated to favour

DNA digestion (e.g., 45 minutes at 37°C). The addition of the restriction enzyme digestion did not result in an increase in the percent linkage between the *stx*/*eae* targets (Figure 4A). However, for the *stx* assay, there was an increase in mean fluorescence amplitude (8150 with enzyme versus 5818 without enzyme, Figure 4B) and more distinct partitions between positive and negative droplets. This difference in fluorescence amplitude was not observed for the *eae* gene (2548 with enzyme versus 2595 without enzyme). The mean fluorescence intensity of the negative droplets was slightly higher when enzyme was used, with a mean value of 1654 (enzyme) versus1563 (no enzyme) for the *stx* assay, and 603 (enzyme) versus 577 (no enzyme) for the *eae* assay, indicating a slight increase in autofluorescence in the enzyme assay. In both the *stx* and *eae* assays, standard deviation of the fluorescence amplitude among the four replicates was lower for the four samples in which enzyme was added. While the addition of enzyme does not seem to impact the linkage value used to identify EHEC samples, it was incorporated in the MuSIC ddPCR assay due to the observation of higher fluorescence and increased amplification of *stx* targets when the enzyme was incorporated.

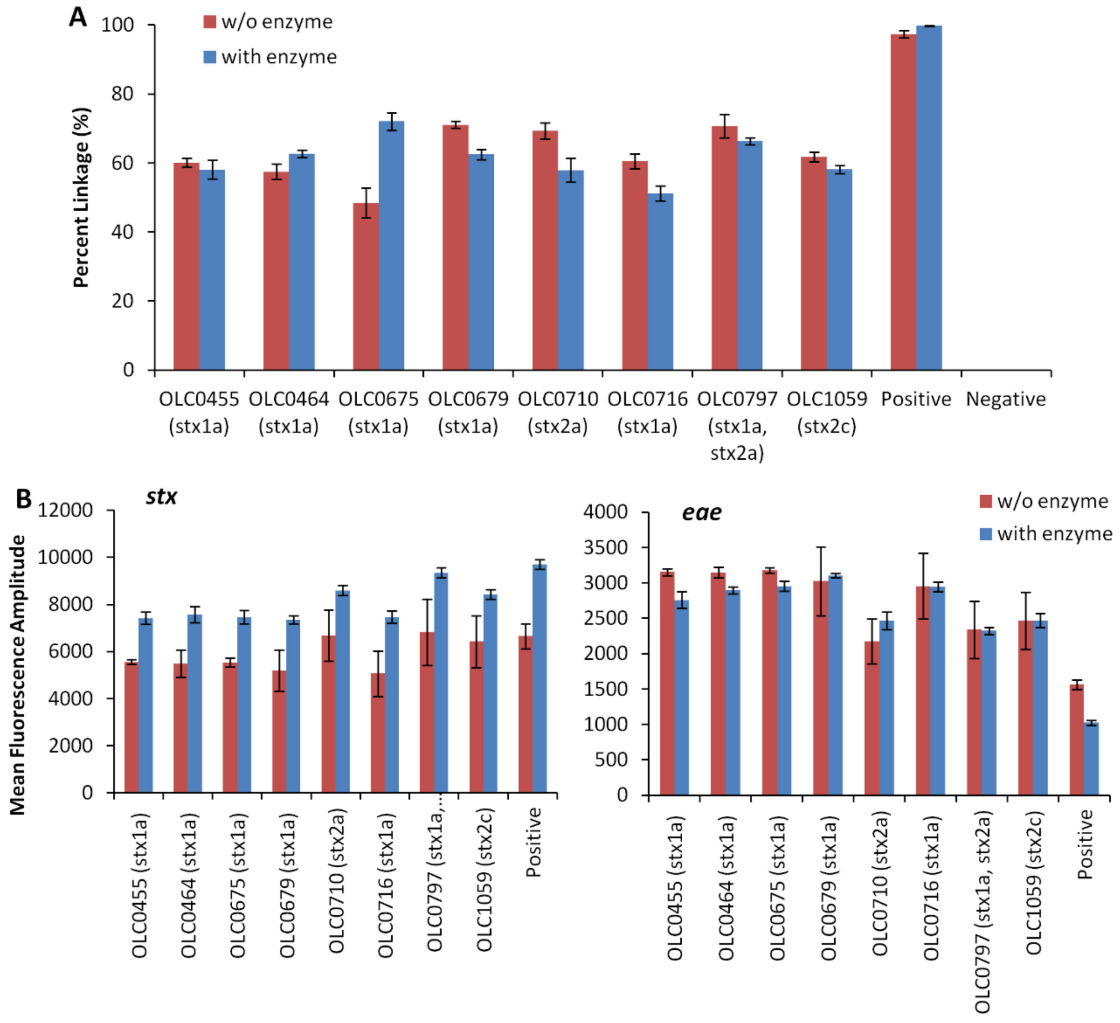

Figure 4. Improved droplet separation with the addition of restriction enzyme in the MuSIC ddPCR assay.

(A)The integration of BamHI restriction enzyme digestion in MuSIC ddPCR was assessed using a panel of EHEC strains with various serological profiles. Association of EHEC virulence targets was assessed by calculating a normalized value for linkage, based on the values provided by the QuantaSoft™ software (Percent linkage). Only minor differences in the proportion of droplets with amplification of both *stx*/*eae* targets was observed in assays with restriction enzyme (blue bars) compared to reactions without restriction enzyme (red bars). Error bars represent the standard deviation of 4 technical replicates. (B) An increase in the mean fluorescence amplitude of positive droplets for the *stx* assay (left panel) relative to assays conducted without the enzyme was observed. The use of the restriction enzyme did not seem to affect amplification of the *eae* target (right panel).

3.4.2. Evaluation of the MuSIC ddPCR Method

3.4.2.1. Distinguishing EHEC from mixed cultures of eae-negative STEC and eae-positive *E. coli*

Performance of the MuSIC ddPCR assay for distinguishing samples contaminated with EHEC from negative samples with mixtures of *eae*-negative STEC and *eae*-positive *E. coli* was evaluated. Overnight cultures of 11 EHEC strains (OLC0456, OLC0467, OLC0639, OLC0728, OLC0986, OLC1069, OLC1070, OLC1256, OLC1264, OLC2284, OLC2285) were diluted to concentrations of approximately 100 cfu/mL and 5 µL were added to each assay. For mixed cultures, overnight cultures of six strains of *eae*-negative STEC (OLC0669, OLC1335, OLC1535, OLC1685, OLC2238, OLC2250) were mixed at a 1:1 ratio with an overnight culture of an *eae*-positive *E. coli* (OLC0684), then diluted to approximately 100 cfu/mL. Samples containing EHEC could be accurately distinguished from mixed samples of *eae*-negative STEC and *eae*-positive *E. coli* based on the linkage between the *eae* and *stx* targets (Figure 5). This percent linkage was consistently above 39% for EHEC positive samples and below 1.99% for the mixed cultures. Concentrations of the EHEC and the mixed cultures used in these analyses were between 10 and 1300 cfu/µL (data not shown).

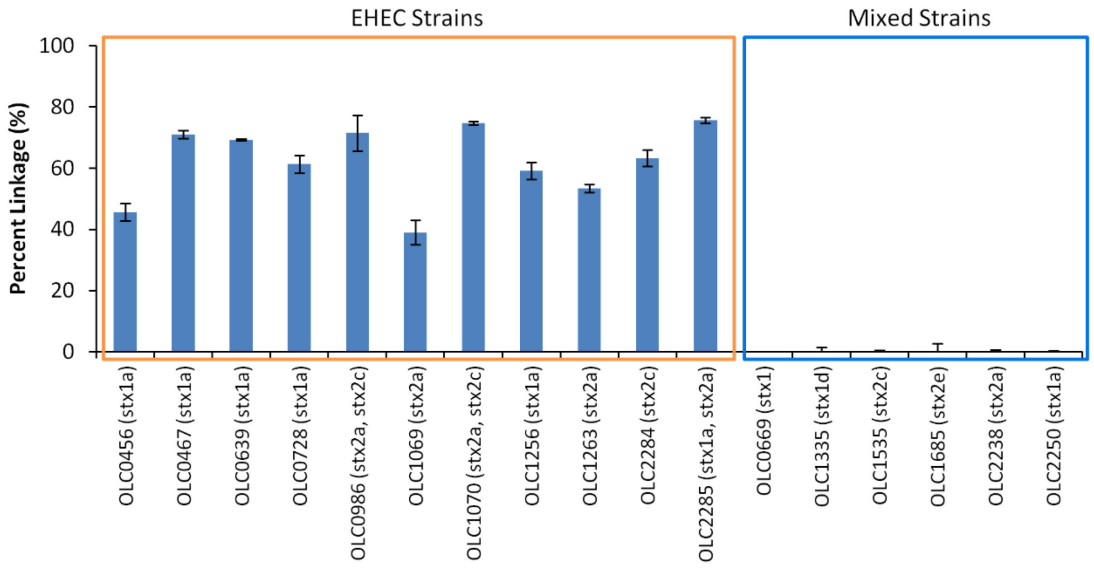

Figure 5. Specific detection of EHEC and not mixtures of *eae*-negative STEC and *eae*-positive *E. coli* based on linkage of *eae* and *stx* targets using MuSIC ddPCR. The performance of the MuSIC ddPCR assay was evaluated with a panel of eleven EHEC strains with varying serological profiles and with mixed cultures containing an *eae*-positive (OLC0684) with *eae*-negative STEC. Association of EHEC virulence targets was assessed by calculating a normalized value for linkage, based on the values provided by the QuantaSoft™ software (Percent linkage). All positive samples had a percent linkage above 30% while the 6 mixed cultures did not show linkage between the targets. Error bars represent the standard deviation of 4 replicates.

3.4.2.2. Performance of MuSIC ddPCR in simulated enrichment broths

The MuSIC ddPCR assay is intended to be applied to the detection of EHEC in enrichment broths with high concentrations of bacteria (approximately 10^5 cfu/µL), in which EHEC may be present at very low relative proportions (e.g. 0.01% of the population). Given that the maximum amount of enrichment broth that could be added to a ddPCR mixture is 5 µL, addition of undiluted enrichment broth to a ddPCR reaction (~500,000 bacteria) would result in the compartmentalization of ~25 non-target bacterial

cells in each of the 20,000 droplets. To determine if high levels of non-target cells in each of the droplets would inhibit the MuSIC ddPCR assay, EHEC strains (OLC0464, OLC0710 and OLC0797) and mixed cultures (1:1) of *eae*-negative STEC (OLC1335 or OLC1535) and *eae*-positive *E. coli* (OLC0684) were combined with different amounts of a non-pathogenic *E. coli* strain (OLC1543). The final ratios of EHEC or mixed cultures relative to the background *E. coli* were 1:10, 1:100, 1:1,000, and 1:10,000. The STEC + background samples were diluted in nutrient broth to generate an optimal target concentration for the ddPCR assay (approximately 100 STEC/µL of sample or 1 STEC per 40 droplets) while maintaining the ratio of target cells to background. While the number of target cells remained consistent for each assay, the number of background cells per droplet varied, with up to 1 bacterial cell/4 droplets in the 1:10 sample, 2.5 cells/droplet in the 1:100 sample, and 25 cells/droplet in the 1:1000 and 1:10,000 samples. Lower concentrations of STEC (approximately 10 STEC/ µL) were added for the 1:10,000 sample to avoid the need for a 10-fold concentration of the sample. For the mixed cultures, equal volumes of the overnight cultures were combined, resulting in lower concentrations of each of the targets for these samples as well (approximately 50 cfu of each strain/µL of the sample).

For all of the EHEC samples, greater than 50% linkage between *eae* and *stx* targets was observed (Figure 6A). In contrast, in mixed samples of bacteria with targets in different cells, percent linkage values were below 0.15%. The concentration of the *eae* target in the EHEC strains was determined to be 115 to 130 copies/µL of test sample, with the exception of the 1:10,000 samples, which ranged in concentration between 21 and 40 copies/µL (Figure 6B). The concentration of the *eae* target in the mixed cultures

was 38 to 52 copies/μL of test sample, with the exception of the 1:10,000 samples, which ranged in concentration between 8 and 9 copies/μL. These studies indicate that EHEC can be accurately detected in the presence of high levels of background microbiota, which could be presented in undiluted enrichment broths where target EHEC are present at relative concentrations as low as 1 target cell for every 10,000 non-target cells.

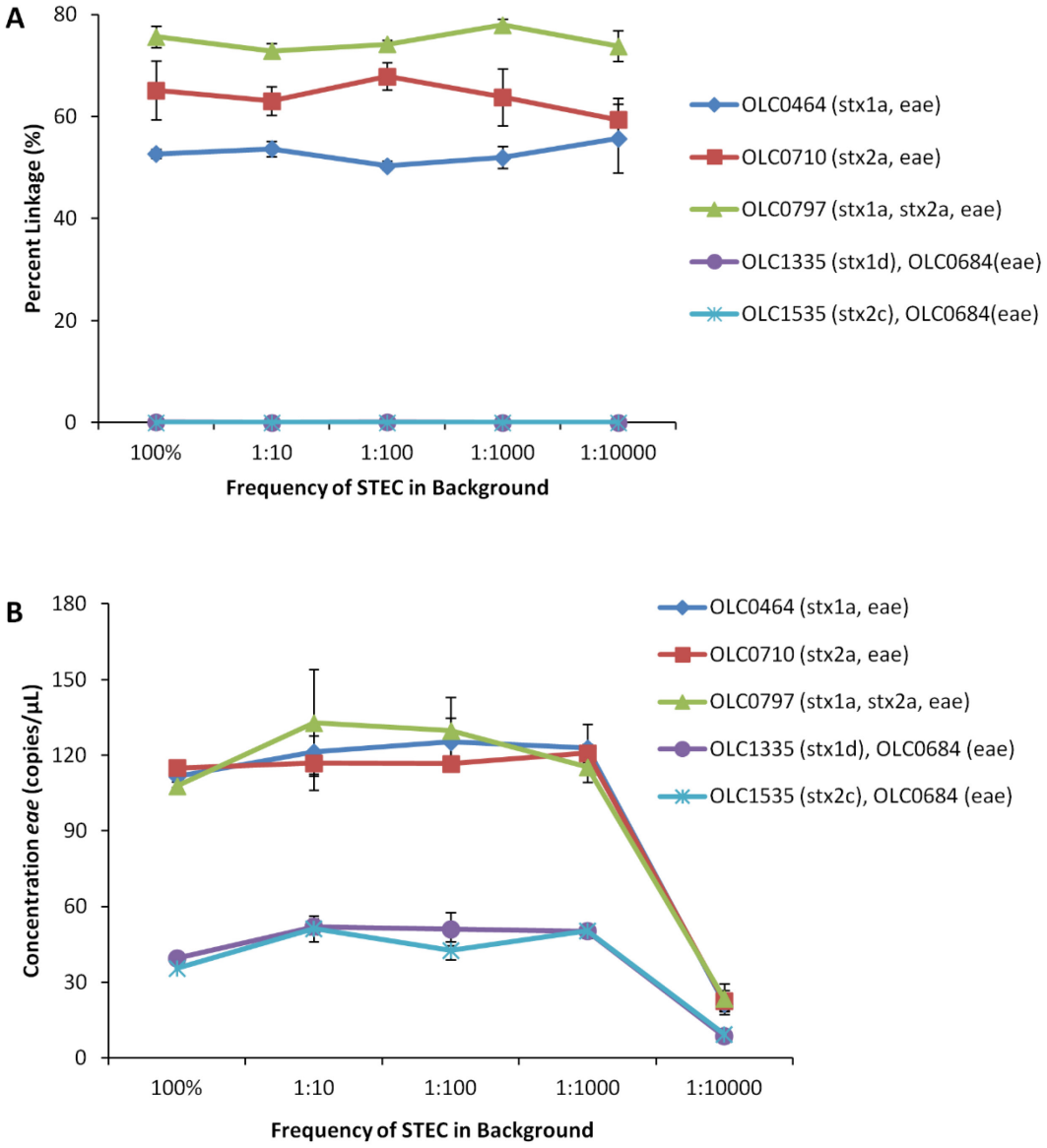

Figure 6. EHEC strains accurately detected at relative proportions of 1 EHEC pathogen in 10,000 non-target *E. coli* (1:10,000) using MuSIC ddPCR.
(A) To simulate enrichment conditions, EHEC cultures were added to generic *E. coli* at relative proportions of 1:10, 1:100, 1:1000 and 1:10,000. Association of EHEC virulence targets was assessed by calculating a normalized value for linkage, based on the values provided by the QuantaSoft™ software (Percent linkage). The EHEC samples were accurately detected (>50% linkage of *eae/stx*) in samples containing up to 10,000 non-target *E. coli* relative to target EHEC. The *eae/stx* genes were not linked in mixed cultures (OLC1335, OLC1535 mixed with OLC0684 (*eae*)) at any ratio. (B). Concentrations of the *eae* target in the samples determined based on values provided by

the QuantaSoft™ software. Error bars represent the standard deviation of 3 technical replicates.

3.4.2.3. MuSIC ddPCR with raw ground beef/pork and lettuce enrichments

Detection of EHEC in enrichment broths derived from food samples (ground beef/pork, leafy greens) was also evaluated to determine performance of the MuSIC ddPCR in the presence of typical food microbiota and inhibitors that may be present in these samples. Overnight cultures of EHEC strains OLC0679, OLC0710, OLC1059 and OLC1263 in mTSB were added to overnight ground beef/pork and produce enrichment broths at ratios of 1:100, 1:1000 (by volume). Mixed cultures of OLC0669 or OLC0335 with OLC0684 were diluted to relative proportions of 1:10 in the enrichment broths. Percent linkage between the *eae* and *stx* targets was greater than 23% in all of the 1:1000 EHEC samples, and greater than 43% in all of the 1:100 samples (Figure 7A). In contrast, in all of the mixed samples, percent linkage between these targets was lower than 0.45% (Figures 7A and B).

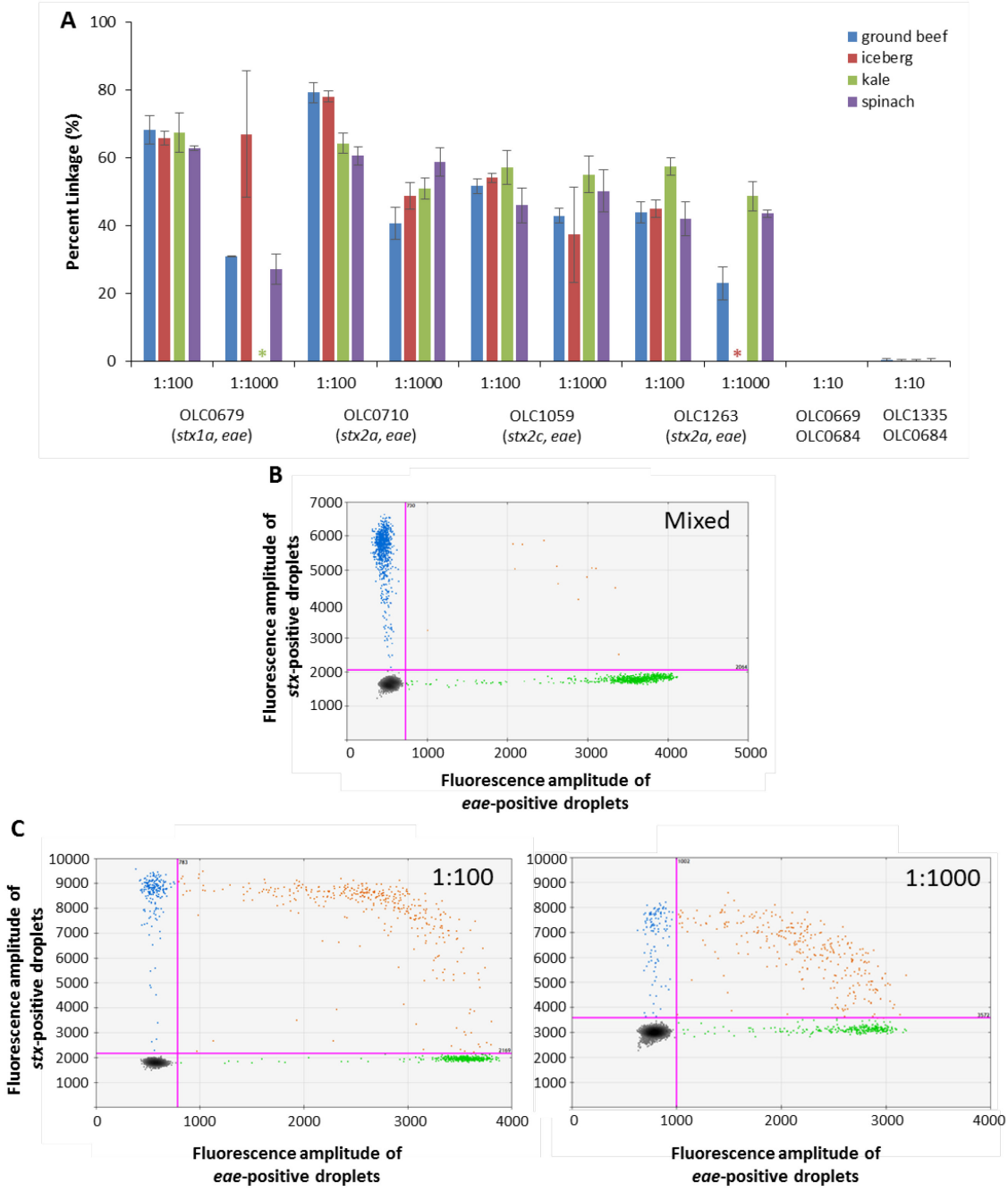

Figure 7. EHEC accurately detected at concentration of 1 volume of STEC pathogen in 1000 volume of overnight beef and lettuce enrichment (1:1000) using MuSIC ddPCR.

(A)The STEC *eae* positive samples were positively detected with greater than 23% linkage for samples with as little as 0.1% of pathogen relative to non-target microorganisms in the ground beef/pork (blue), iceberg lettuce (red), kale (green) and spinach (purple) enrichment broths. Stars indicate samples that were excluded due to missing data. Error bars represent the standard deviation of 3 technical replicates. (B) A representative 2D plot generated by the QuantaSoft™ analysis software for the 1:10

mixture of OLC0684 and OLC1335 with spinach enrichment broth is shown. Droplets positive for *stx* amplification are blue, droplets positive for *eae* amplification are green, and droplets positive for both targets are orange. (C) Representative 2D plots generated by the QuantaSoft analysis software for strain OLC1059 with spinach enrichment broth at relative proportions (target:background) of 1:100 (left) and 1:1000 (right) are shown.

The concentration of the *eae* target was determined to be between 64 to 262 copies/µL of test sample in all samples included in the analysis (data not shown). Ratios of 1:1000 for samples OLC0679 in iceberg lettuce, and OLC1263 in kale were excluded from the analysis due to concentrations lower than 5 copies/µL. In spinach and kale samples, autofluorescence seemed to interfere with the *stx* assay when the enrichment broths were minimally diluted (Figure 7C). Mean fluorescence amplitude intensity of both negative and positive droplets was higher in the *stx* assay for EHEC samples with relative proportions of 1:1000 of EHEC to enrichment broth, than in samples with proportions of 1:100.

3.5 Discussion

The MuSIC ddPCR assay represents a novel approach for the detection of two genetic targets within a single, intact, bacterial cell. To our knowledge, this is a new application of ddPCR technology. In this study, MuSIC ddPCR has been applied to the specific detection of EHEC in enrichment broths, and was shown to be able to distinguish samples contaminated with EHEC from samples containing mixed cultures of *eae*-negative STEC and *eae*-positive *E. coli*, even in samples with low proportions of target EHEC (e.g., 0.01% of the total bacterial population). This method is intended for samples

in which target EHEC are present at high concentrations (e.g. 10-500 cfu/µL), and would be appropriate for screening enrichment broths derived from foods.

One of the early challenges in the development of this method was the lot-to-lot variability in the performance of the degenerate primers used in the original implementation of the method. While this degeneracy may not impact standard quantitative PCR methods, for the ddPCR assay, the reduced concentrations of specific primers likely led to the exhaustion of these reagents in individual droplets. Specific primers performed more consistently, even in samples in which there were up to 3 mismatches between primers and primer binding sites. Note that this assay is not appropriate for the detection of the *stx2f* subtype of the *stx2* gene. Due to the high variability of the *stx2f* subtype relative to the other *stx2* subtypes, none of the methods currently deployed for the detection of EHEC are capable of detecting this subtype (B. Blais, Deschênes, *et al.*, 2014). Given the evidence that EHEC with this toxin variant are associated with clinical infections (Friesema *et al.*, 2015; Jenkins *et al.*, 2003; van Duynhoven *et al.*, 2008), improvement of current methods through the addition of specific *stx2f* primers and probes may be of value.

The use of intact bacterial cells was required to detect linkage between the *eae*/*stx* targets indicative of the presence of EHEC. These targets were not linked when gDNA extracts were used as template in the assay, probably due to fragmentation of the chromosomal DNA during its preparation (Figure 3). The *stx* and *eae* genes are widely dispersed on the *E. coli* chromosome (typically over 1 Mb); therefore, it is highly

probable that chromosomal breakage between these targets would occur during the extraction procedure (Brisco, Latham, Bartley, & Morley, 2010; Malentacchi *et al.*, 2015), and that the fragments would segregate into different droplets. In the case of the intact cell assay, the contents of the entire cell would be present within a droplet, and linked targets would remain in the droplet, even if the gDNA became degraded after the cell lysis. This is consistent with the higher linkage values observed for intact cells compared to gDNA (Figure 3). Optimization of droplet ddPCR often requires development of conditions in which there is a clear distinction between positive and negative partitions (Huggett *et al.*, 2013). While this partitioning could be easily achieved with the gDNA extracts, more variability in the fluorescence amplitude intensity was observed when cell cultures were used.

In the conception of this method, it was predicted that amplification of two targets would be observed for every droplet containing an intact EHEC cell. In practice though, dual amplification was lower than predicted, ranging from 23-79% of the droplets expected to be positive for both targets, with the lowest linkages observed for undiluted food enrichment broth samples. This was not due to reagent limitations, or the use of intact cells, as dual target amplification was observed in 87 to 99.9% of control samples of *E. coli* transformed with a plasmid containing both targets. Restriction digestion of gDNA templates is commonly recommended for ddPCR methods to relieve tertiary structure and improve target accessibility (Hindson *et al.*, 2011) and so this approach was evaluated. While conditions for the restriction digestion were not optimal due to the need to apply heat to lyse cells at the beginning of the reaction, thereby partially inactivating

the restriction enzymes, significant increases in the fluorescent intensities of the *stx*-positive droplets were observed (Figure 4B) indicating an improvement in the amplification of targets. This improvement may be due to reduction in tertiary structure near the *stx* genes, improving accessibility target sites. In this study, this did not translate into an increased proportion of droplets in which dual-amplification of the targets was observed; nonetheless, the use of thermostable restriction enzymes should be explored to determine if further improvement to the partitioning of the positive and negative droplets could be achieved. Another possible explanation for the low proportion of droplets with dual-target amplification is that degraded DNA released from dead cells provided single-target templates for this reaction. Removal of free gDNA could potentially be achieved by adding a DNase step before the cell lysis. Although variability in percentage linkage was high in the experiments conducted in this study, linkage in non-target mixed culture samples was consistently less than 2%, and EHEC-positive samples could be easily identified using a conservative cut-off value of >10% linkage.

The use of cultures of intact bacterial cells and food enrichments in the MuSIC ddPCR assay instead of purified gDNA presented a number of challenges, likely due to presence of debris and particles in these samples. In this study, up to 6% of samples were lost due to blockages within capillaries which prevented droplet formation. In addition, droplet counts were not consistent. Under ideal conditions, droplet counts should approach 20,000, but when using cell cultures and enrichment broths, droplet counts varied from 10,000 to as high as 28,000. This is not surprising as the ddPCR reagents were developed for purified samples, and would not be expected to be optimal for these

samples. Even with the use of purified gDNA, higher concentrations can increase viscosity of the sample, changing the average volume of the droplets (Hindson *et al.*, 2011). In most cases, the problems with droplet generation were overcome with the use of 3 to 4 technical replicates per sample. The performance of the assay was also variable depending on the broth used for growth of the bacteria. Mean fluorescence amplitude was higher when cells were suspended in nutrient broth relative to BHI, mTSB and PBS (not shown). Partitioning of positive and negative droplets was also impacted in enrichments broths generated from leafy-green produce such as spinach or kale (Figure 7C), where dark green pigments were observed in the enrichment broths.

Despite apparent inefficiencies with the use of intact cells in the MuSIC ddPCR assay, the application of the percent linkage value, with positives defined as samples with greater than 10% linkage between *stx* and *eae* targets, provided a robust metric for distinguishing EHEC-positive samples from mixed cultures containing targets in different cells, even in samples containing high levels of non-target bacteria (e.g. 10,000-fold excess). In samples of mixed strains that would have generated false positives by traditional PCR-based methods, linkage between the two targets was extremely low, less than 0.5 % in most samples. The use of undiluted food enrichments had some impact on the performance of the assay. For example, the higher degree of variability in percent linkage values for EHEC mixed at 1:1000 with food enrichment broths relative to the 1:100 mixtures (Figure 7A) may be due to the lower dilution used to achieve the optimal concentration of EHEC in the 1:1000 samples. Nonetheless, even in these samples, accurate identification of EHEC was achieved. Use of a positive control will be important

for evaluating the impact of inhibition in different food matrices. This proof-of-concept study shows promising results for the use of MuSIC ddPCR with food enrichments broths containing food debris and high relative proportions of background bacteria. The performance of the method was robust in food-enrichment samples where concentration of target cells was between 50 and 2500 cfu/reaction (detection limit of 10 cfu/µL in the original enrichment broth). While this is a limited dynamic range for detection compared to other PCR-based approaches, in practice testing of up to three dilutions of the enrichment broths will enable accurate detection of EHEC-positive samples.

False-positive detection of EHEC is a significant challenge for food-microbiology laboratories (M. Z. Alonso, Padola, Parma, & Lucchesi, 2011; Delannoy *et al.*, 2016; Feng *et al.*, 2010; Krause, Zimmermann, & Beutin, 2005; Shelton *et al.*, 2006). For example, in a study including 1739 beef enrichment broths, approximately half of the 180 enrichment broths positive for *stx* and *eae* markers were false-positives, and did not contain EHEC (Delannoy *et al.*, 2016). The work required for the analysis of these false-positive samples is onerous, as many colonies would need to be screened to confirm either the presence or absence of EHEC in the sample. To achieve higher specificity, the Canadian (B. Blais, Martinez, *et al.*, 2014), ISO/CEN TS13136:2012 Technical specification (ISO, 2012) and the US MLG5B.05 (USDA-FSIS, 2014) methods also detect O-serogroup specific markers for priority serovars, that vary among jurisdictions. Unfortunately, serogroup markers can also be present in non-pathogenic strains, and not all of the clinically-important EHEC fall within the priority serogroups (EFSA, 2013). New screening methods that use additional genes, associated with typical EHEC, have

been effective in reducing false-positive detection of EHEC in food enrichment broths (Delannoy et al., 2016, 2013), but these rely on the detection of a number of different genes known to be associated with EHEC, none of which (individually) are present in all EHEC variants. Specific detection of samples containing typical EHEC, which are *eae*- and *stx*-positive, would enable identification of emerging pathotypes in foods.

The MuSIC ddPCR EHEC assay enables accurate detection of EHEC in enrichment broths based on detection of two diagnostic genetic markers (*stx* and *eae*) and determination that these occur in a single cell. While this method is somewhat more complex than standard PCR approaches, the benefit in reducing false-positive detection of EHEC outweigh the challenges associated with the implementation of the method. A more extensive validation of the assay with priority food types (e.g. leafy-green produce and raw meat) will be undertaken to enable deployment to food-testing laboratories. Integration of additional targets such as the *stx2f* toxin subtype and/or the *aaiC* and *aggR* markers associated with the seropathotype responsible for the 2011 European O104 outbreak (EFSA, 2013) may also be of value. The reduction in false-positive detection of EHEC associated with the application of the MuSIC ddPCR EHEC assay will enable high throughput screening for EHEC in food samples, ultimately reducing consumer exposure to EHEC-contaminated products.

4 Chapter: Inhibition of Shiga toxin-producing *E. coli* (STEC) and *Shigella* by Bacteria Present in Food Enrichment Cultures

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Key words: Bacteriocin, bacteriophage, STEC, *Shigella*, food

4.1 Abstract

Current microbiological methods for identification of Shiga toxin-producing *E. coli* (STEC) and *Shigella* spp. in foods require an initial enrichment step. However, these organisms can be difficult to grow in enrichment cultures due to the presence of competing non-target bacteria in the food matrix. Bacteria present in the food matrix often outcompete target pathogens due to faster growth rates, or through production or presence of antibiotics such as bacteriocins or bacteriophages. The purpose of this study was to determine the frequency of occurrence of *Shigella* /STEC inhibitors produced by non-target bacteria present in food enrichment cultures. We evaluated production of antibiotic compounds in cell-free extracts from 199 bacterial isolates recovered from food samples, and 333 food enrichment broths. Cell-free extracts produced by 23 (12%) of the

isolates tested inhibited growth of at least one of the *Shigella* or STEC strains used in this study. Of the 333 enrichment broths tested, cell-free extracts from 25 (7.5%) of the samples inhibited growth of at least one of the indicator strains tested. *E. coli* was the most common bacterial isolate that inhibited growth of the *Shigella* and STEC strains. Meat products were the most common food enrichment that had antibiotic-producing bacteria that inhibited the growth of *Shigella* and STEC. It was also discovered that the inhibiting compounds were largely protein based (34 of the 48 positive samples, 71%) as they were affected by proteolytic enzymes. The inhibitors in the remaining 14 samples were determined to be bacteriophages as they were not affected by proteolytic enzymes and plaques were observed. Improvements to the *Shigella* and STEC methods should mitigate the impact of these antibiotic-producing organisms in enrichment cultures.

4.2 Introduction

Foodborne illnesses due to Shiga toxin-producing *E. coli* (STEC) and *Shigella* are still an important public health concern around the world (Adam & Pickings, 2016; EFSA, 2013; Gould *et al.*, 2013; The *et al.*, 2016; Thomas *et al.*, 2015). In Canada, it is estimated that there are approximately 1200 domestically-acquired cases of Shigellosis and approximately 33 000 illnesses attributed to STEC per year (Thomas *et al.*, 2013). Infections by both STEC and *Shigella* can result in complications such as hemolytic-uremic syndrome (HUS), hemorrhagic colitis and can lead to the development of Reiter's syndrome (Donnenberg & Whittam, 2001; Perelle *et al.*, 2004; Smith *et al.*, 2014; The *et al.*, 2016; Warren *et al.*, 2006). These complications can have long term effects and are sometimes fatal (Smith *et al.*, 2014; The *et al.*, 2016).

Current methods used to detect foodborne STEC and *Shigella* in Canada, USA and Europe involve an enrichment step followed by screening of enrichments for characteristic virulence genes (Burton Blais, Martinez, McIlwham, Mohajer, & Gauthier, 2014; ISO, 2012; USDA-FSIS, 2014). An enrichment step is necessary as levels of target bacteria are typically low in the food samples. The enrichment procedure is intended to favour the growth of the target pathogens while limiting the growth of non-target or background bacteria present in the food sample. In many enrichment cultures, the non-target bacteria can outcompete the target pathogen. This can lead to false-negative samples in cases where the pathogen dies or is present at a very low concentration after enrichment and cannot be detected or isolated in downstream analyses.

One mechanism by which background bacteria can outcompete STEC and *Shigella* is through microbial antagonism via the production of antibiotics such as bacteriocins or other antimicrobial compounds. Bacteriocins are normally narrow spectrum antimicrobial peptides that kill closely related bacteria to reduce competition for resources. (Kim, Tarr, & Penfold, 2014; Yang *et al.*, 2014). There are 3 classes of bacteriocins produced by Gram-positive bacteria: lantibiotics, non-lanthionine and heat unstable (Yang *et al.*, 2014). Bacteriocins produced by Gram-negative bacteria can be classified as colicins or microcins (Duquesne, Destoumieux-Garzón, Peduzzi, & Rebuffat, 2007; Mader *et al.*, 2015; Yang *et al.*, 2014). It is believed that more than 99% of bacteria encode at least one bacteriocin gene (Yang *et al.*, 2014). Other antibiotic compounds such as aminoglycosides (streptomycin, neomycin, kanamycin, gentamicin), tetracyclines and aminocoumarins (Mohr, 2016) are produced by certain bacterial groups such as the orders Actinomycetales and Bacillales as a means to reduce competition by

inhibiting growth or destroying competitor bacteria (Challinor & Bode, 2015; Mohr, 2016).

Colicins and microcins are bacteriocins produced by and targeting Gram-negative bacteria. Colicins are large bacteriocins (25-80 kDa) typically plasmid-encoded and involve a structural gene, immunity gene and a lysis gene. These proteins are induced by the SOS response and sometimes by environmental stress (Bayramoglu *et al.*, 2017; Duquesne *et al.*, 2007). It is important that the colicin and lysis gene are not induced unnecessarily as the lysis and release of the colicin causes suicide of the cell. Lysing of the cell and colicin release only occurs when the lysis protein has reached a certain concentration threshold (Bayramoglu *et al.*, 2017; Ghazaryan, Tonoyan, Ashhab, Soares, & Gillor, 2014).

Expression of the lysis gene is tightly controlled, and not all cells in a population produce the lysis and colicin genes at the same time. In a culture, about 0.1% of the cells produce colicin and cause lysis under normal conditions and this is increased to 3% during nutrient starvation and 50% after SOS response (Bayramoglu *et al.*, 2017). The immunity gene is continuously being expressed at small levels so that a bacterium closely related to the cell releasing colicin can be protected (Ghazaryan *et al.*, 2014). Colicin receptors on bacterial cell membranes are usually receptors that uptake nutrients such as iron and vitamin B12 (Ghazaryan *et al.*, 2014; Kim *et al.*, 2014). The Tol and Ton transporters translocate the colicin into the cell. Once inside the cell, colicin acts by degrading DNA/RNA, inhibiting protein synthesis, forming channels in cell membranes or digesting peptidoglycan precursors (M. Z. Alonso *et al.*, 2011; Mader *et al.*, 2015; Yang *et al.*, 2014).

Microcins are smaller than 10 kDa and are highly stable to heat, pH and protease (Duquesne *et al.*, 2007; Yang *et al.*, 2014). Microcin genes are plasmid- or chromosome-encoded, and gene clusters include a microcin precursor, self-immunity factors, secretion proteins and modification enzymes (Duquesne *et al.*, 2007). Microcins are secreted from the cell through an ABC transporter or efflux pumps and attach to specific receptors on the targets cell (Duquesne *et al.*, 2007; Yang *et al.*, 2014). Some receptors include TonB-dependent iron-uptake machineries, ompF, SdaC and SbmA of other bacteria (Duquesne *et al.*, 2007). Once inside the target cell, they act through pore formation, DNA/RNA degradation, protein synthesis inhibition or DNA replication inhibition (Duquesne *et al.*, 2007; Yang *et al.*, 2014).

Another mechanism of microbial antagonism is through the production of bacteriophages that can infect and kill bacteria (Penadés, Chen, Quiles-Puchalt, Carpena, & Novick, 2015). Bacteriophages have two cycles of viral reproduction: the lysogenic and lytic cycles. In the lysogenic cycle, the bacteriophage genome is integrated into the host's genome as a prophage and is propagated through replication of the host's chromosome (Parasion, Kwiatek, Gryko, Mizak, & Malm, 2014; Penadés *et al.*, 2015). In this cycle, the bacteriophages do not kill the host cell and use the host's machinery to produce new phages. The phages are then released though programmed cell lysis to infect new cells (Penadés *et al.*, 2015). Bacteriophages in the lysogenic cycle can be induced into the lytic cycle when the bacteria undergo stress or through induction of the SOS response. Most phages can only affect a subset of bacteria within a species, and specificity of the phage depends on the receptors it binds to (Koskella & Meaden, 2013; Parasion, Kwiatek, Gryko, Mizak, & Malm, 2014).

The purpose of this study was to evaluate the prevalence of *Shigella* spp. and STEC inhibitors produced by bacteria commonly found in foods and to identify likely mechanisms of inhibition. Cell-free extracts derived from bacteria isolated from enrichment broths were tested for inhibitory activity against STEC and *Shigella*. Food enrichments in modified Tryptone Soya Broth (mTSB) for STEC or *Shigella* broth were also evaluated. Samples containing inhibitors were characterized to determine likely mechanisms of inhibition. Results of this study will inform the development of improved methods for the recovery and detection of STEC and *Shigella* spp. from foods.

4.3 Materials and Methods

4.3.1. Growth and Maintenance of Bacterial Strains.

A selection of predominantly Enterobacteriaceae strains, mainly isolated from foods enrichment broths, were selected for this study (Appendix 2). Seven STEC isolates representing priority STEC and five *Shigella* strains were used as the indicator strains for testing sensitivity to inhibition from cell-free extracts (Table 3). All strains were stored at -80°C in 15% glycerol and were plated on Brain-Heart Infusion agar (BHI) (OXOID, Nepean, ON, Canada) overnight (14–16 h) at 37°C prior to use.

Table 3. List of STEC and Shigella strains used in this study

Strain name	Genus	Species	Serotype
OLC0024	*Shigella*	*sonnei*	
OLC0603	*Shigella*	*flexneri*	
OLC0608	*Shigella*	*dysenteriae*	

OLC1597	*Shigella*	*flexneri*	
OLC2340	*Shigella*	*sonnei*	
OLC0455	*Escherichia*	*coli*	O111:H11
OLC0464	*Escherichia*	*coli*	O26:H11
OLC0675	*Escherichia*	*coli*	O145:NM
OLC0679	*Escherichia*	*coli*	O103:H2
OLC0710	*Escherichia*	*coli*	O121:H19
OLC0716	*Escherichia*	*coli*	O45:H2
OLC0797	*Escherichia*	*coli*	O157:H7

4.3.2. Preparation of Cell-free Extracts.

Bacterial isolates from foods (Appendix 2) were grown overnight at 37°C in 10 mL of Nutrient broth (OXOID) and broths were filtered using a 0.22 µm Vacuum filter (EMD Millipore Steriflip™ Sterile Disposable Vacuum Filter Units; Thermo Fisher Scientific, Ottawa). The isolates had previously had their whole genome sequenced and the genera were determined based on 16S rDNA sequence (data not shown). The cell-free extracts were stored at 4°C for up to 3 months and at -20°C for longer storage.

Three hundred and thirty-three food enrichment broths derived from analyses conducted using either the MFLP-26 method for detection of *Shigella* (Kingombe *et al.*, 2006a) or the MFLP-30 method for detection of STEC (Microbiological Methods Committee, 2012) were tested for the presence of *Shigella* and STEC inhibitors (Appendix 3). In the MFLP-26 method, samples are enriched in a 1:10 dilution of sample to *Shigella* broth (*Shigella* broth base (OXOID) with Tween-80 (Sigma, Markham, ON,

Canada)) containing 0.5 µg/mL of Novobiocin (Sigma). The *Shigella* broth is enriched for 20 hours at 42°C in a CO_2 incubator or CO_2 jar system. Similarly, the MFLP-30 method requires enrichment using a 1:10 dilution of sample to modified Tryptone Soya Broth (mTSB, OXOID) with 20 µg/mL of Novobiocin. The mTSB is enriched at 42°C for 18-24 hours.

Most of the food products were sampled in the fall of 2016 and the winter of 2017. Enrichment broths were stored at 4°C prior to use. Cell-free extracts were prepared by centrifugation at 500 x g for 5 minutes, transferring the supernatant to a new tube and centrifugation at 14 000 x g for 10 minutes. The supernatant was then filtered and stored at 4°C or -20°C as described above. The pellets from the high-speed spin were resuspended in 30% glycerol and stored at -80°C.

4.3.3. Detection of Inhibitors in Cell-free Extracts.

The methods for evaluating the inhibitory activity of cell-free extracts were modified from methods developed by Arici *et al.*(2004) and Vijayakumar *et al.* (2015)(Figure 8). *Shigella* and STEC were grown overnight in 10 mL of Nutrient Broth (OXOID) at 37°C to obtain a concentration of approximately 10^8 cells/mL. The overnight *Shigella* or STEC culture was added at a concentration of approximately 1 x 10^6 cells/mL into 0.5% Nutrient Agar (soft agar) and poured into a petri dish. The soft agar consisted of Nutrient broth (OXOID) with 0.5% bacteriological agar (Sigma). Once the plates were solidified, 3 µL spots of the cell-free extracts were added and left to dry before incubating overnight at 37°C. To enable high-throughput analyses, a multichannel pipette was used to generate 36 spots on square petri plates.

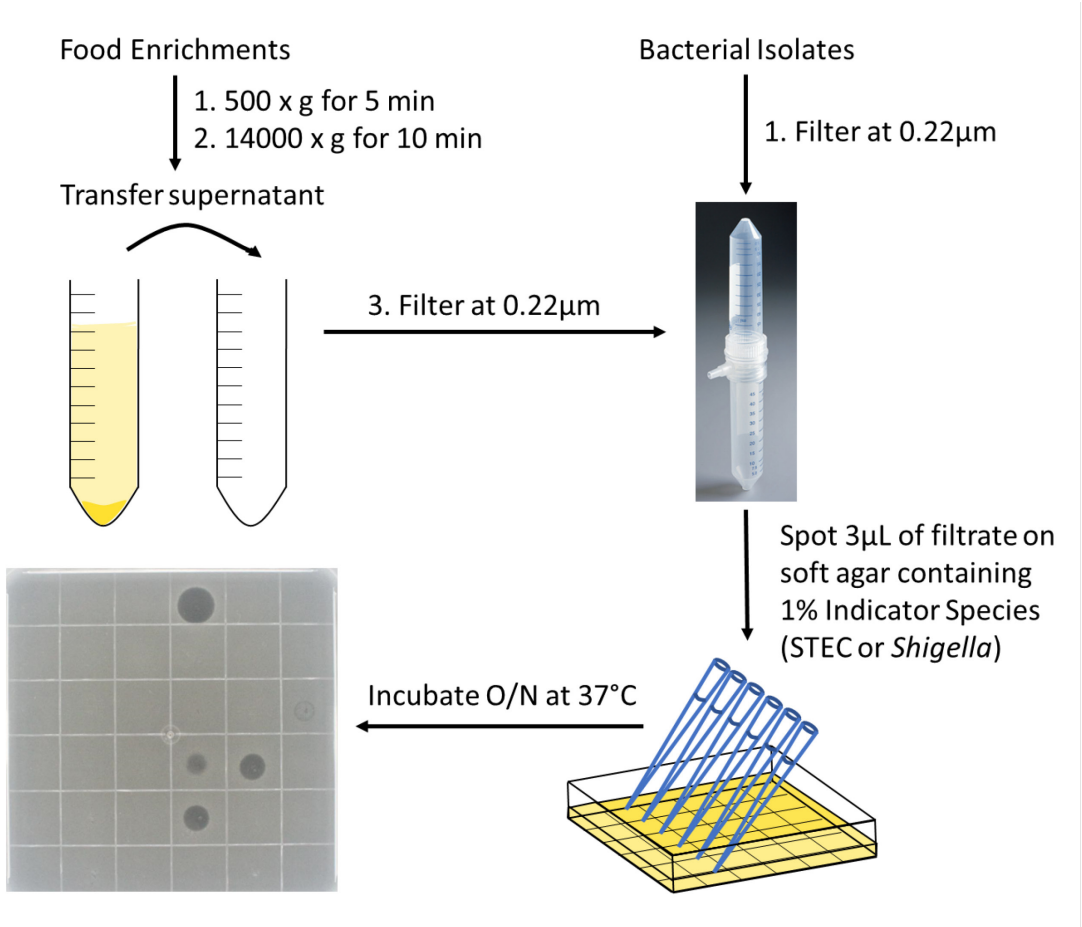

Figure 8. Method for detecting inhibitors from Cell-free extracts.

Food enrichments were incubated according to MLFP-26 or MLFP-30 methods (Kingombe *et al.*, 2006a; Microbiological Methods Committee, 2012). An aliquot of 50 mL was taken and centrifuged first at 500 x g to remove large food particles and then at 14000 x g to remove bacterial cells and debris. The supernatant was then filtered with a 0.22 µm Vacuum filter to ensure the extracts were cell-free. The bacterial isolates were grown overnight in 10 mL of Nutrient broth at 37°C. The overnight culture was then filtered directly with a 0.22 µm Vacuum filter. Filtrates were now considered cell-free extracts and were spotted on soft agar with the indicator species. Spots were left to dry, and plates were incubated overnight at 37°C. The following day, pictures were taken to observe the clearings and the diameter of the clearings were measured in millimeters. The plate in this figure had 35 different cell-free extracts and a negative control spotted in a 6 x 6 grid.

4.3.4. Isolation of Bacteria producing Inhibitors from Food Enrichments: Triple Agar Overlay Method.

The method from Henning *et al.* (2015) was used to isolate the bacterium from a subset of the food enrichment broths that were inhibitory to at least one indicator organism. Dilutions of the enrichment broths were spread plated on nutrient agar (OXOID) then immediately overlaid with a thin nutrient agar sandwich layer. The plates were incubated at 37°C overnight before a 0.5% nutrient agar containing ~1 x 10^6 cells/mL of the indicator species (*Shigella* or STEC) was layered on top. The triple layer agar was incubated overnight at 37°C.

The triple agar plates were inverted and placed onto the petri dish cover and colonies that contained clearings were cut out with a sterile loop and streaked onto a new nutrient agar plate. The streaked plates were incubated overnight at 37°C and colonies were transferred onto new nutrient agar plates in duplicates. The two plates were incubated at 37°C for 4 hours and then one of the duplicate plates was overlaid with 0.5% nutrient agar containing 1% of the indicator species. The plates were again incubated at 37°C overnight. Finally, 400 µL of nutrient broth was added to the colonies that were confirmed to cause inhibition and the culture was grown at 37°C for 4 hours. Testing of strains for production of inhibitors, and maintenance of cultures was done as described above.

4.3.5. Testing cell-free extracts for proteinaceous properties, bacteriophage and pH.

Different proteolytic enzymes were used to assess the proteinaceous nature of the inhibitory cell-free extracts (Elayaraja, Annamalai, Mayavu, & Balasubramanian, 2014). Proteinase K (Thermo Fisher Scientific) and trypsin (Sigma) were added to the extracts at a final concentration of 1mg/mL or 1X. The extracts were incubated for 2 hours at 30°C before spotting on soft agar as described in the Detection of Inhibitors in Cell-free Extracts section above. The presence of bacteriophage was identified using the dilution method from Hockett *et al.* (2017). Cell-free extracts were serially diluted by a factor of ten, three times in nutrient broth. Dilutions were spotted on soft agar as described above. Bacteriophage presence was confirmed if individual plaques were visible in the diluted samples. The pH of the cell-free extract was measured with pH Indicator strips (Thermo Fisher Scientific)

4.3.6. Whole Genome Sequencing (WGS) Analysis

WGS analysis was used to identify species of bacterial isolates. Genomic DNA was isolated from overnight cultures grown on brain heart infusion (BHI) agar using the Promega, Maxwell 16 cell DNA purification kit (Promega, Madison, WI). Sequencing libraries were constructed using the Nextera XT DNA sample preparation kit (Illumina, Inc., San Diego, CA) and paired-end sequencing was performed on the Illumina MiSeq platform (Illumina, Inc.), using a 600 cycle MiSeq reagent kit (v3). Sequencing errors in reads were corrected using Quake version 0.3 with a k-mer size of 15 (Kelley, Schatz, & Salzberg, 2010) and assembled *de novo* using

SPAdes v3.1.1(Bankevich *et al.*, 2012). Contigs shorter than 1000 bp were excluded from the analysis.

To determine the species of bacterial isolates the following analysis was done. The NCBI 16S rRNA microbial BLAST database (downloaded from ftp://ftp.ncbi.nih.gov/blast/db/16SMicrobial.tar.gz) was converted to FASTA format using blastdbcmd (Camacho et al., 2009) and custom Perl code. 16S rRNA sequences from FASTQ-formatted raw reads were extracted and mapped against the 16S rRNA database to determine the genus of samples using the GeneSipprV2 pipeline (https://github.com/OLC-Bioinformatics/geneSipprV2).

4.4 Results

4.4.1. Inhibition of growth of *Shigella* and STEC by cell-free extracts derived from bacterial isolates.

A total of 199 cell-free extracts from bacterial isolates recovered from food were tested in triplicate to detect inhibition of growth of five strains of *Shigella* and seven strains of STEC. A total of 23 of the 199 samples tested (12%) impacted growth of at least one of the indicator organisms (*Shigella* and EHEC) used in this study. Most of the isolates were Enterobacteriaceae (194/199), except for five strains (Pseudomonadaceae and Aeromonadaceae; Figure 9A). The three main genera evaluated were *Escherichia*, *Enterobacter* and *Hafnia* (122(61%), 31(16%) and 19(10%) respectively). Cell-free extracts produced by 21 *E. coli* isolates (11% of all isolates, 17% of *E. coli*) inhibited growth of *Shigella* and cell-free extracts from two *Enterobacter* spp. isolates (1% of all isolates, 6.5% of *Enterobacter* isolates) inhibited growth of STEC (Figures 9B). Most of the antimicrobial-producing *E. coli* affected *S. sonnei* (18, 86%), with a smaller

proportion (7, 33%) affecting *S. flexneri*. In most samples, both strains of *S. sonnei* (OLC0024 and OLC2340) were affected (16 out of 18) whereas only 3 out of the 7 extracts affecting *S. flexneri* inhibited both of the strains used in this study (Figure 10). The antimicrobial-producing *Enterobacter* spp. affected *E. coli* O45 (2, 100%) and *E. coli* O103 (1, 50%) (Figure 10). None of the isolates evaluated in this study inhibited growth of *S. dysenteriae. E. coli* isolates were able to show inhibitory activity against *S. sonnei* and *S. flexneri* while *Enterobacter* isolates were able to show inhibitory activity against certain EHEC strains.

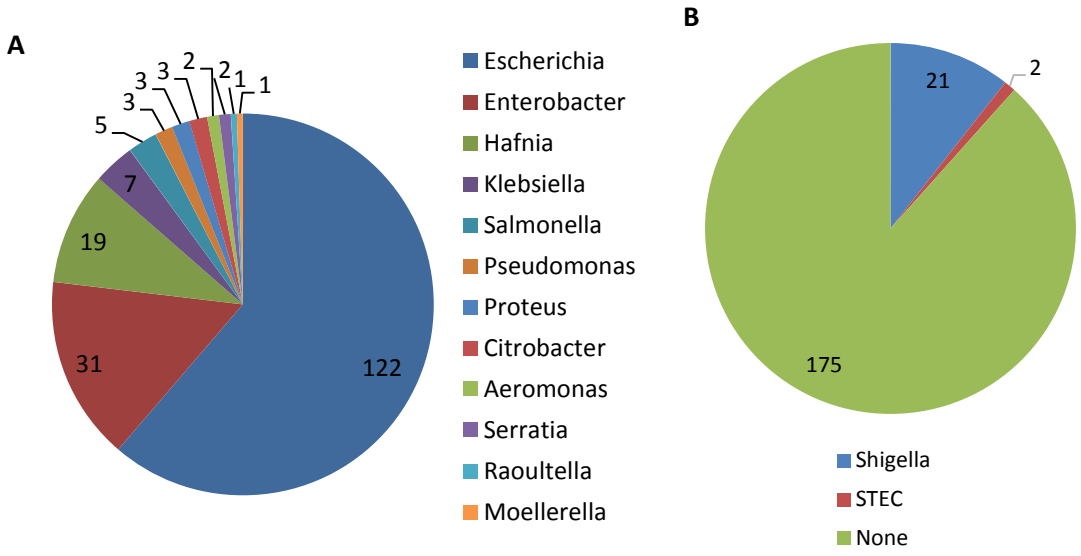

Figure 9. Species and inhibitory activity of food isolates tested against *Shigella* and STEC.

A: Species distribution of food isolates tested for *Shigella* and STEC for inhibition. B: Number of bacterial isolates that inhibited *Shigella* and STEC. In total 199 bacterial isolates were tested on *Shigella* (2 *Shigella sonnei*, 2 *Shigella flexneri* and 1 *Shigella dysenteriae*) and STEC (1 each of O26, O45, O103, O111, O121, O145, O157) samples.

The inhibitory activity varied among cell free extracts and was described based on diameter of the zone of inhibition and strength of the clearing (Figure 10A). Samples were designated very weak (3mm diameter) to very strong (11mm diameter) (Figure 10; Appendix 4). The inhibitory activity of cell-free extracts from *E. coli* on *S. sonnei* was mostly strong and very strong whereas inhibitory activity on *S. flexneri* was largely designated as medium or weak. Similarly, the cell-free extracts from *Enterobacter* spp. that inhibited STEC produced medium or weak inhibition of growth. The pH of the Cell-free extract from the food isolates were between 6.5 and 7.5.

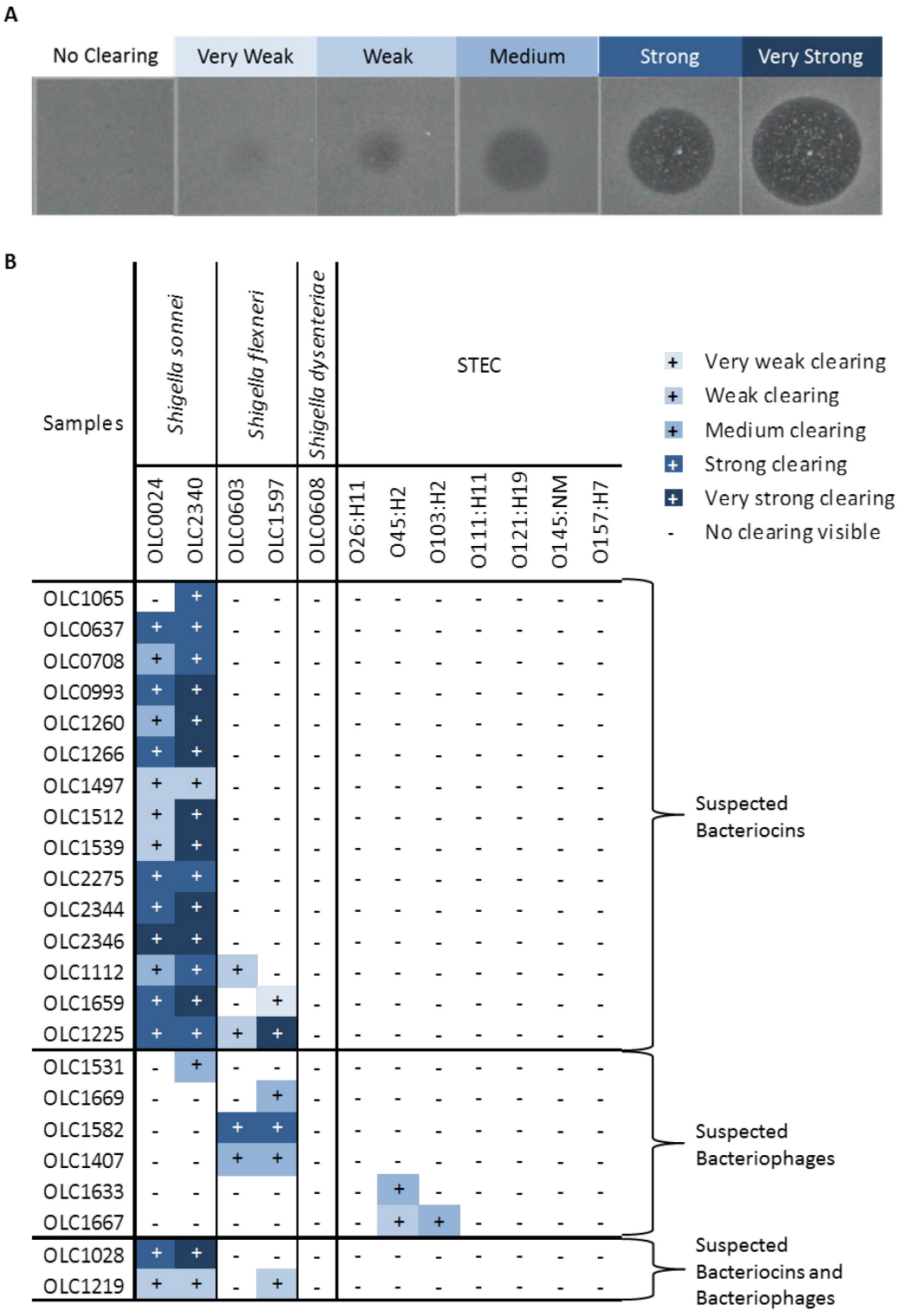

Figure 10. Strengths of inhibitory activity of bacterial isolates.

A: Representative image of the different strengths of the inhibitory activity observed in the samples used in this study. B: Strengths of inhibitory activity of bacterial isolates on

Shigella and STEC. The isolates are grouped based on likely inhibitor present in the sample (suspected bacteriocins or bacteriophages).

4.4.2. Inhibition of *Shigella* and STEC from cell-free extracts from food enrichments.

Cell-free extracts derived from 333 food-enrichment broths were evaluated to determine prevalence of inhibitors to *Shigella* spp. or STEC in food enrichments. Food products were sampled between the fall 2016 and winter 2017 and were representative of the types of foods currently tested in regulatory food testing programs (Figure 11). Of the food products tested, there were 120 food products enriched in mTSB only, 93 enriched in mTSB and *Shigella* broth and 27 enriched in *Shigella* broth only. Categories of food products used in the enrichments included fruits (46 (14%)), salads and coleslaws (78 (23%)), meats (85 (26%)) and vegetables, cheese and other (124 (37%)) (Figure 11). The meats and cheese were only enriched in mTSB and the rest were enriched in *Shigella* broth or both broths.

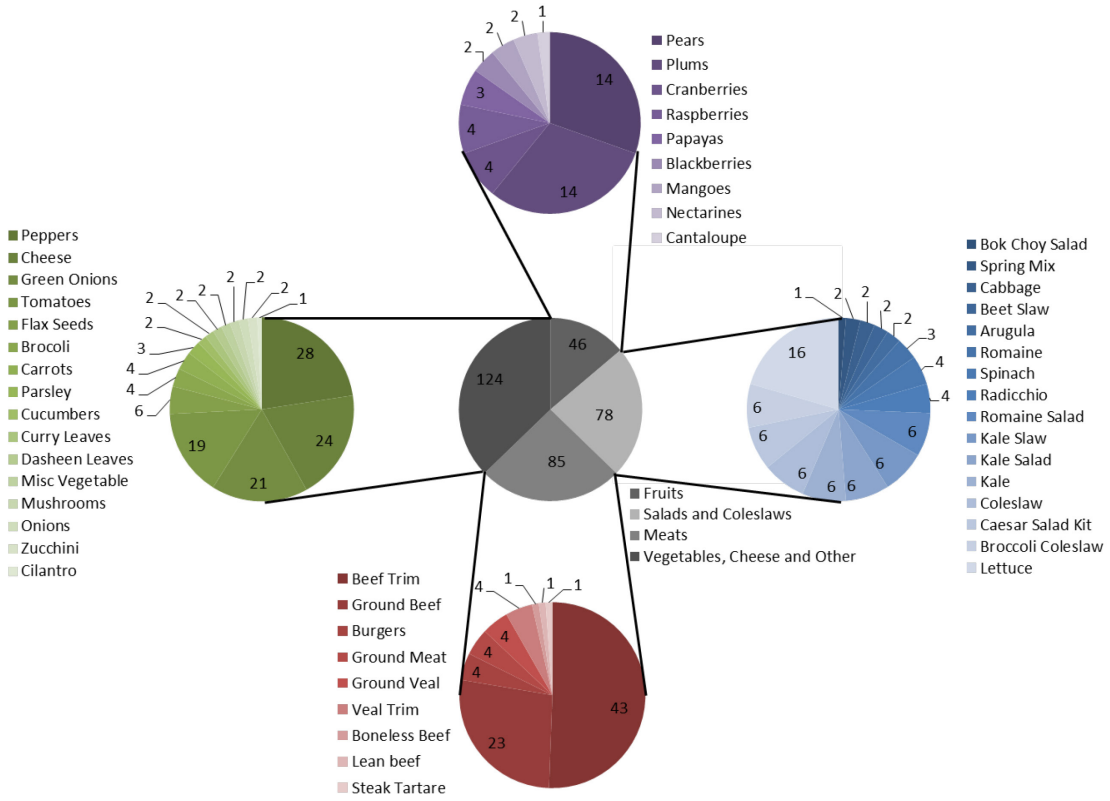

Figure 11. Food product enrichment cultures tested for production of inhibitors. Food samples used to make the enrichment broths. Grey pie chart: Categories. Purple pie chart: Fruits. Blue pie chart: Salads and Coleslaws. Red pie chart: Meats. Green pie chart: Vegetables, Cheese and Other. Total food enrichment samples tested was 333 samples.

Of the 333 enrichment broths tested, cell-free extracts from 25 (7.5%) of the samples inhibited growth of at least one of the *Shigella* or STEC strains used in this study. Twenty-one samples (6%) inhibited growth of *Shigella* spp. and 7 (2%) inhibited growth of STEC (Figure 12). Among the 25 cell-free extracts containing inhibitors, 21 (84%) affected *S. sonnei*, 6 (24%) affected *S. flexneri* and 7 (28%) affected STEC (Figure 12). One of the samples inhibited growth of all STEC strains tested in this study and 3 samples inhibited both *Shigella* and STEC strains (Figure 12). None of the samples affected growth of *S. dysenteriae*.

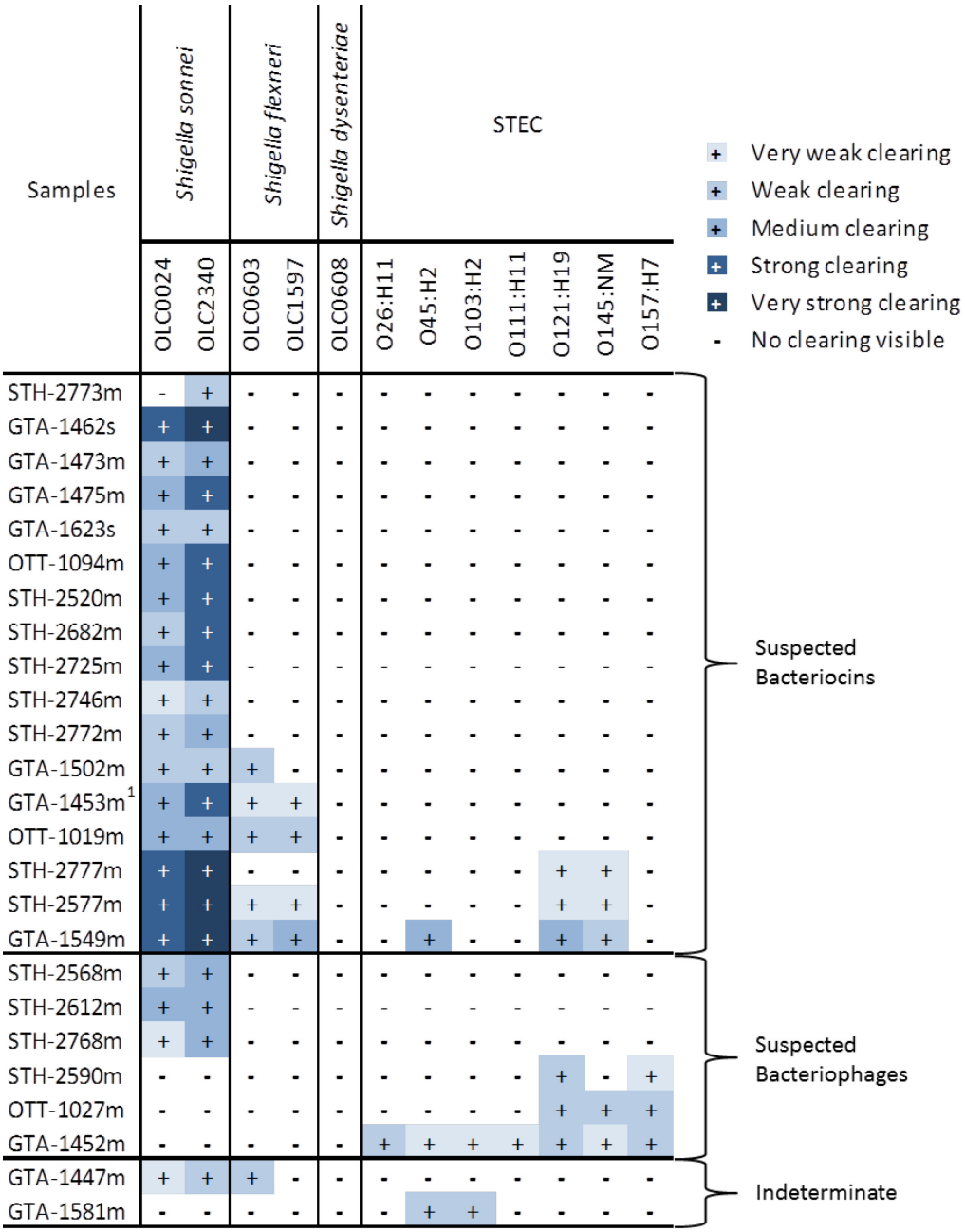

Figure 12. Strengths of inhibitory activity of food enrichments on *Shigella* and STEC.

The enrichments are grouped based on likely inhibitor present in the sample (suspected bacteriocins or bacteriophages). [1] For these samples, there were indeterminate results for *S. flexneri*. Samples were deemed to be indeterminate if observed inhibition of untreated samples was weak and impacts of proteolytic enzymes and dilutions could not be observed. m: mTSB broth. s: *Shigella* broth.

Cell-free extracts derived from meat enrichment broths were most likely to contain inhibitors to both *Shigella* (17 (81%); Figure 13A) and STEC (5 (71%); Figure 13B). Approximately 26% of the raw meat enrichments inhibited growth of *Shigella* or STEC. In contrast, only 6 (2.4%) of the 248 cell-free extracts from the other categories of samples, largely produce, contained inhibitors. In samples where products were enriched in both mTSB and *Shigella* broth, inhibition was typically only observed in one of the broths. For two of these samples, inhibitory compounds were only produced in *Shigella* broth, not mTSB and for two samples inhibitory compounds were recovered from the mTSB but not the *Shigella* broth. Similar to observations with the bacterial cell-free extracts, strength of inhibition varied among the extracts and among indicator organisms tested (Figure 10A, Figure 12). Inhibitory activity against *S. sonnei* tended to be strong, whereas inhibitory activity against *S. flexneri* and STEC was weaker. To ensure that inhibition was not due to factors such as high acidity, the pH of the cell-free extracts was also measured. All the food enrichments had relatively neutral pH values between 6.0-7.5.

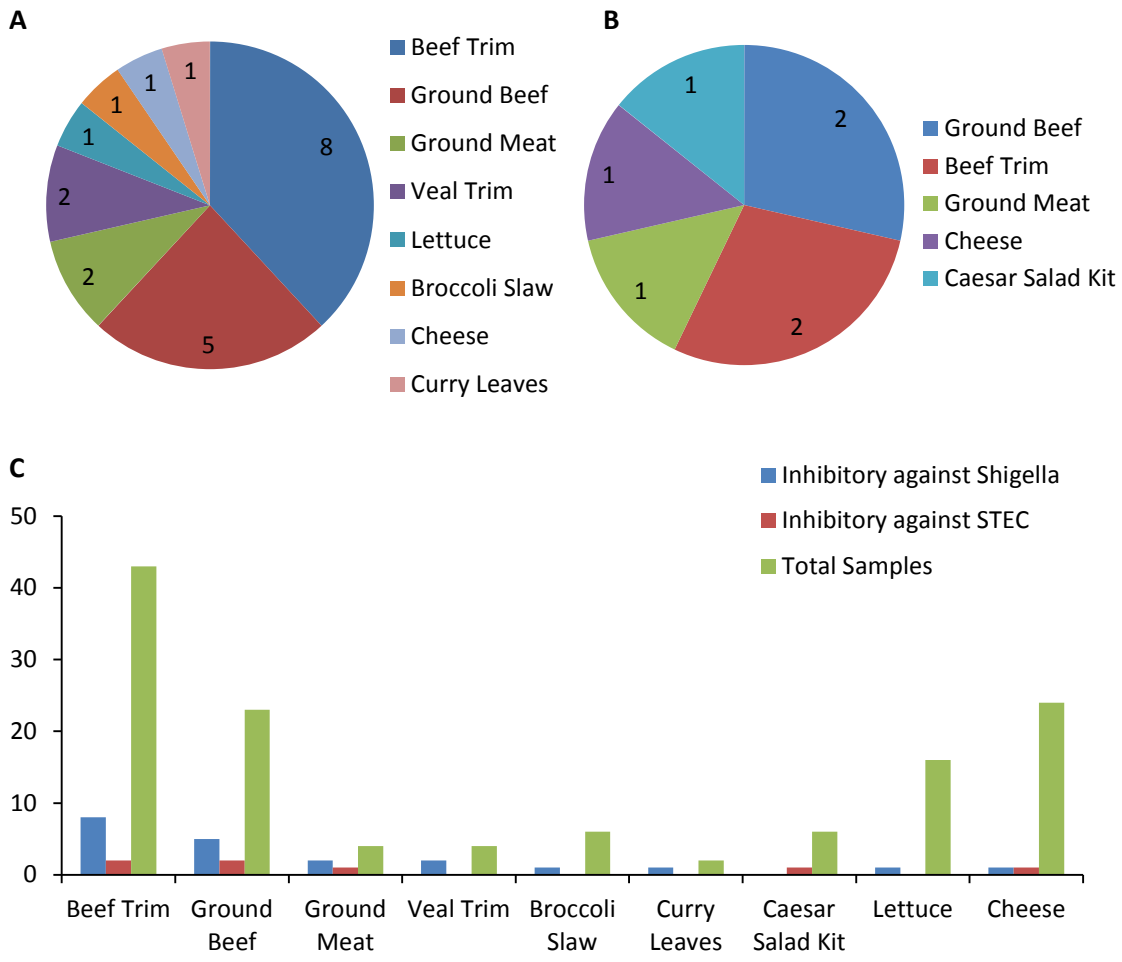

Figure 13. Inhibitory activity of food enrichments on *Shigella* and STEC.
A: Food enrichment broths inhibiting growth of *Shigella*. Note that 19 of the 21 samples containing inhibitors were enriched in mTSB (90%) B: Food enrichment broths inhibiting growth of STEC. Note that all samples containing inhibitors were enriched in mTSB. C: Food products that were inhibitory against *Shigella* and STEC relative to total number tested.

4.4.3. Characterization of inhibitory properties of cell-free extracts.

All the cell-free extracts causing inhibition in at least one of the indicator *Shigella* or STEC strains were digested with the proteolytic enzymes proteinase-K and trypsin to determine if inhibition was reduced following removal of the protein components of the

cell-free extracts (Figure 15). The inhibitory activity of the cell-free extracts from most of the food isolates (17 (74%)) and the food enrichments (15 (60%)) were affected by at least one proteolytic enzyme (Figure 14). For the food enrichment broths, there were 2 samples that were indeterminate for all inhibited strains and one sample indeterminate for *S. flexneri*. The problem with these samples was likely associated with prolonged storage as inhibitory activity was found to generally decrease over time during storage at 4°C. For the cell-free extract from OLC1219, the proteinase treatment reduced inhibition for *S. sonnei* and not for *S. flexneri.* Most of the samples that inhibited *Shigella* (particularly *S. sonnei*) were affected by proteolytic enzymes, (Figures 10 and 12). Samples that inhibited STEC were generally not affected by proteolytic enzymes and suspected to be bacteriophage.

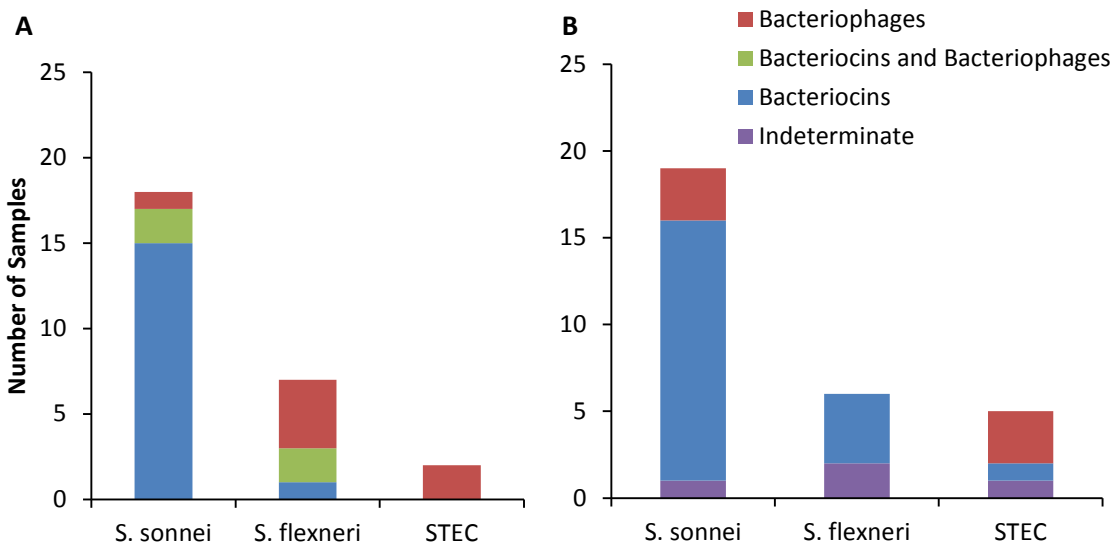

Figure 14. Characterization of inhibitors in isolate and food enrichment cell-free extracts

Cell-free extracts from food isolates and enrichments were evaluated to characterize inhibitors present. To assess the possibility of the inhibiting factor being caused by bacteriocins, which are protein-based, the cell-free extracts from the isolates and enrichments were treated with two proteolytic enzymes (proteinase-K, trypsin). To assess

the possibility of the inhibiting factor being caused by bacteriophages, the cell-free extracts were diluted to detect the presence of plaques. A: Number of food isolates that are suspected bacteriocins (Blue bars), bacteriophages (Red bars) or both (Green bars). B: Number of food enrichments that are suspected bacteriocins (Blue bars), bacteriophages (Red bars) or both (Green bars). Indeterminate are samples where the clearing was very weak and it could not be determined if it was affected by the proteolytic enzymes or had plaques (Purple bars).

To identify bacteriophages, the inhibitory cell-free extracts from the food isolates and food enrichments were diluted to assess the presence of plaques. Plaques were observed in dilutions derived from eight of the cell-free extracts from bacterial isolates (Figure 14A and 15). Six of these samples were not affected by the proteolytic enzymes and for strain OLC1219 plaques were observed with *S. flexneri* but not *S. sonnei.* The strain OLC1028 was not completely affected by proteolytic enzymes and only showed plaques when treated with proteolytic enzymes. After treatment with proteolytic enzymes, the clearing went from very strong to weak and plaques were present. There were no plaques present in the dilutions. Plaques were observed for six of the food enrichment broths (Figure 14B, 15).

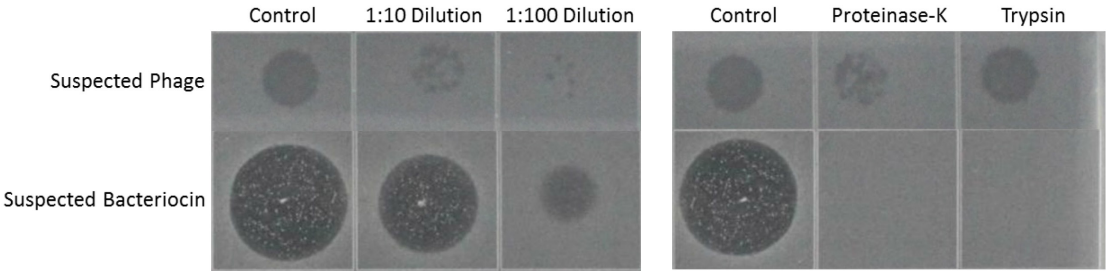

Figure 15. Characterization of cell-free extracts.
The Cell-free extract in the top row is suspected to contain bacteriophage as plaques are formed when diluted and it is minimally affected by Proteinase-K and trypsin. The Cell-free extract used in the second row is suspected to contain a bacteriocin as plaques are not visible in the dilution and it is affected by both Proteinase-K and Trypsin.

4.4.4. Recovery of bacteria producing inhibitory compounds from food enrichment broths.

For a subset of 4 of the enrichment broths (STH-2577, STH-2682, STH-2725 and STH-2768) producing inhibitory compounds, the bacteria causing the inhibition were isolated using the triple overlay method (Henning *et al.*, 2015). One isolate was recovered from each of the samples, with 2 isolates from (STH-2768). The strains were STH-2577, STH-2682, STH-2725 and STH-2768. The *E. coli* isolated from STH-2577 inhibited the same *Shigella* and STEC strains as the enrichment (OLC 0024, 603, 1597, 2340, 675, 710). The *E. coli* isolated from STH-2682 inhibited *S. sonnei* as the original enrichment broth. The *E. coli* isolated from STH-2725 only inhibited *S. sonnei* and not the *S. flexneri* or STEC strains. Two *E. coli* were isolated from STH-2768 and they both inhibited *S. sonnei* as the original enrichment broth. The one *E. coli* had stronger activity than the other. *E. coli isolated* from STH-2577, STH-2682 and STH-2725 were affected by proteolytic enzymes. *E. coli* isolated from STH-2768 showed plaques and were not affected by proteolytic enzymes.

4.5 Discussion

4.5.1. Inhibition of *Shigella* and STEC from cell-free extracts derived from bacterial isolates.

Recovery of the *Shigella* spp. and STEC from foods can be extremely challenging, in part due to problems culturing pathogens to detectable levels relative to

non-target organisms present in different foods. This study was done to evaluate the prevalence of *Shigella* spp. and STEC inhibitors produced by bacteria present in foods. Cell-free extracts from 199 Gram-negative bacterial isolates (Appendix 2) were tested for inhibitory activity on a panel of 7 STEC and 5 *Shigella* strains. Gram-negative organisms would typically be highly represented in food-enrichment cultures aimed at recovery of *Shigella*/STEC as most methods integrate antibiotics such as novobiocin to reduce growth of Gram-positive bacteria, but generally do not include selective agents that reduce growth of Gram-negative bacteria (Figure 9A, (Robson & Baddiley, 1977)). Extracts from 17% of the *E. coli* tested were found to inhibit growth of *Shigella* spp., particularly *S. sonnei*. *E. coli* was also shown to inhibit *Shigella* spp. in previous studies, however in those studies, *Klebsiella*, *Proteus*, *Enterobacter*, *Citrobacter* and *Salmonella* also caused inhibition (Uyttendaele *et al.*, 2001). Growth of STEC was affected by extracts from only 2 of the 36 *Enterobacter* strains tested (Figure 9A). This is different than seen in previous investigations where *Hafnia alvei*, *Brochothrix thermosphacta* and *Pediococcus acidilactici* present in meats were shown to inhibit STEC (G Duffy, Whiting, & Sheridan, 1999). To get a better understanding of the inhibition against STEC and *Shigella*, the compounds causing the inhibition (ex. suspected bacteriocins and bacteriophages) should be characterized.

Shigella sonnei was the pathogen most commonly affected by antibiotic compounds, followed by *Shigella flexneri* (Figure 10B). This is different than other studies that showed *S. flexneri* affected by organic acids more than *S. sonnei* (Hentges, 1969; Uyttendaele et al., 2001; Zhang et al., 2011), however, it is an important finding as *S. sonnei* and *S. flexneri* are the most common *Shigella* species that cause outbreaks in

North America (Centers for Disease Control and Prevention (CDC), 2016; Shanmugakani & Ghosh, 2012; The *et al.*, 2016; Thompson, Duy, & Baker, 2015). Most of the inhibitors were affected by proteolytic enzymes and are likely to be bacteriocins (Figure 10B, 14A) as they did not show the formation of plaques when diluted (Figure 15). *Shigella dysenteriae* was not affected by any of the samples, so it is possible that *S. dysenteriae* has a tolerance or resistance to bacteriocins (G. Alonso, Vílchez, & Rodríguez Lemoine, 2000). A tolerance or resistance would be obtained by mutations in the bacteriocin receptors or translocation system.

In this study, there were two bacterial isolates that had two types of antimicrobial activity on *Shigella* (Figure 10B, 14A). Samples that have multiple antimicrobials against *Shigella* are of concern as the *Shigella* would be at a lower concentration since it would have two substances affecting its growth. The pH range of *Shigella* growth is between 4.8-9.3 and the range of STEC growth is 4.0-10.0 (Food and Drug Administration, 2011). The pH of the bacterial isolate cell-free extracts (6.5-7.5) was well within these ranges and should not have impacted the growth of *Shigella* or STEC.

4.5.2. Inhibition of *Shigella* and STEC from cell-free extracts from food enrichment broths.

Food enrichments in mTSB and *Shigella* broths were tested for inhibition of both STEC and *Shigella.* While *Shigella* broth would not be used for detection of STEC, and mTSB would not be used for detection of *Shigella*, similar analyses with both types of enrichment broths enabled comparison of antibiotic production by organisms growing in the two media. Inhibition was never observed in both broths, even though the

enrichments were from the same food sample (Figure 12). This was seen in a curry leave (GTA1462) sample and a Caesar salad sample (GTA-1581). This shows that the food itself was not the cause of inhibition. There were more food enrichment broths that were enriched in mTSB than *Shigella* broth as meat and cheese products were not tested for *Shigella* spp. (Figure 11). Most of the samples that inhibited *Shigella* spp. (18 of 21) were among the 120 samples only enriched in mTSB, and were more likely to be derived from raw meat products. Given the high proportion of *E. coli* isolates producing antibiotics affecting *Shigella*, and the known association of *E. coli* with raw meat products (Barco, Belluco, Roccato, & Ricci, 2015), it is possible that the relatively high prevalence of *Shigella* inhibition in mTSB enrichments was due to the bacterial food matrix and not to differences in the enrichment broth. Paired enrichments of meat products in both mTSB and *Shigella* broth could be done to confirm this. Finally, the pH of the cell-free extracts from the food enrichment cultures was between 6.0-7.5, well within normal ranges for growth of *Shigella* or STEC and would not affect their growth.

Similar to what was observed with the bacterial isolates, *Shigella sonnei* strains were most sensitive to inhibition, followed by *Shigella flexneri* and STEC (Figure 12). The observation of similar trends between the bacterial isolates and enrichments indicates that the bacterial isolates used in this study are representative of the bacteria that cause inhibition in food enrichments. Most enrichments affected a few species of *Shigella* and/or STEC however there were some samples that showed a broader activity. For instance, there was one enrichment culture that was a suspected bacteriophage and it inhibited all 7 STEC strains (GTA-1452, Figure 12). This inhibition is likely to be due to the presence of a bacteriophage that has a wide spectrum of activity or multiple

bacteriophage targeting a few strains. There were also three enrichment broths that inhibited both STEC and *Shigella* strains (GTA-1549, STH-2577 and STH-2777). This is likely indicative of an inhibitor that has a larger spectrum of activity or potentially more than one inhibitor in the samples.

The bacteria responsible for production of antibiotics against *Shigella* in four of the food enrichment broths were isolated using a triple overlay method. The isolates were sequenced and the species were determined to be *E. coli.* Cell-free extracts from the isolated *E. coli* strains had similar inhibitory activities compared to the extracts from the food samples from which they were isolated. Recovery of *E. coli* isolates from food samples provides further evidence that the presence of *E. coli* can cause inhibition of *Shigella* growth. Faecal contamination is common in meat samples during the slaughter process (Barco et al., 2015) and *E. coli* is an indicator of faecal matter contamination (Odonkor & Ampofo, 2013; Tallon, Magajna, Lofranco, & Leung, 2005). *Shigella*-contamination would be likely to occur through contamination of food with faecal matter as *Shigella* is most commonly spread by the faecal oral route. To confirm this, more food samples contaminated with faecal matter or samples of animal faecal matter could be examined for inhibitors against *Shigella*.

This study provides evidence that production of antibiotics such as bacteriocins and bacteriophages by microbiota present in food samples can inhibit growth of *Shigella* and STEC. Presence of species such as *E. coli* and *Enterobacter* may be of greatest concern; however, a more comprehensive study is needed to isolate and characterize species most commonly associated with production of inhibitors to *Shigella* spp. and STEC. This could be done by isolating more of the inhibitors from the inhibiting food

enrichments. Cell-free extracts from isolates and food enrichments were found to inhibit growth of target organisms with the agar diffusions assay. Some inhibition may have been below the limit of detection of this method. Further studies are needed to better understand how the presence of antibiotic-producing organisms would impact growth of *Shigella* and STEC during enrichment culture. This could be done by performing growth curves of the inhibitors with the *Shigella* and STEC strains they inhibit. A better understanding of the impact of microbial antagonism on detection of pathogens in enrichment culture will enable the development of more effective methods for recovery of pathogens from foods.

5 Chapter: Discussion

Despite advances in sanitation, microbiology, molecular biology and technology, foodborne illnesses are still a concern in developed countries (Centers for Disease Control and Prevention (CDC), 2016; Public Health Agency of Canada, 2013; Thomas *et al.*, 2013; WHO, 2014). Improvements to current methods for detecting EHEC and *Shigella* in foods will reduce false positives and false negatives, leading to a more accurate assessment of foods and a safer food supply.

5.1 EHEC Method

The MuSIC ddPCR method developed in Chapter 3 is able to reduce false positives in testing food samples for EHEC. Widespread adoption of this methodology can reduce the burden of EHEC on society by limiting the number of false positives and enabling more efficient detection of EHEC in food samples. The resources and time

gained from the reduction of false positives, and the fact that this approach is amenable to high-throughput automation, could mean that more samples could be tested and potentially more contaminated products could be detected before they are consumed. In addition, the MuSIC ddPCR provides an estimate of relative proportion of EHEC to background bacteria, aiding estimation of the number of colonies that would need to be screened to find the EHEC or mixed culture (data not shown). This is valuable information as it could inform the investigator how many resources would be needed to find the EHEC or mixed culture in a sample.

To address the problem of false negatives in methods for detection of EHEC, Chapter 4 describes the investigation of the impact of background bacteria on EHEC growth. Studies on the production of EHEC-inhibitors by bacteria in the food enrichment cultures could eventually provide enough information to modify the enrichment broth to prevent competing bacteria from inhibiting EHEC growth. A better understanding of the frequency of bacteriocins and bacteriophages targeting EHEC in enrichment cultures, and species that produce these antimicrobials will lead to strategies for mitigating their impact in enrichment cultures. This would improve recovery of EHEC in a positive sample by reducing the number of colonies to be screened to find the EHEC bacteria.

A number of recent methods have been developed to improve detection of low numbers of EHEC in an enrichment culture with immunocapture, gold nanoparticles, flow cytometry and other magnetic capture methods (Chen, Shi, Gehring, & Paoli, 2014; Kanki, Seto, & Kumeda, 2014; C.H. Li, Bai, Selvaprakash, Mong, & Chen, 2017; Park *et al.*, 2017). These methods appear to be promising but the EHEC may still be outcompeted to a level where it is still undetectable with these concentration methods.

Improvement of the enrichment broth and reducing competing background bacteria will allow the concentration of EHEC to be higher and allow for these new methods to work.

5.2 *Shigella* Method

The results from the inhibition study of bacteria in foods done in Chapter 4, may suggest that the difficulty of detecting *Shigella* might be partly caused by *Shigella* being outcompeted by other bacteria secreting bacteriocins or encoding bacteriophages. It may even be possible that the cause of VBNC *Shigella* may be from bacteriocins, bacteriophages and organic acids. The study in Chapter 4, suggests that *S. sonnei* is affected heavily by bacteriocins and not very much by bacteriophages. *S. flexneri* is affected more by bacteriophages and not very much by bacteriocins. This is different than other studies which showed that *S. flexneri* was mostly affected by organic acids (Uyttendaele *et al.*, 2001; Zhang *et al.*, 2011). By further studying these inhibitors, the enrichment step of the *Shigella* method can be improved. With an improved enrichment step, other molecular assays can be used to improve the detection of *Shigella* in foods.

5.3 Future studies

To ensure that the methods developed in this study can be integrated into Canada's food testing programs, improvements to methods must be rigorously evaluated. For example, the MuSIC ddPCR method in Chapter 3 still needs validation studies and further optimizations before it could be used as a diagnostic test for EHEC. The method could be optimized to increase the percent linkage by removing free gDNA in the samples. It is possible that degraded free-gDNA from dead cells are causing the reduction of percent linkage by having fragments of the genes in different droplets. There could be

many possible ways to remove the free-gDNA. One way would be to perform a high-speed spin to separate the cells from the DNA. This was attempted, however the centrifugation also caused more cells to burst and more free-gDNA and a reduction in the percent linkage (data not shown). It might also be possible to treat the enrichment samples with a DNase to remove the free-gDNA and then deactivate the DNase before lysis and PCR. This may be possible with a heat in activated DNase. Another protocol that may work is the Bio-Rad iQ-Check Free DNA Removal Solution. With the free-gDNA removed, the percent linkage could be closer to 100%.

The MuSIC ddPCR method has not been tested with real samples contaminated with EHEC and mixed cultures. In chapter 3, the method was only tested with food samples that were spiked with EHEC and only with MuSIC ddPCR. It was not compared to the current methods as it was a proof of concept study. Now that it has been shown to work, validation studies with a direct comparison of the current methods of testing and MuSIC ddPCR would be of great value. This would determine if the MuSIC ddPCR method correctly identified the false-positives of the current method as negative samples. The method would need to be validated alongside the current method with either spiked samples or real samples. Real samples would be of greater value as the MuSIC ddPCR would be tested with contaminated samples and samples with mixed cultures that are commonly seen. However, a very small portion of samples tested are contaminated with pathogens and are true positive. Spiked samples are necessary for validation studies to ensure that there are enough samples tested, enough of a variety of strains tested and a wide enough variety of food products tested.

After the method has been validated, there are a few other considerations before the method should be implemented. ddPCR costs more than regular PCR or real-time PCR and is much more difficult to perform. The number of samples tested is very large and only a small portion of those samples are actually positive (Bosilevac & Koohmaraie, 2011; Wasilenko *et al.*, 2014). It would not be cost effective to test all of the samples currently tested using MuSIC ddPCR. To address this problem, the screening PCR of the current method should still be performed and then positive samples further tested using MuSIC ddPCR. This would add excessive time to the analysis, as the MuSIC ddPCR could be done the same day as the initial PCR screening. The formation of the droplets is the most difficult part in ddPCR. To allow the method to run smoother, the MuSIC ddPCR method could also be tested using the automated droplet generator. This would improve the reliability of the MuSIC ddPCR as it would minimize the need for technical expertise with the ddPCR technology. With the combination of the first PCR screening with MuSIC ddPCR screening and the automated droplet generation, true positive samples would be found faster and excess work will not be wasted on false-positives.

Further studies investigating the inhibition of bacteriocins and bacteriophages on *Shigella* and EHEC would also be of value. The studies discussed in chapter 4, show that there are inhibitors in food enrichment broths and isolates from food enrichments that can inhibit *Shigella* and EHEC. The impact of the presence of these microbial antagonists of *Shigella* and EHEC in food enrichment broth cultures remains unknown. Growth curves done with the bacteria that produce the bacteriocins or bacteria that have the bacteriophages along with the *Shigella* or EHEC strains they inhibit could be done to look at the effect of the inhibitors on growth and detection. It would also be interesting to

determine if low concentrations of the inhibitors could still impact the isolation and detection of *Shigella* and EHEC.

Shigella is spread through the faecal oral route and the samples that caused most of the inhibition in Chapter 4 were meat samples which usually have higher levels of faecal contamination. To further investigate this, foods contaminated with faecal matter and faecal matter samples should be tested for inhibitors. This would provide information on whether faecal contaminated products are an important cause of false negatives for *Shigella* testing methods. Knowing the source may help determine a way to prevent inhibitors from affecting the detection and isolation of STEC and *Shigella,* and may inform the development of more rigorous validation schemes to ensure that *Shigella* methods are suitable for investigating food outbreaks.

The results of these studies show potential for improving the current *Shigella* and EHEC methods. The MuSIC ddPCR method developed provides a novel way to reduce the false positives that occur in the current methods for detection of EHEC. The study on microbial antagonisms provides new information on the prevalence of bacteriocins and bacteriophages against *Shigella* and EHEC in food enrichments. This work shows that Gram-negative bacteria cause problems with the detection of *Shigella* and EHEC and finding ways to reduce the background Gram-negative bacteria during the enrichment could improve the detection of EHEC and *Shigella* in food. This information is valuable for future experiments done to improve the enrichment step in the detection methods for both *Shigella* and EHEC.

6 Appendices

6.1 Appendix 1: Construction of a control plasmid

Supplementary Figure. Construction of a control plasmid. A plasmid comprising fragments of genes encoding *stx1* (blue box), *stx2* (open blue box) and *eae* (green box) inserted into the "Best-Fit" pIDTSMART-KAN Vector was constructed. Sequences for the gene fragments were obtained from the *E. coli* O157:H7 Sakai strain (GenBank: BA000007.2). Location and direction of primers (half arrows) and probes (lines) used in the MuSIC ddPCR assay are indicated. F:forward, R:reverse

6.2 Appendix 2: List of bacterial isolates used in Chapter 4

Name	Family	Genus	Species	Serotype	Food
OLC0625	Enterobacteriaceae	*Escherichia*	*coli*	O8:H27	N/A
OLC0637	Enterobacteriaceae	*Escherichia*	*coli*	O26:H11	N/A
OLC0673	Enterobacteriaceae	*Salmonella*	*enterica*	*abaetetuba*	Liquid Egg
OLC0708	Enterobacteriaceae	*Escherichia*	*coli*	O183:H18	N/A
OLC0993	Enterobacteriaceae	*Escherichia*	*coli*	O146:H21	N/A
OLC1028	Enterobacteriaceae	*Escherichia*	*coli*	O111:H8	Bovine
OLC1065	Enterobacteriaceae	*Escherichia*	*coli*	O146:H21	N/A
OLC1075	Enterobacteriaceae	*Escherichia*	*coli*	O113:H4	N/A
OLC1080	Enterobacteriaceae	*Klebsiella*	*pneumoniae ssp*	*pneumoniae*	Ground Beef
OLC1081	Enterobacteriaceae	*Escherichia*	*coli*		Ground Beef
OLC1082	Enterobacteriaceae	*Hafnia*	*alvei*		Beef Trim
OLC1083	Enterobacteriaceae	*Escherichia*	*coli*	O21:H21	Ground Beef
OLC1084	Enterobacteriaceae	*Klebsiella*	*pneumoniae ssp*	*pneumoniae*	Ground Beef
OLC1085	Enterobacteriaceae	*Escherichia*	*coli*	O102:H38	Ground Beef
OLC1087	Enterobacteriaceae	*Proteus*	*mirabilis*		Ground Beef
OLC1088	Enterobacteriaceae	*Escherichia*	*coli*	O8:H45	Ground Beef
OLC1089	Enterobacteriaceae	*Escherichia*	*coli*	-:H9	Ground Beef
OLC1090	Pseudomonadaceae	*Pseudomonas*	*lundensis*		Beef Trim
OLC1091	Aeromonadaceae	*Aeromonas*	*sobria*		Beef Trim
OLC1092	Enterobacteriaceae	*Escherichia*	*coli*	O21:H11	Beef Trim
OLC1093	Enterobacteriaceae	*Hafnia*	*alvei*		Beef Trim
OLC1094	Enterobacteriaceae	*Serratia*	*proteamaculans*		Ground Beef
OLC1095	Pseudomonadaceae	*Pseudomonas*	*lundensis*		Ground Beef

OLC1096	Enterobacteriaceae	*Enterobacter*			Ground Beef
OLC1097	Enterobacteriaceae	*Serratia*	*fonticola*		Ground Beef
OLC1098	Enterobacteriaceae	*Hafnia*	*alvei*		Ground Beef
OLC1099	Enterobacteriaceae	*Proteus*			Ground Beef
OLC1100	Enterobacteriaceae	*Proteus*	*mirabilis*		Ground Beef
OLC1101	Enterobacteriaceae	*Raoultella*	*planticola*		Beef Trim
OLC1102	Enterobacteriaceae	*Escherichia*	*coli*	O174:H2	Beef Trim
OLC1103	Enterobacteriaceae	*Escherichia*	*coli*	-:H25	Beef Burger
OLC1104	Enterobacteriaceae	*Hafnia*	*alvei*		Ground Beef
OLC1105	Enterobacteriaceae	*Escherichia*	*coli*	O9:H30	Ground Beef
OLC1106	Enterobacteriaceae	*Escherichia*	*coli*	O132:H28	Ground Beef
OLC1107	Enterobacteriaceae	*Klebsiella*	*pneumoniae ssp*	*pneumoniae*	Beef Trim
OLC1108	Enterobacteriaceae	*Hafnia*	*alvei*		Ground Beef
OLC1110	Enterobacteriaceae	*Escherichia*	*coli*	O107:H54	Beef Trim
OLC1111	Enterobacteriaceae	*Enterobacter*	*kobei*		Ground Beef
OLC1112	Enterobacteriaceae	*Escherichia*	*coli*	O157:H7	Veal Meat
OLC1113	Enterobacteriaceae	*Escherichia*	*coli*	O157:H7	Beef Burger
OLC1114	Enterobacteriaceae	*Escherichia*	*coli*	O157:H7	Beef Burger
OLC1115	Enterobacteriaceae	*Escherichia*	*coli*	O157:H7	Beef Burger
OLC1116	Enterobacteriaceae	*Escherichia*	*coli*	O157:H7	Beef Burger
OLC1117	Enterobacteriaceae	*Escherichia*	*coli*	O157:H7	Beef Burger
OLC1118	Enterobacteriaceae	*Escherichia*	*coli*	O157:H7	Beef Burger
OLC1135	Enterobacteriaceae	*Citrobacter*	*freundii*	O150:H8	Ground Beef
OLC1136	Enterobacteriaceae	*Citrobacter*	*Braakii*	O138:H48	Ground Beef
OLC1137	Enterobacteriaceae	*Hafnia*	*Alvei*	O48:H10	Ground Beef
OLC1210	Enterobacteriaceae	*Hafnia*	*alvei*		Ground Beef
OLC1211	Enterobacteriaceae	*Enterobacter*			Ground Beef

OLC1212	Enterobacteriaceae	*Escherichia*	*coli*	O157:H7	Ground Beef
OLC1213	Enterobacteriaceae	*Escherichia*	*coli*	O22:H8	Ground Beef
OLC1214	Enterobacteriaceae	*Escherichia*	*coli*	O148:H53	Ground Beef
OLC1215	Enterobacteriaceae	*Escherichia*	*coli*	O8:H21	Beef Trim
OLC1216	Enterobacteriaceae	*Hafnia*	*alvei*		Beef Trim
OLC1217	Enterobacteriaceae	*Enterobacter*	*kobei*		Beef Trim
OLC1218	Enterobacteriaceae	*Escherichia*	*coli*	O18ab:H14	Beef Trim
OLC1219	Enterobacteriaceae	*Escherichia*	*coli*	O7:H18	Beef Trim
OLC1220	Enterobacteriaceae	*Escherichia*	*coli*	-:H7	Ground Beef
OLC1221	Enterobacteriaceae	*Escherichia*	*coli*	O157:H7	Beef Trim
OLC1222	Enterobacteriaceae	*Hafnia*	*alvei*		Ground Beef
OLC1223	Enterobacteriaceae	*Hafnia*	*alvei*		Ground Beef
OLC1224	Enterobacteriaceae	*Citrobacter*	*freundii*		Ground Beef
OLC1225	Enterobacteriaceae	*Escherichia*	*coli*	O9:H19	Ground Beef
OLC1226	Enterobacteriaceae	*Escherichia*	*coli*	O8:H21	Ground Beef
OLC1227	Enterobacteriaceae	*Klebsiella*			Ground Beef
OLC1228	Enterobacteriaceae	*Hafnia*	*alvei*		Beef Trim
OLC1229	Enterobacteriaceae	*Moellerella*	*wisconsensis*		Beef Trim
OLC1230	Enterobacteriaceae	*Escherichia*	*coli*	OUT:H7	Beef Trim
OLC1231	Enterobacteriaceae	*Hafnia*	*alvei*		Ground Beef
OLC1232	Enterobacteriaceae	*Hafnia*	*alvei*		Ground Beef
OLC1233	Enterobacteriaceae	*Hafnia*	*alvei*		Ground Beef
OLC1234	Enterobacteriaceae	*Escherichia*	*coli*	O153/O178:H7	Beef Trim
OLC1235	Enterobacteriaceae	*Hafnia*	*alvei*		Beef Trim
OLC1236	Enterobacteriaceae	*Escherichia*	*coli*	O21:H25	Beef Trim
OLC1237	Enterobacteriaceae	*Klebsiella*	*pneumoniae ssp*	*pneumoniae*	Beef Trim
OLC1238	Enterobacteriaceae	*Escherichia*	*coli*	-:H8	Ground Beef

OLC1239	Enterobacteriaceae	*Hafnia*	*alvei*		Ground Beef
OLC1240	Enterobacteriaceae	*Klebsiella*	*pneumoniae ssp*	*pneumoniae*	Ground Beef
OLC1246	Enterobacteriaceae	*Hafnia*	*alvei*		Ground Beef
OLC1247	Pseudomonadaceae	*Pseudomonas*			Ground Beef
OLC1248	Enterobacteriaceae	*Hafnia*	*alvei*		Ground Beef
OLC1249	Aeromonadaceae	*Aeromonas*	*salmonicida*		Ground Beef
OLC1260	Enterobacteriaceae	*Escherichia*	*coli*	-:H8	N/A
OLC1266	Enterobacteriaceae	*Escherichia*	*coli*	O22:H8	N/A
OLC1272	Enterobacteriaceae	*Escherichia*	*coli*	O25:H12	Beef Trim
OLC1276	Enterobacteriaceae	*Hafnia*	*alvei*		Beef Trim
OLC1277	Enterobacteriaceae	*Escherichia*	*coli*	O9:H18	Beef Trim
OLC1304	Enterobacteriaceae	*Escherichia*	*coli*	O148:H30	Beef Trim
OLC1305	Enterobacteriaceae	*Escherichia*	*coli*	O157:H7	Ground Beef
OLC1307	Enterobacteriaceae	*Escherichia*	*coli*	-:H7	Ground Beef
OLC1308	Enterobacteriaceae	*Escherichia*	*coli*	O21:H11	Beef Trim
OLC1310	Enterobacteriaceae	*Escherichia*	*coli*	O28ac/O42:H8	Beef Trim
OLC1311	Enterobacteriaceae	*Escherichia*	*coli*	O150:H8	Beef Trim
OLC1342	Enterobacteriaceae	*Escherichia*	*coli*	O157:H7	N/A
OLC1346	Enterobacteriaceae	*Escherichia*	*coli*	O21:H11	Beef Trim
OLC1348	Enterobacteriaceae	*Escherichia*	*coli*	-:H16	Beef Trim
OLC1350	Enterobacteriaceae	*Escherichia*	*coli*	O154:H4	Beef Trim
OLC1352	Enterobacteriaceae	*Escherichia*	*coli*	O21:H25	Beef Trim
OLC1353	Enterobacteriaceae	*Escherichia*	*coli*	O184:H48	Ground Beef
OLC1354	Enterobacteriaceae	*Escherichia*	*coli*	O18ab:H14	Ground Beef
OLC1359	Enterobacteriaceae	*Escherichia*	*coli*	O157:H7	N/A
OLC1404	Enterobacteriaceae	*Escherichia*	*coli*	O107:H27	Beef Trim
OLC1405	Enterobacteriaceae	*Escherichia*	*coli*	O110:H2	Beef Trim

OLC1407	Enterobacteriaceae	*Escherichia*	*coli*	O13:H11	Beef Trim
OLC1410	Enterobacteriaceae	*Escherichia*	*coli*	O18ab:H14	Beef Trim
OLC1417	Enterobacteriaceae	*Escherichia*	*coli*	-:H21	Beef Trim
OLC1419	Enterobacteriaceae	*Escherichia*	*coli*	O9:H30	Ground Beef
OLC1425	Enterobacteriaceae	*Escherichia*	*coli*	O6:H49	Beef Trim
OLC1430	Enterobacteriaceae	*Escherichia*	*coli*	O18ab:H14	Beef Trim
OLC1431	Enterobacteriaceae	*Escherichia*	*coli*	O25:H12	Ground Beef
OLC1433	Enterobacteriaceae	*Escherichia*	*coli*	O21:H11	Beef Trim
OLC1491	Enterobacteriaceae	*Escherichia*	*coli*	-:H2	Beef Trim
OLC1494	Enterobacteriaceae	*Escherichia*	*coli*	-:H2	Ground Beef
OLC1497	Enterobacteriaceae	*Escherichia*	*coli*	-:H25	Beef Trim
OLC1498	Enterobacteriaceae	*Escherichia*	*coli*	-:H21	Beef Trim
OLC1501	Enterobacteriaceae	*Escherichia*	*coli*	-:H14	Ground Beef
OLC1502	Enterobacteriaceae	*Escherichia*	*coli*	O18ab:H14	Ground Beef
OLC1506	Enterobacteriaceae	*Escherichia*	*coli*	-:H27	Beef Trim
OLC1507	Enterobacteriaceae	*Escherichia*	*coli*	-:H42	Ground Beef
OLC1510	Enterobacteriaceae	*Escherichia*	*coli*	-:H7	Ground Beef
OLC1511	Enterobacteriaceae	*Escherichia*	*coli*	-:H9	Beef Trim
OLC1512	Enterobacteriaceae	*Escherichia*	*coli*	-:H7	Ground Beef
OLC1519	Enterobacteriaceae	*Escherichia*	*coli*	O21:H11	Beef Trim
OLC1521	Enterobacteriaceae	*Escherichia*	*coli*	O88:H25	Beef Trim
OLC1523	Enterobacteriaceae	*Escherichia*	*coli*	O51:H10	Ground Beef
OLC1527	Enterobacteriaceae	*Escherichia*	*coli*	O8:H19	Ground Beef
OLC1530	Enterobacteriaceae	*Escherichia*	*coli*	O18ab:H14	Beef Trim
OLC1531	Enterobacteriaceae	*Escherichia*	*coli*	O45:H51	Ground Beef
OLC1538	Enterobacteriaceae	*Escherichia*	*coli*	-:H7	Beef Trim
OLC1539	Enterobacteriaceae	*Escherichia*	*coli*	O51:H10	Beef Trim

OLC1541	Enterobacteriaceae	*Escherichia*	*coli*	O112ac:H16	Ground Beef
OLC1542	Enterobacteriaceae	*Escherichia*	*coli*	-:H21	Beef Trim
OLC1543	Enterobacteriaceae	*Escherichia*	*coli*	O87:H7	Beef Trim
OLC1545	Enterobacteriaceae	*Enterobacter*	*kobei*		Beef Trim
OLC1547	Enterobacteriaceae	*Escherichia*	*coli*	-:H2	Ground Beef
OLC1548	Enterobacteriaceae	*Enterobacter*	*kobei*		Ground Beef
OLC1555	Enterobacteriaceae	*Escherichia*	*coli*	O51:H10	Ground Beef
OLC1557	Enterobacteriaceae	*Escherichia*	*coli*	-:H4	Ground Beef
OLC1558	Enterobacteriaceae	*Escherichia*	*coli*	O39:H7	Beef Trim
OLC1560	Enterobacteriaceae	*Escherichia*	*coli*	-:H8	Ground Beef
OLC1566	Enterobacteriaceae	*Escherichia*	*coli*	O6:H49	Ground Beef
OLC1568	Enterobacteriaceae	*Escherichia*	*coli*	O83:H7	Ground Beef
OLC1569	Enterobacteriaceae	*Enterobacter*	*cloacae*	*cloacae*	Ground Beef
OLC1571	Enterobacteriaceae	*Escherichia*	*coli*	-:H23	Ground Beef
OLC1573	Enterobacteriaceae	*Escherichia*	*coli*		Ground Beef
OLC1575	Enterobacteriaceae	*Enterobacter*	*kobei*		Ground Beef
OLC1579	Enterobacteriaceae	*Escherichia*	*coli*	O71:H27	Beef Trim
OLC1580	Enterobacteriaceae	*Escherichia*	*coli*	-:H10	Beef Trim
OLC1581	Enterobacteriaceae	*Enterobacter*	*cloacae*	*cloacae*	Ground Beef
OLC1582	Enterobacteriaceae	*Escherichia*	*coli*	O154:H51	Ground Beef
OLC1583	Enterobacteriaceae	*Escherichia*	*coli*	-:H20	Beef Trim
OLC1584	Enterobacteriaceae	*Escherichia*	*coli*	-:H25	Ground Beef
OLC1585	Enterobacteriaceae	*Escherichia*	*coli*	O21:H25	Beef Trim
OLC1602	Enterobacteriaceae	*Salmonella*	*enterica*	*mishmar*	N/A
OLC1614	Enterobacteriaceae	*Escherichia*	*coli*	O6:H1	N/A
OLC1633	Enterobacteriaceae	*Enterobacter*	*asburiae*		Lettuce
OLC1634	Enterobacteriaceae	*Enterobacter*	*cloacae*	*cloacae*	Lettuce

OLC1635	Enterobacteriaceae	*Enterobacter*	*aerogenes*		Lettuce
OLC1638	Enterobacteriaceae	*Enterobacter*	*cloacae*	*cloacae*	Spinach
OLC1641	Enterobacteriaceae	*Enterobacter*	*cloacae*	*cloacae*	Lettuce
OLC1642	Enterobacteriaceae	*Escherichia*	*coli*	-:H25	Lettuce
OLC1645	Enterobacteriaceae	*Enterobacter*	*cloacae*	*cloacae*	Lettuce
OLC1646	Enterobacteriaceae	*Escherichia*	*coli*	-:H14	Lettuce
OLC1647	Enterobacteriaceae	*Escherichia*	*coli*	O50/O2:H14	Lettuce
OLC1648	Enterobacteriaceae	*Enterobacter*	*cloacae*	*cloacae*	Arugula
OLC1649	Enterobacteriaceae	*Enterobacter*	*kobei*		Arugula
OLC1650	Enterobacteriaceae	*Enterobacter*	*cloacae*	*cloacae*	Spinach
OLC1651	Enterobacteriaceae	*Klebsiella*	*pneumoniae*		Beef Trim
OLC1652	Enterobacteriaceae	*Escherichia*	*coli*	-:H11	Beef Trim
OLC1653	Enterobacteriaceae	*Escherichia*	*coli*	-:H14	Beef Trim
OLC1659	Enterobacteriaceae	*Escherichia*	*coli*	O8:H19	Cooking Greens
OLC1660	Enterobacteriaceae	*Enterobacter*	*cloacae*	*cloacae*	Cooking Greens
OLC1661	Enterobacteriaceae	*Enterobacter*	*cloacae*	*cloacae*	Cooking Greens
OLC1664	Enterobacteriaceae	*Escherichia*	*coli*	O159:H21	Spinach
OLC1665	Enterobacteriaceae	*Enterobacter*	*cloacae*	*cloacae*	Arugula
OLC1667	Enterobacteriaceae	*Enterobacter*	*cloacae*	*cloacae*	Spinach
OLC1668	Enterobacteriaceae	*Escherichia*	*coli*	O6:H49	Spinach
OLC1669	Enterobacteriaceae	*Escherichia*	*coli*	O63:H6	Arugula
OLC1670	Enterobacteriaceae	*Escherichia*	*coli*	-:H27	Ground Beef
OLC1673	Enterobacteriaceae	*Enterobacter*	*cloacae*	*cloacae*	Lettuce
OLC1674	Enterobacteriaceae	*Enterobacter*	*cloacae*	*cloacae*	Romaine
OLC1675	Enterobacteriaceae	*Enterobacter*	*cloacae*	*cloacae*	Lettuce
OLC1676	Enterobacteriaceae	*Escherichia*	*coli*	O83:H7	Spinach
OLC1677	Enterobacteriaceae	*Enterobacter*	*cloacae*	*cloacae*	Spinach

OLC1678	Enterobacteriaceae	*Escherichia*	*coli*		Lettuce
OLC1687	Enterobacteriaceae	*Enterobacter*	*cancerogenus*		Kale
OLC1688	Enterobacteriaceae	*Enterobacter*	*cloacae*	*cloacae*	Kale
OLC1760	Enterobacteriaceae	*Salmonella*	*enetrica Kentucky*	*kentucky*	Egg Environmental
OLC1813	Enterobacteriaceae	*Salmonella*	*enterica poona*	*poona*	Cantaloupe
OLC1900	Enterobacteriaceae	*Salmonella*	*enterica*	l:8,20:-:z6	Egg Environmental
OLC2079	Enterobacteriaceae	*Enterobacter*	*cloacae*	*cloacae*	Collard Greens
OLC2080	Enterobacteriaceae	*Escherichia*	*coli*	O134:H52	Kale
OLC2082	Enterobacteriaceae	*Enterobacter*	*cloacae*	*cloacae*	Spring Mix
OLC2126	Enterobacteriaceae	*Escherichia*	*coli*		Spinach
OLC2127	Enterobacteriaceae	*Enterobacter*	*kobei*		Spinach
OLC2275	Enterobacteriaceae	*Escherichia*	*coli*	O146:H21	N/A
OLC2344	Enterobacteriaceae	*Escherichia*	*coli*	O163:H19	N/A
OLC2346	Enterobacteriaceae	*Escherichia*	*coli*	O157:H16	Ground Pork

OUT: O-untypeable

6.3 Appendix 3: List of food enrichment broths used in Chapter 4

Name	Food	Broth
GTA-1445	Beef Trim	mTSB
GTA-1446	Beef Trim	mTSB
GTA-1447	Lettuce	mTSB
GTA-1447	Lettuce	*Shigella* Broth
GTA-1448	Kale	mTSB
GTA-1448	Kale	*Shigella* Broth
GTA-1449	Bok Choy Salad	*Shigella* Broth
GTA-1450	Beef Trim	mTSB
GTA-1452	Ground Beef	mTSB
GTA-1453	Beef Trim	mTSB
GTA-1454	Beef Trim	mTSB
GTA-1462	Curry Leaves	mTSB
GTA-1462	Curry Leaves	*Shigella* Broth
GTA-1469	Beef Trim	mTSB
GTA-1472	Beef Trim	mTSB
GTA-1473	Ground Beef	mTSB
GTA-1475	Beef Trim	mTSB
GTA-1476	Broccoli	mTSB
GTA-1476	Broccoli	*Shigella* Broth
GTA-1479	Cheese	mTSB
GTA-1480	Coleslaw	mTSB
GTA-1480	Coleslaw	*Shigella* Broth
GTA-1482	Cabbage	mTSB
GTA-1482	Cabbage	*Shigella* Broth
GTA-1483	Zucchini	mTSB
GTA-1483	Zucchini	*Shigella* Broth
GTA-1484	Carrots	mTSB
GTA-1484	Carrots	*Shigella* Broth
GTA-1485	Parsley	mTSB
GTA-1485	Parsley	*Shigella* Broth
GTA-1493	Ground Beef	mTSB
GTA-1494	Beef Trim	mTSB
GTA-1495	Beef Trim	mTSB
GTA-1497	Mushrooms	mTSB
GTA-1497	Mushrooms	*Shigella* Broth
GTA-1498	Cranberries	mTSB

GTA-1498	Cranberries	*Shigella* Broth
GTA-1502	Beef Trim	mTSB
GTA-1503	Beef Trim	mTSB
GTA-1504	Beef Trim	mTSB
GTA-1509	Tomatoes	mTSB
GTA-1509	Tomatoes	*Shigella* Broth
GTA-1521	Dasheen Leaves	mTSB
GTA-1521	Dasheen Leaves	*Shigella* Broth
GTA-1548	Ground Beef	mTSB
GTA-1549	Beef Trim	mTSB
GTA-1550	Pears	mTSB
GTA-1550	Pears	*Shigella* Broth
GTA-1551	Spring Mix	mTSB
GTA-1551	Spring Mix	*Shigella* Broth
GTA-1552	Spinach	mTSB
GTA-1552	Spinach	*Shigella* Broth
GTA-1556	Pears	mTSB
GTA-1556	Pears	*Shigella* Broth
GTA-1557	Plums	mTSB
GTA-1557	Plums	*Shigella* Broth
GTA-1559	Plums	mTSB
GTA-1559	Plums	*Shigella* Broth
GTA-1561	Green Onions	mTSB
GTA-1561	Green Onions	*Shigella* Broth
GTA-1562	Peppers	mTSB
GTA-1562	Peppers	*Shigella* Broth
GTA-1563	Pepper	mTSB
GTA-1563	Pepper	*Shigella* Broth
GTA-1564	Radicchio	mTSB
GTA-1564	Radicchio	*Shigella* Broth
GTA-1567	Kale Salad	mTSB
GTA-1567	Kale Salad	*Shigella* Broth
GTA-1568	Coleslaw	mTSB
GTA-1568	Coleslaw	*Shigella* Broth
GTA-1569	Broccoli Coleslaw	mTSB
GTA-1569	Broccoli Coleslaw	*Shigella* Broth
GTA-1570	Broccoli Coleslaw	mTSB
GTA-1570	Broccoli Coleslaw	*Shigella* Broth
GTA-1571	Beef Trim	mTSB
GTA-1574	Red Pear	mTSB
GTA-1574	Red Pear	*Shigella* Broth

GTA-1581	Caesar Salad Kit	mTSB
GTA-1581	Caesar Salad Kit	*Shigella* Broth
GTA-1582	Broccoli	mTSB
GTA-1582	Broccoli	*Shigella* Broth
GTA-1583	Mangoes	mTSB
GTA-1583	Mangoes	*Shigella* Broth
GTA-1590	Papayas	mTSB
GTA-1590	Papayas	*Shigella* Broth
GTA-1595	Beet Slaw	mTSB
GTA-1595	Beet Slaw	*Shigella* Broth
GTA-1608	Beef Trim	mTSB
GTA-1609	Ground Beef	mTSB
GTA-1610	Beef Trim	mTSB
GTA-1613	Caesar Salad Kit	mTSB
GTA-1613	Caesar Salad Kit	*Shigella* Broth
GTA-1614	Plums	mTSB
GTA-1614	Plums	*Shigella* Broth
GTA-1615	Arugula	mTSB
GTA-1615	Arugula	*Shigella* Broth
GTA-1618	Beef Trim	mTSB
GTA-1622	Tomatoes	mTSB
GTA-1622	Tomatoes	*Shigella* Broth
GTA-1623	Broccoli Coleslaw	mTSB
GTA-1623	Broccoli Coleslaw	*Shigella* Broth
GTA-1624	Kale Salad	mTSB
GTA-1624	Kale Salad	*Shigella* Broth
GTA-1625	Pears	mTSB
GTA-1625	Pears	*Shigella* Broth
GTA-1626	Lettuce	mTSB
GTA-1626	Lettuce	*Shigella* Broth
GTA-1630	Tomatoes	mTSB
GTA-1630	Tomatoes	*Shigella* Broth
GTA-1641	Kale Slaw	mTSB
GTA-1641	Kale Slaw	*Shigella* Broth
GTA-1645	Cucumbers	mTSB
GTA-1645	Cucumbers	*Shigella* Broth
GTA-1646	Tomatoes	mTSB
GTA-1646	Tomatoes	*Shigella* Broth
GTA-1660	Beef Trim	mTSB
GTA-1661	Beef Trim	mTSB
GTA-1665	Radicchio	mTSB

GTA-1665	Radicchio	*Shigella* Broth
GTA-1666	Romaine Salad	mTSB
GTA-1666	Romaine Salad	*Shigella* Broth
GTA-1667	Green Onions	mTSB
GTA-1667	Green Onions	*Shigella* Broth
GTA-1669	Peppers	mTSB
GTA-1669	Peppers	*Shigella* Broth
GTA-1671	Plums	mTSB
GTA-1671	Plums	*Shigella* Broth
GTA-1672	Romaine Salad	mTSB
GTA-1672	Romaine Salad	*Shigella* Broth
GTA-1679	Cranberries	mTSB
GTA-1679	Cranberries	*Shigella* Broth
GTA-1680	Caesar Salad Kit	mTSB
GTA-1680	Caesar Salad Kit	Shigella Broth
GTA-1681	Plums	mTSB
GTA-1681	Plums	*Shigella* Broth
GTA-1720	Misc Vegetable	mTSB
GTA-1720	Misc Vegetable	*Shigella* Broth
GTA-1725	Kale Salad	mTSB
GTA-1725	Kale Salad	*Shigella* Broth
GTA-1726	Peppers	mTSB
GTA-1726	Peppers	*Shigella* Broth
GTA-1732	Cantaloupe	*Shigella* Broth
GTA-1736	Kale Slaw	mTSB
GTA-1736	Kale Slaw	*Shigella* Broth
GTA-1737	Romaine	mTSB
GTA-1737	Romaine	*Shigella* Broth
GTA-1746	Lettuce	mTSB
GTA-1747	Green Onion	mTSB
GTA-1747	Green Onion	*Shigella* Broth
GTA-1753	Grape Tomatoes	*Shigella* Broth
GTA-1759	Papaya	*Shigella* Broth
GTA-1830	Raspberries	mTSB
GTA-1830	Raspberries	*Shigella* Broth
GTA-1843	Kale	mTSB
GTA-1843	Kale	*Shigella* Broth
GTA-1844	Peppers	mTSB
GTA-1844	Peppers	*Shigella* Broth
GTA-1845	Pears	mTSB
GTA-1845	Pears	*Shigella* Broth

GTA-1846	Kale	mTSB
GTA-1846	Kale	*Shigella* Broth
GTA-1851	Lettuce	mTSB
GTA-1851	Lettuce	*Shigella* Broth
GTA-1852	Lettuce	mTSB
GTA-1852	Lettuce	*Shigella* Broth
GTA-1859	Peppers	mTSB
GTA-1859	Peppers	*Shigella* Broth
GTA-1860	Tomatoes	mTSB
GTA-1860	Tomatoes	*Shigella* Broth
GTA-1861	Onions	mTSB
GTA-1861	Onions	*Shigella* Broth
GTA-1877	Peppers	mTSB
GTA-1877	Peppers	*Shigella* Broth
GTA-1878	Tomatoes	mTSB
GTA-1878	Tomatoes	*Shigella* Broth
GTA-1879	Lettuce	mTSB
GTA-1879	Lettuce	*Shigella* Broth
GTA-2166	Ground Beef	mTSB
GTA-2167	Boneless beef	mTSB
GTA-2171	Coleslaw	mTSB
GTA-2171	Coleslaw	*Shigella* Broth
GTA-2172	Kale slaw	mTSB
GTA-2172	Kale slaw	*Shigella* Broth
GTA-2173	Peppers	mTSB
GTA-2173	Peppers	*Shigella* Broth
GTA-2174	Green Onions	mTSB
GTA-2174	Green Onions	*Shigella* Broth
GTA-2177	Peppers	mTSB
GTA-2177	Peppers	*Shigella* Broth
GTA-2180	Romaine salad	mTSB
GTA-2180	Romaine salad	*Shigella* Broth
GTA-2184	Ground Beef	mTSB
GTA-2185	Beef Trim	mTSB
GTA-2186	Ground Beef	mTSB
GTA-2189	Beef Trim	mTSB
OTT-FD-2016-MI-777	Lean Beef	mTSB
OTT-FD-2016-MI-1013	Burgers	mTSB
OTT-FD-2016-MI-1018	Ground Beef	mTSB
OTT-FD-2016-MI-1019	Ground Beef	mTSB
OTT-FD-2016-MI-1027	Cheese	mTSB

OTT-FD-2016-MI-1034	Burgers	mTSB
OTT-FD-2016-MI-1056	Cheese	mTSB
OTT-FD-2016-MI-1057	Cheese	mTSB
OTT-FD-2016-MI-1058	Cheese	mTSB
OTT-FD-2016-MI-1058	Cheese	mTSB
OTT-FD-2016-MI-1065	Lettuce	*Shigella* Broth
OTT-FD-2016-MI-1076	Burgers	mTSB
OTT-FD-2016-MI-1084	Cheese	mTSB
OTT-FD-2016-MI-1129	Beef Trim	mTSB
OTT-FD-2016-MI-1094	Ground Beef	mTSB
OTT-FD-2016-MI-1131	Cheese	mTSB
OTT-FD-2016-MI-1150	Green Onions	mTSB
OTT-FD-2016-MI-1153	Blackberries	mTSB
OTT-FD-2016-MI-1154	Raspberries	mTSB
OTT-FD-2016-MI-1155	Lettuce	mTSB
OTT-FD-2016-MI-1150	Green Onions	*Shigella* Broth
OTT-FD-2016-MI-1153	Blackberries	*Shigella* Broth
OTT-FD-2016-MI-1154	Raspberries	*Shigella* Broth
OTT-FD-2016-MI-1155	Lettuce	*Shigella* Broth
OTT-FD-2016-MI-1211	Lettuce	*Shigella* Broth
OTT-FD-2016-MI-1212	Green Onions	*Shigella* Broth
OTT-FD-2016-MI-1253	Lettuce	*Shigella* Broth
OTT-FD-2016-MI-1254	Green Onions	*Shigella* Broth
OTT-FD-2016-MI-1255	Parsley	*Shigella* Broth
OTT-FD-2016-MI-1276	Green Onions	*Shigella* Broth
OTT-FD-2016-MI-1277	Carrots	*Shigella* Broth
OTT-FD-2016-MI-1279	Green Onions	*Shigella* Broth
OTT-FD-2016-MI-1280	Green Onions	*Shigella* Broth
OTT-FD-2016-MI-1303	Green Onions	*Shigella* Broth
OTT-FD-2016-MI-1304	Romaine	*Shigella* Broth
OTT-FD-2016-MI-1305	Carrots	*Shigella* Broth
OTT-FD-2016-MI-1331	Green Onions	*Shigella* Broth
OTT-FD-2016-MI-1335	Green Onions	*Shigella* Broth
OTT-FD-2016-MI-1337	Cilantro	*Shigella* Broth
OTT-FD-2016-MI-1340	Green Onions	*Shigella* Broth
OTT-FD-2016-MI-1341	Green Onions	*Shigella* Broth
OTT-FD-2016-MI-1342	Green Onions	*Shigella* Broth
STH-FD-2016-MI-2520	Cheese	mTSB
STH-FD-2016-MI-2521	Cheese	mTSB
STH-FD-2016-MI-2522	Cheese	mTSB
STH-FD-2016-MI-2523	Cheese	mTSB

STH-FD-2016-MI-2524	Beef Trim	mTSB
STH-FD-2016-MI-2525	Ground Beef	mTSB
STH-FD-2016-MI-2526	Beef Trim	mTSB
STH-FD-2016-MI-2527	Beef Trim	mTSB
STH-FD-2016-MI-2535	Cheese	mTSB
STH-FD-2016-MI-2536	Peppers	mTSB
STH-FD-2016-MI-2536	Peppers	*Shigella* Broth
STH-FD-2016-MI-2550	Ground Veal	mTSB
STH-FD-2016-MI-2551	Tomatoes	mTSB
STH-FD-2016-MI-2551	Tomatoes	*Shigella* Broth
STH-FD-2016-MI-2552	Peppers	mTSB
STH-FD-2016-MI-2552	Peppers	*Shigella* Broth
STH-FD-2016-MI-2553	Pears	mTSB
STH-FD-2016-MI-2553	Pears	*Shigella* Broth
STH-FD-2016-MI-2554	Plums	mTSB
STH-FD-2016-MI-2554	Plums	*Shigella* Broth
STH-FD-2016-MI-2565	Ground Beef	mTSB
STH-FD-2016-MI-2568	Beef Trim	mTSB
STH-FD-2016-MI-2572	Cheese	mTSB
STH-FD-2016-MI-2573	Cheese	mTSB
STH-FD-2016-MI-2575	Cheese	mTSB
STH-FD-2016-MI-2576	Cheese	mTSB
STH-FD-2016-MI-2577	Ground Meat	mTSB
STH-FD-2016-MI-2582	Flax Seeds	mTSB
STH-FD-2016-MI-2583	Flax Seeds	mTSB
STH-FD-2016-MI-2584	Flax Seeds	mTSB
STH-FD-2016-MI-2585	Flax Seeds	mTSB
STH-FD-2016-MI-2586	Cheese	mTSB
STH-FD-2016-MI-2588	Ground Beef	mTSB
STH-FD-2016-MI-2589	Beef Trim	mTSB
STH-FD-2016-MI-2590	Beef Trim	mTSB
STH-FD-2016-MI-2594	Steak Tartare	mTSB
STH-FD-2016-MI-2605	Flax Seeds	mTSB
STH-FD-2016-MI-2608	Cheese	mTSB
STH-FD-2016-MI-2611	Ground Beef	mTSB
STH-FD-2016-MI-2612	Veal Trim	mTSB
STH-FD-2016-MI-2613	Ground Beef	mTSB
STH-FD-2016-MI-2614	Veal Trim	mTSB
STH-FD-2016-MI-2615	Ground Beef	mTSB
STH-FD-2016-MI-2628	Beef Trim	mTSB
STH-FD-2016-MI-2637	Beef Trim	mTSB

STH-FD-2016-MI-2644	Ground Beef	mTSB
STH-FD-2016-MI-2645	Ground Beef	mTSB
STH-FD-2016-MI-2646	Beef Trim	mTSB
STH-FD-2016-MI-2647	Beef Trim	mTSB
STH-FD-2016-MI-2664	Cheese	mTSB
STH-FD-2016-MI-2669	Beef Trim	mTSB
STH-FD-2016-MI-2682	Beef Trim	mTSB
STH-FD-2016-MI-2688	Beef Trim	mTSB
STH-FD-2016-MI-2698	Cheese	mTSB
STH-FD-2016-MI-2701	Veal Trim	mTSB
STH-FD-2016-MI-2702	Ground Veal	mTSB
STH-FD-2016-MI-2703	Beef Trim	mTSB
STH-FD-2016-MI-2714	Cheese	mTSB
STH-FD-2016-MI-2715	Flax Seeds	mTSB
STH-FD-2016-MI-2717	Ground Beef	mTSB
STH-FD-2016-MI-2721	Burgers	mTSB
STH-FD-2016-MI-2722	Ground Beef	mTSB
STH-FD-2016-MI-2724	Beef Trim	mTSB
STH-FD-2016-MI-2725	Veal Trim	mTSB
STH-FD-2016-MI-2734	Ground Veal	mTSB
STH-FD-2016-MI-2746	Ground Meat	mTSB
STH-FD-2016-MI-2755	Beef Trim	mTSB
STH-FD-2016-MI-2762	Cheese	mTSB
STH-FD-2016-MI-2763	Cheese	mTSB
STH-FD-2016-MI-2768	Beef Trim	mTSB
STH-FD-2016-MI-2770	Ground Meat	mTSB
STH-FD-2016-MI-2771	Beef Trim	mTSB
STH-FD-2016-MI-2772	Ground beef	mTSB
STH-FD-2016-MI-2773	Beef trim	mTSB
STH-FD-2016-MI-2777	Ground Beef	mTSB
STH-FD-2016-MI-2780	Ground Veal	mTSB
STH-FD-2016-MI-2784	Tomatoes	mTSB
STH-FD-2016-MI-2784	Tomatoes	*Shigella* Broth
STH-FD-2016-MI-2785	Peppers	mTSB
STH-FD-2016-MI-2785	Peppers	*Shigella* Broth
STH-FD-2016-MI-2786	Tomatoes	mTSB
STH-FD-2016-MI-2786	Tomatoes	*Shigella* Broth
STH-FD-2016-MI-2787	Peppers	mTSB
STH-FD-2016-MI-2787	Peppers	*Shigella* Broth
STH-FD-2016-MI-2788	Peppers	mTSB
STH-FD-2016-MI-2788	Peppers	*Shigella* Broth

STH-FD-2016-MI-2789	Spinach	mTSB
STH-FD-2016-MI-2789	Spinach	*Shigella* Broth
STH-FD-2016-MI-2790	Pears	mTSB
STH-FD-2016-MI-2790	Pears	*Shigella* Broth
STH-FD-2016-MI-2791	Plum	mTSB
STH-FD-2016-MI-2791	Plum	*Shigella* Broth
STH-FD-2016-MI-2792	Nectarines	mTSB
STH-FD-2016-MI-2792	Nectarines	*Shigella* Broth
STH-FD-2016-MI-2794	Beef Trim	mTSB
STH-FD-2016-MI-2798	Ground Meat	mTSB

6.4 Appendix 4: Size and description of inhibitory Cell-free extracts

	Shigella sonnei				*Shigella flexneri*			
	OLC0024		OLC2340		OLC0603		OLC1597	
	Description	Average Diameter (mm)	Description	Average Diameter (mm)	Description	Average Diameter (mm)	Description	Average Diameter (mm)
OLC0637	Strong	6.92	Strong	9.00				
OLC0708	Medium	6.00	Strong	6.67				
OLC0993	Strong	6.75	Very Strong	10.17				
OLC1028	Strong	8.00	Very Strong	10.17				
OLC1065			Strong	8.00				
OLC1112	Medium	6.42	Strong	7.33	Weak	4.42		
OLC1219	Weak	4.67	Weak	4.54			Weak	4.72
OLC1225	Strong	6.83	Strong	8.00	Weak	4.67	Very Strong	10.50
OLC1260	Medium	6.50	Very Strong	9.25				
OLC1266	Strong	7.00	Very Strong	9.33				
OLC1407					Medium	5.83	Medium	5.25
OLC1497	Weak	4.94	Weak	4.83				
OLC1512	Weak	4.50	Very Strong	11.33				
OLC1531			Medium	5.06				
OLC1539	Weak	4.67	Very Strong	8.56				
OLC1582					Strong	6.33	Strong	6.42
OLC1659	Strong	6.50	Very Strong	8.10			Very Weak	3.50
OLC1669							Medium	5.50
OLC2275	Strong	7.83	Strong	7.67				

OLC2344	Strong	7.80	Very Strong	10.17				
OLC2346	Very Strong	8.83	Very Strong	10.83				
GTA-1447m	Very Weak	4.47	Weak	5.40	Weak	4.94		
GTA-1462s	Strong	6.97	Very Strong	9.50				
GTA-1473m	Weak	4.56	Medium	4.80				
GTA-1475m	Medium	5.00	Strong	6.13				
GTA-1502m	Weak	4.00	Weak	4.47	Weak	4.35		
GTA-1549m	Strong	8.85	Very Strong	9.96	Weak	4.78	Medium	4.73
GTA-1623s	Weak	4.57	Weak	5.71				
OTT-1019m	Medium	5.17	Medium	5.17	Weak	4.58	Weak	4.50
OTT-1094m	Medium	5.67	Strong	7.56				
STH-2520m	Medium	5.50	Strong	7.28				
STH-2568m	Weak	4.00	Medium	5.33				
STH-2577m	Strong	8.67	Very Strong	10.39	Very Weak	4.50	Very Weak	4.50
STH-2612m	Medium	5.08	Medium	6.11				
STH-2682m	Weak	4.67	Strong	5.86				
STH-2725m	Medium	6.39	Strong	7.28				
STH-2746m	Very Weak	4.33	Weak	4.54				
STH-2768m	Very Weak	4.17	Medium	5.33				
STH-2772m	Weak	4.50	Medium	5.67				
STH-2773m			Weak	4.33				
STH-2777m	Strong	7.17	Very Strong	8.83				

m: mTSB

s: *Shigella* broth

	O26:H11		O45:H2		O103:H2		O111:H11		O121:H19		O145:NM		O157:H7	
	Description	Average Diameter (mm)	Description	Average Diameter (mm)	Description	Average Diameter (mm)	Description	Average Diameter (mm)	Description	Average Diameter (mm)	Description	Average Diameter (mm)	Description	Average Diameter (mm)
OLC-1633			Medium	5.00										
OLC-1667			Weak	4.17	Medium	5.00								
GTA-1452m	Weak	4.72	Very Weak	4.50	Very Weak	4.75	Very Weak	4.67	Weak	4.83	Very Weak	4.67	Weak	4.72
GTA-1549m			Medium	4.75					Medium	5.75	Weak	4.58		
GTA-1581m			Weak	4.25	Weak	4.67								
OTT-1027m									Weak	4.33	Weak	4.17	Weak	4.08
STH-2577m									Very Weak	3.00	Very Weak	3.00		
STH-2590m									Weak	4.50			Very Weak	4.67
STH-2777m									Very Weak	4.83	Very Weak	3.00		

m: mTSB

s: *Shigella* broth

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