PREVALENCE OF SHIGA-TOXIN PRODUCING *ESCHERICHIA COLI* IN ALBERTA BEEF CATTLE AND CHARACTERISTICS OF NON-PATHOGENIC MICROCIN-PRODUCING *ESCHERICHIA COLI* CHAMPION BATTLING BACTERIAL VIRULENCE

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ABSTRACT

Zoonotic pathogens, like Shiga toxin-producing *Escherichia coli* (STEC) are a food safety and health risk. Among STEC, O157 is considered a major pathogen; however, globally the number of outbreaks with new emerging STEC strains is rising. Here, quantitative data on STEC O157 were gathered for western-Canada beef cattle and compared to emerging STEC. Quantitative data revealed; O178 is 4-times more numerous than O157 and high Shiga-toxin events are often not attributed to O157. Results suggest current surveillance likely misses emerging STEC due to restricted O-serogroup screening. Secondly, an alternate STEC mitigation strategy was investigated as an alternative approach to use of antibiotics which is controversial in human and animal therapeutics. Competition experiments identified a strong *E. coli* O103F which incapacitated STEC growth by producing a diffusible antimicrobial, most likely a microcin. The antimicrobial revealed tremendous potential for use along the farm-to-fork continuum or in human intervention to mitigate STEC.

CONTRIBUTIONS OF AUTHORS

The work from this thesis resulted in three manuscripts that have been published (Chapter III, IV and V) and one manuscript currently in preparation for publication (Appendix IV).

Publication list:

Chapter III - Paquette S-J, Stanford K, Thomas J, Reuter T: **Quantitative surveillance of shiga toxins 1 and 2, *Escherichia coli* O178 and O157 in feces of western-Canadian slaughter cattle enumerated by droplet digital PCR with a focus on seasonality and slaughterhouse location**. *Plos One*. 2018; **13**(4):e0195880

The experimental design, laboratory work and first draft of the manuscript were performed by myself. I consulted with my supervisors (Dr. James Thomas and Dr. Tim Reuter), and committee member (Dr. Kim Stanford) during the course of the project to ensure the thoroughness of the experimental design or if I encountered any problems requiring troubleshooting. Dr. Stanford provided statistical analysis guidance and reviewed the manuscript. Dr. Thomas provided guidance with experimental design and reviewed the manuscript. Dr. Reuter provided guidance with experimental design, troubleshooting and manuscript revisions. The majority of the work on this manuscript was performed by myself with guidance from my co-authors.

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Similarly to the first manuscript, experimental design, laboratory work and manuscript writing were performed by myself with support from my co-authors.
I consulted with my supervisors (Dr. Thomas and Dr. Reuter), co-authors (Dr. Zaheer) and committee member (Dr. Stanford) on experimental design, troubleshooting and

manuscript revisions. Experimental design was constructed mainly by myself with input from my co-authors to ensure the thoroughness of the experiments. The laboratory work was performed primarily by myself with the help of my laboratory colleagues when needed. I drafted the first version of the manuscript and my co-authors provided revisions which I incorporated into the manuscript.

Chapter V - Paquette S-J, Reuter T: **Properties of an antimicrobial molecule produced by an *Escherichia coli* champion**. *Antibiotics.* 2019; **9**(1):6

The experimental design, laboratory work and manuscript writing were performed by myself. I consulted with my supervisor Dr. Reuter for troubleshooting and manuscript revisions.

Appendix IV - *Escherichia coli*: using physiological cues to turn on antimicrobial molecule production.

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A/E	attaching and effacing lesion
AIEC	adherent invasive *E. coli*
AMMO	cell free supernatant collected after 12 h of growth of *E. coli* O103F in EC, containing **A**nti**M**icrobial **Mo**lecule(s)
AMR	antimicrobial resistance
bp	base pair
CCR	carbon catabolite repression
CDI	contact-dependent growth inhibition
CE	competitive exclusion
CFU	colony forming units
DAEC	diffusely adherent *E. coli*
ddPCR	droplet digital polymerase chain reaction
DEC	diarrheagenic *E. coli*
DFM	direct-fed microbials
DNA	deoxyribonucleic acid
EAEC	enteroaggregative *E. coli*
EC	*E. coli* broth
E. coli	*Escherichia coli*
EHEC	enterohemorrhagic *E. coli*
EID	emerging infectious diseases
EIEC	enteroinvasive *E. coli*
EPEC	enteropathogenic *E. coli*
ETEC	enterotoxigenic *E. coli*
ExPEC	extraintestinal pathogenic *E. coli*
Gb3	globotriaosylceramide
GEC	generic *E. coli*
GHG	greenhouse gas
GIT	gastro intestinal tract
h	hours
H-	flagellar antigen
HC	hemorrhagic colitis
HCl	hydrochloric acid
HGT	horizontal gene transfer
HUS	hemolytic uremic syndrome
K-	capsular antigen
kDa	kilodaltons
LB	Luria-Bertani
LBA	Luria-Bertani agar
LEE	locus of enterocyte effacement
lpf	long polar fimbriae
MAC	MacConkey agar
MH	Mueller-Hinton II agar
MLST	multilocus sequence typing
NaOH	sodium hydroxide

NMEC	neonatal meningitis-associated *E. coli*
O-	somatic antigen
OD	optical density
PAI	pathogenicity island
PCR	polymerase chain reaction
PFGE	pulse field gel electrophoresis
qPCR	real-time polymerase chain reaction
RNA	ribonucleic acid
saa	STEC autoagglutinating adhesin
Sab	STEC autotransporter mediating biofilm formation
SEPEC	sepsis-causing *E. coli*
SPENT	cell free supernatant collected after 12 h growth of *E. coli* O157A in EC
STEAEC	shiga-toxin-producing enteroaggregative *E. coli*
STEC	shiga-toxin producing *E. coli*
Stx	shiga toxin
SubAB	subtilase cytotoxin
T3SS	type III secretion system
T6SS	type VI secretion system
TSA	Tryptic Soy agar
TSB	Tryptic Soy broth
UPEC	uropathogenic *E. coli*
USA	United States of America
USDA-FSIS	United States Department of Agriculture Food Safety and Inspection Service
WGS	whole genome sequencing
WHO	World Health Organization

CHAPTER I

1.1 Background: Human Microbiome

Often referred to or regarded as a human organ [1, 2], the human microbiome is the collection of all the microbes associated with the human body, such as those found along the gastro intestinal tract [3]. The microbiome has a crucial role in human immunity and nutrition among others [3] and out-number human cells by at least 10-fold. In fact, the human microbiome is estimated to weigh 1.5 kg, equivalent to the human brain [2]. However, there are many unknowns about the residents of our microbiomes but the advent of next generation sequencing is helping to tackle this dilemma. Recently, a project was undertaken to expand our knowledge about our microbe residents but even with all the data generated there still remains many questions [4], especially regarding our ancestral microbiome and human evolution [2] and the role of the microbiome in human disease [3, 4].

The human microbiome has adapted and evolved to changes in lifestyle and food choices of modern humans and in many cases can help protect them from diseases but pathogens have also evolved. This evolution has led to emergence of diseases that can tax both our health systems and food supply lines. Often times, these pathogens have evolved from non-pathogenic bacteria that are well tailored to humans or livestock and are equally capable of responding to intestinal cues and immune system defenses to grow in their human hosts [5]. One such bacterium is *Escherichia coli* (*E. coli*), which has been recognized for having a beneficial role in people but can also cause severe human diseases, ranging from meningitis to severe diarrhea and death [6].

1.2 *Escherichia coli*

Bacterium coli commune was first isolated and characterized by German microbiologist and pediatrician Theodor Escherich [7-9]. Theodor Escherich was studying the intestinal flora of infants when he identified the rod shape bacterium [9]. Over the years, the bacterium has been identified by various researchers and given different names but it wasn't until 1954 that the designation *Escherichia coli* was coined [8].

Escherichia coli is a rod-shaped, gram-negative facultative anaerobe that is part of the gastrointestinal microbiota in humans, livestock, other warm-blooded animals and reptiles [10]. It is classified as a part of the family *Enterobacteriaceae*, class *Gammaproteobacteria* [11]. *E. coli* is a fast-growing bacterium and under optimal conditions (nutrient rich medium and a temperature of 37°C) can replicate in ~20 minutes [8, 11]. This quality (rapid growth) and many others have allowed us to designate *E. coli* as a "model organism" for scientific research [7]. *E. coli* has been used to unravel many scientific concepts, from the genetic code, to transcription, translation and replication [7, 12, 13]. Furthermore, it is an extensively used organism in pharmaceutical production and genetic engineering [7].

E. coli is quite diverse with various genotypic and phenotypic traits across strains that are often characterized by their serotypes. Serotyping is based on the Kaufmann method which separates strains using 3 types of antigens: somatic (O), capsular (K) and flagellar (H) and their combinations with O- and H- typing deemed the standard [14]. Currently, ~182 O-groups and 53 H-antigens are recognized [15] and over 700 serotypes have been identified using O and H antigens [11, 16].

E. coli genome sizes vary across strains; for example non-pathogenic *E. coli* MG1655 have a genome size of ~4.6 million base pairs (bp), while pathogenic O157:H7 EDL933 have a genome size of ~5.5 million bp [17]. In fact, genomes of pathogenic *E. coli* can be up to 1 million bp larger than their commensal counterparts [18]. Analyzing the pan-genome of *E. coli* revealed that *E. coli* has a core genome of approximately 2200 genes which is shared among all strains and an accessory genome or flexible genome, leading to various characteristics unique to specific isolates or groups such as toxin genes [16, 18-20].

Examining pathogenic *E. coli* genomes revealed that they have potential to encode ~5000 genes with less than half part of the core genome, leading to significant genetic diversity among *E. coli* strains, in many cases acquired through horizontal gene transfer (HGT) [18]. Coding DNA fragments can be readily transferred between prokaryotes through mechanisms such as conjugation, transformation and transduction mediated through the use of mobile genetic elements, such as plasmids, transposons and bacteriophages [8]. Acquisition and loss of genes allow *E. coli* to alter its genetic composition, resulting in strains displaying unique phenotypes that either benefit or have no effect on its human hosts (commensal) or cause harm (pathogenic).

1.3 *E. coli:* Non-pathogen vs Pathogen

E. coli strains associated with humans can be divided into 3 main subsets: commensal, extraintestinal pathogenic (ExPEC) and diarrheagenic/enteric (DEC) [6]. Commensal *E. coli* are often identified along mammalian digestive tracts and have a symbiotic host interaction. They colonize early on (infants) and have a critical role in mammalian intestines by producing vitamin B and K, out-competing invading pathogens

and removing oxygen thus preventing the killing of obligate anaerobes in the gut [7, 10, 21]. Also, recent data suggest that *E. coli* proteins may influence control of host appetite by signaling meal termination through satiety pathways [22]; further demonstrating that commensal *E. coli* can have many different and vital roles as part of the human gut microbiome.

ExPEC are a group of *E. coli* pathogens that cause diseases in humans outside the digestive tract, whereas enteric pathogenic *E. coli* cause gastrointestinal infections [6]. Traditionally, ExPEC are divided by their ability to cause clinical disease; e.g.: urinary tract infections, (uropathogenic *E. coli* – UPEC), neonatal meningitis-associated *E. coli* (NMEC), and sepsis-causing *E. coli* (SEPEC) [23]. However, it has been observed that ExPEC isolates are not necessarily disease specific and can in fact cause infections at multiple body sites. ExPEC can cause a variety of diseases from urinary tract diseases, infective peritonitis, pelvic inflammatory disease, skin and soft tissue infections, neonatal meningitis and hospital-acquired pneumonia, blood infections and sepsis [23, 24].

Enteric or diarrheagenic *E. coli* (DEC) pathogens on the other hand cause gastrointestinal diseases [6, 24, 25]. DEC can be sub-divided into different pathotypes (Table 1.1) that cause diarrhea disease, the original 6 - enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC) or shiga-toxin producing *E. coli* (STEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli (EIEC) and* diffusely adherent *E. coli* (DAEC) [25, 26] and the new and emerging pathotypes: adherent invasive *E. coli* (AIEC), associated with Crohn's disease [8] and shiga-toxin-producing enteroaggregative *E. coli* (STEAEC) identified in the German

outbreak [27]. One of the most notable DEC pathogens is STEC O157:H7 [7, 11]; STEC are a foodborne cause of severe disease [28].

Table 1.1. Pathotypes of diarrheagenic *Escherichia coli* (DEC) and their defining characteristics.*

Pathotypes	Defining characteristics
Enterotoxigenic *E. coli* (ETEC)	Strains contain a heat stable enterotoxin or heat-labile enterotoxin groups
Enteropathogenic *E. coli* (EPEC)	Strains contain the locus of enterocyte effacement (LEE) pathogenicity island
Enterohemorrhagic *E. coli* (EHEC) / Shiga-toxin producing *E. coli* (STEC)	Produce a Shiga-like toxin
Enteroaggregative *E. coli* (EAEC)	Display aggregative adherence patterns
Enteroinvasive *E. coli* (EIEC)	Strains are closely related to *Shigella* spp.
Diffusely adherent *E. coli* (DAEC)	Display diffuse adherence patterns
Adherent invasive *E. coli* (AIEC)	Ability to invade and adhere to epithelial cells
Shiga-toxin-producing enteroaggregative *E. coli* (STEAEC)	Produce a Shiga-like toxin and display aggregative adherence patterns

* adapted from Nataro and Kaper,1998 [26], Robins-Browne et al, 2016 [25] and Mora et al, 2011 [27].

1.4 STEC

STEC are characterized based on production of one or more shiga-toxins (Stx), a major virulence factor for this pathotype [16, 29]. Over 400 STEC serotypes have been identified [8, 30] but only a subset have been associated with human disease. STEC O157:H7 is the most recognized STEC but non-O157 STEC are also implicated in

outbreaks worldwide [7, 28]. STEC can cause diverse disease etiology ranging from mild diarrhea to severe infections such as hemorrhagic colitis (HC) or hemolytic uremic syndrome (HUS) [31, 32]. It is estimated that annually, STEC causes: 2,801,000 acute illnesses, 3890 cases of HUS, 270 cases of end-stage renal disease and 230 deaths, worldwide [28]. However, a European study examining STEC outbreaks and sporadic cases of disease from 2007 to 2010 concluded that STEC infections are most likely under-reported and burden of this often food-borne pathogen is in all likelihood higher than currently estimated [33].

1.5 Reservoir and Transmission of STEC

Cattle are considered a major reservoir for STEC [8, 34] and over 400 STEC serotypes have been identified in cattle [30]. As, a host for STEC, cattle are often implicated as a disseminator along the farm to fork chain [35] and cattle that shed high numbers of STEC in their feces are thought to be the main contributor of animal to animal transmission [36]. However, STEC have also been identified in other animals such as swine, sheep, deer, rabbits, dogs and insects suggesting that STEC may also have alternate reservoirs [8, 34]. In fact, in the case of the STEAEC German outbreak strain, humans are alleged to be the reservoir [37].

Transmission of STEC to humans occurs via three possible modes: **I**) by ingesting contaminated food and/or water, **II**) by person to person contact such as in daycares and, **III**) by zoonosis or animal contact (Figure 1.1). Contamination of raw and/or processed agricultural products such as beef and unpasteurized milk is one of the main ways that STEC enter the food chain [35, 36]. In the United States of America (USA) and Canada the most common food source implicated in STEC infections is beef [36, 38, 39] which

has been implicated in outbreaks in the USA [40], Canada [41] and France [42]. However, some STEC outbreaks have been implicated in various different types of food contamination that are not only linked to ground beef [43]. In fact, one of the largest STEC outbreaks was in 1996 in Sakai, Japan and the food source was radish sprouts and more recently the German outbreak with STEAEC was linked to fenugreek sprouts [44]. Fresh produce is being increasingly associated with STEC outbreaks and human infections [45] and the STEC O157 was linked to spinach contamination in 2006 [46] and with romaine lettuce in the USA and Canada in 2018 [47].

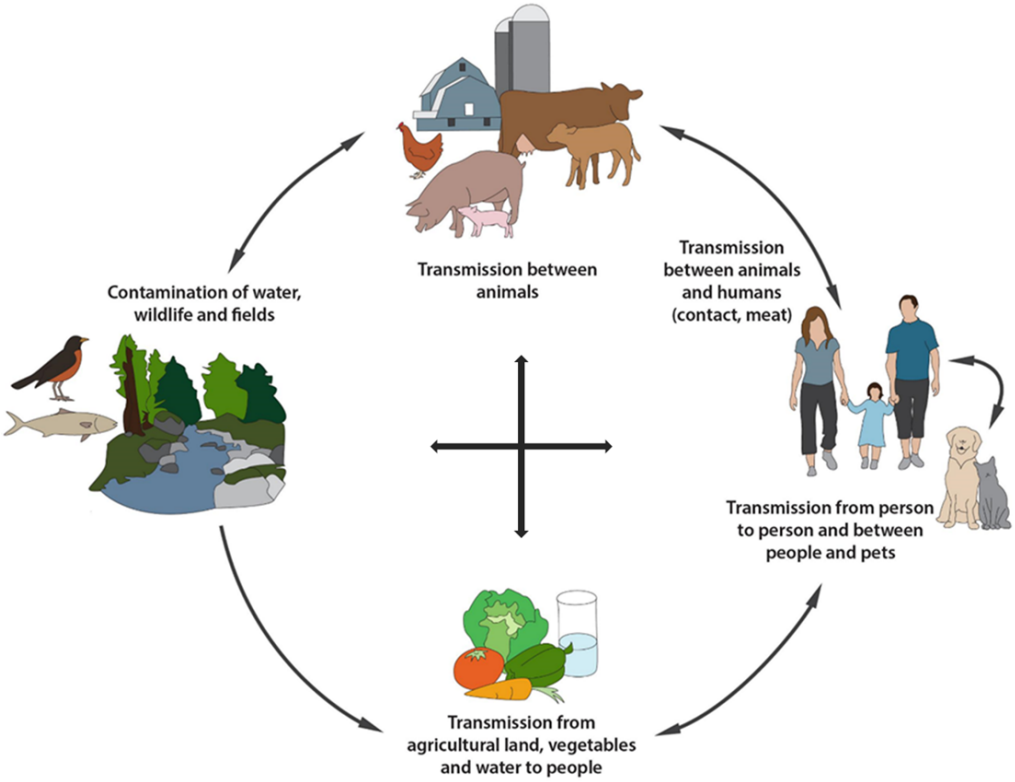


Figure 1.1. Transmission routes of STEC to humans.
* Adapted from Rhouma et al, 2016 [48].

Waterborne infections have occurred worldwide and are of concern due to the possibility of widespread contamination affecting numerous people in a short period of

time [49]. In 1998, an O157:H7 outbreak occurred in Alpine, Wyoming, USA due to drinking insufficiently-chlorinated water from the town's water supply [49]. In Canada, a water-borne outbreak of *E. coli* O157:H7 and *Campylobacter jejuni* was seen in Walkerton, Ontario; over 2000 people were infected and seven people died [50]. In Korea, an outbreak at a children's camp was caused by various DEC strains, including STEC which infected 188 students and was linked to the water reservoir used by the camp for drinking and food preparation [51]. Outbreaks of this nature often point out deficiencies in water supply lines [50, 51] and can lead to changes in water regulations [50].

Transmission of bacterial pathogens from person to person can have a significant role in disease dissemination and can account for 15-20% of cases within outbreaks [52, 53]. Often person to person transmission is the main route in which disease is transmitted in households, work environments and daycare facilities [36, 52-55]. Secondary infections mainly affect children (1 to 6 yrs. old) due to their immature immune systems and poor hygiene practices [34]. Social interactions and common use of space by contaminated and/or infected individuals likely result in spread of most types of infectious agents. Proof of infectious routes requires both good epidemiological evidence as well as molecular confirmation which is provided by use of microbial source tracking tools [56]. Further compounding the issue of human to human transmission is the occurrence of asymptomatic carriers. During the 2011 German STEAEC outbreak, asymptomatic carriers were considered to have had a substantial impact on dissemination during the outbreak [37].

Direct animal to human transmission or zoonosis, is another concern for STEC dissemination and infections have occurred due to contact with farm animals and animals at petting zoos [34, 57]. A study examining outbreaks found that animal contact was

identified frequently as a possible source of STEC infections [52]. Transmission of STEC infections is lower compared to other zoonotic diseases but the low infective dose estimated to be 10-100 cells of STEC is a major concern [36, 58].Compounding this issue is that STEC can survive in soil and water for weeks and possibly months; therefore removing infected animals does not lower the risk of infection and transmission to humans may still be possible without direct animal contact [55, 59].

1.6 STEC Serogroups and Emerging STEC O178

Historically, O157:H7 is the most recognized STEC [7] and has been traced to many outbreaks, globally, including the recent romaine lettuce outbreak in the USA and Canada in 2018 [47]. It is considered to be the most virulent STEC serotype but non-O157 STEC serogroups have been linked to human diseases and outbreaks [32], such as the STEC O121 flour outbreak that occurred in Canada [60]. A recent review, discussed that in North America STEC O157:H7 diseases have decreased over the past 20 yrs, most likely due to safety improvements in the farm to fork chain [61]. However, the review also stated that severe infections with non-O157 STEC and emerging hybrid STEC strains are increasing. A review by Castro and coworkers [12] mentioned that in the USA in 2010, O157 infections decreased while non-O157 infections increased. Shiga toxin (*stx*) virulence genes can be transferred between negative *stx E. coli* and STEC populations, potentially creating new STEC pathogens with increased virulence potential [62]. This phenomenon was recently seen with the STEAEC German outbreak strain, where the combination of EAEC and STEC strains resulted in an exceptionally virulent strain [63].

Non-O157 STEC frequently associated with human disease includes the serotypes O26:H11, O103:H2, 0111:H8 and O145:H28. In 2011, the United States Department of

Agriculture Food Safety and Inspection Service (USDA-FSIS) made testing mandatory for what is called the "top 6" non-O157 STEC serogroups (O26, O45, O103, O111, O121 and O145) in beef products [64]. Together with O157 they are considered the "top 7" STEC adulterants in beef products [36]. In Argentina, non-O157 STEC are predominantly isolated in cattle [65]. It is estimated that 20 to 50% of STEC infections were due to non-O157 strains [66]; for example in Canada and the USA, O157:H7 is the serotype implicated in causing most cases of HUS while non-O157 infections were more common in continental Europe, Australia and Latin America [67, 68].

Recently, in Argentina and Germany another non-O157 serogroup has emerged and has been implicated in cases of human diseases, i.e., STEC O178 [69]. STEC O178 has been isolated in beef in both of these countries and cattle in South America and Europe have been identified as a reservoir for this STEC serogroup. *E. coli* O178 has been implicated in various cases of sporadic disease in Germany and Brazil and has been implicated in severe HUS infections in Argentina and Belgium. Human infections with STEC O178 are infrequently reported which may be due to STEC O178 being less virulent, less common or possibly due to complexities associated with screening and typing of uncommon STEC, so human cases with this STEC may not be reported [70, 71].

STEC O178 possesses several virulence factors associated with an increasingly detected STEC group negative for the locus of enterocyte effacement (LEE), which is a virulence factor found in the top 7 STEC [72]. However, LEE negative STEC are still able to cause severe human disease [65, 69]. The main virulence factor for all STEC is production of the Stx and STEC O178 isolates have been found to produce one or both of

either Stx_1 or Stx_2. Despite the STEC O178 being LEE-negative it has been implicated in cases of HUS and LEE-negative STEC have different virulence factors such as long polar fimbriae (lpf) Protein LPFAO113, STEC autoagglutinating adhesin (saa) and subtilase cytotoxin (SubAB), which are thought to contribute to infection [69]. SubAB is thought to be a significant factor in LEE-negative STEC's virulence and pathogenicity to humans. Some STEC O178 have all 3 of these factors, which suggest they have the virulence potential to cause human disease and should be monitored as part of routine investigations [65, 69].

1.7 STEC Virulence Factors

The main and crucial virulence factor for STEC is presence of the *stx* gene [29]. Another important factor is the presence or absence of the LEE pathogenicity island (PAI) [12] and the genes associated with each virulence profile. All 3 will be discussed below.

1.7.1 Shiga Toxin Genes

Stx is a part of a class of AB_5 toxins [73, 74]. The toxin is composed of one A subunit which inhibits protein synthesis and five B subunits which bind the toxin to the globotriaosylceramide (Gb3) receptor on target cells [12]. There are 2 types of Stx, Stx_1 which resembles the Stx toxin produced by *Shigella dysenteriae* and Stx_2 which is immunologically different [73]. Stx_1 and Stx_2 can be further separated into subtypes, based on sequencing and toxicity (Table 1.2). Despite both Stx_1 and Stx_2 having the same mode of action, Stx_2 appears to be more toxic; purified Stx_2 is 1000 times more toxic to human endothelial cells then Stx_1 [75] and Stx_{2a} and Stx_{2c} are often associated with HUS [74]. *Stx_1* and *stx_2* genes are also regulated by two different promotors; *stx_1*

genes are controlled by the iron-regulated authentic promoter while *stx*$_2$ is regulated by the phage late gene promoter [75].

Table 1.2. Stx subtypes and toxicity.

Toxin subtype(s)	Linked with serious human disease
Stx_{1a}	Yes
Stx_{1c}	No - immunologically distinct
Stx_{1d}	No - immunologically distinct, less potent
Stx_{2a}	Yes
Stx_{2b} (originally, VT-2d or Stx_{2d})	No
Stx_{2c}	Yes
Stx_{2d} (Stx_{2dact})	Yes - more toxic after incubation with elastase, less toxic to Vero cells
Stx_{2e}	No - binds to Gb4, associated with porcine disease
Stx_{2f}	No - originally isolated in STEC from pigeons, immunologically distinct
Stx_{2g}	No

*Adapted from Melton-Celsa, 2014 [73].

Once an STEC adheres to the intestines the Stx toxin needs to be transported across the intestinal epithelium and into the blood stream to reach the target cells [73, 76]; however, this mechanism is not yet well understood. It has been suggested that micropinocytosis of the toxin may occur in cells that do not have Gb3 receptors [73] and research into the German outbreak strain showed that Stx may be packaged into outer membrane vesicles which may transport Stx to its target [77]. Humans have many Gb3 receptors on renal cells and Stx has been found bound to renal cells during autopsy [78].

Once, Stx binds to Gb3 receptors it is internalized and subsequently transported to the Golgi apparatus, where it is directed to the endoplasmic reticulum and then the cytosol [12, 73]. During the transfer process in the cell the toxin is nicked by a furin (a protease) activating the toxin; once in the cytosol the toxin inactivates the 28S ribosome by removing an adenine residue, leading to protein synthesis inhibition and eventually to cell death (Figure 1.2).

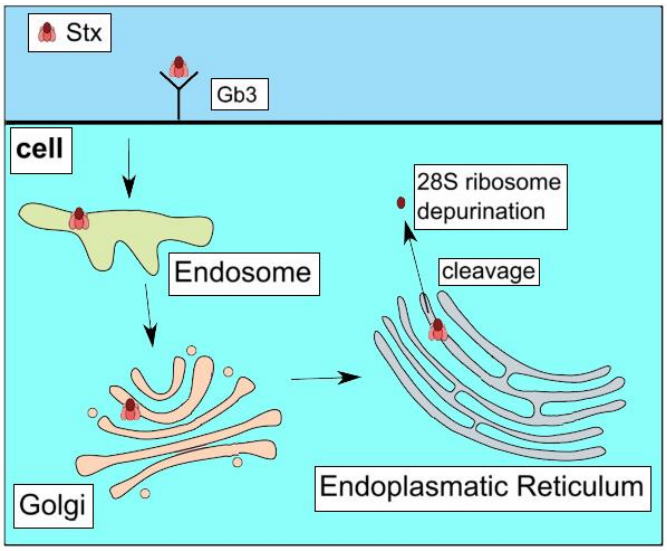


Figure 1.2. Shiga toxin target sites in the cell.
*Adapted from Krause et al, 2018 [74].

1.7.2 LEE STEC

LEE PAI encodes for several key colonization features of LEE positive STEC including the type III secretion system (T3SS) and the ability to produce the attaching and effacing lesion (A/E) which is key to LEE positive STEC pathogenesis [79, 80]. The T3SS is conserved in many bacteria and acts as a "molecular syringe" that secretes proteins into target cells and modifies the host's cytoskeleton [81-83]. One of the proteins encoded for by the LEE is Tir, which acts as the receptor for intimin [84]. Intimin is an adhesion factor encoded by the *eae* gene also found on the LEE PAI which is needed for bacteria to attach to the surface of target cells [82, 84]. Tir and the T3SS secreted effectors activate various

signaling cascades which results in changes in the intestinal target cell physiology [84]. The A/E lesion is the result of this process, where STEC attaches to an intestinal cell, destroys the microvilli (effacing) and induces pedestal formation [81, 85]. The pedestal is where STEC pathogens "sit" on the intestinal cell (Figure 1.3). The STEC O157:H7 generates many A/E lesions, suggesting it can adhere intimately to intestinal epithelial cells and when the *eae* gene is knocked out, O157 cannot colonize the intestine, demonstrating the significance of intimin in adhering bacteria to the intestine [26, 79]. Despite the importance of the LEE PAI and intimin in infection, it does not appear to be crucial for STEC infection since LEE negative STEC are still able to cause severe diseases (HUS and HC), suggesting that other adherence factors are also involved in STEC infection [79].

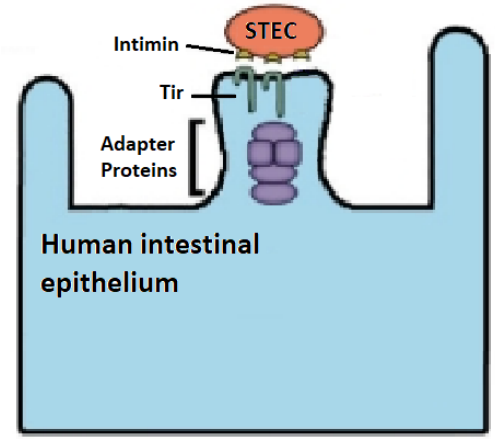


Figure 1.3. Diagram of STEC adhesion to intestinal epithelial cells using LEE PAI effectors that create the pedestal for STEC.
*Adapted from Farfan et al, 2012 [79].

1.7.3 Non-LEE STEC

There is an increasing number of non-LEE STEC infections in humans which suggests that these STEC use other virulence factors for adherence to the human intestine [79]. Non-LEE STEC are thought to have evolved separately from LEE containing STEC and have gained virulence factors in parallel to LEE STEC [74].

The understanding of how non-LEE STEC colonize the intestine is limited but several factors have been identified in LEE and non-LEE STEC, as well as factors unique to non-LEE STEC. One of these factors is the long polar fimbriae protein, which has been identified in both types of STEC [83]; it enhances adherence to epithelial cells as mutations in this gene have shown a reduction in adherence [79]. Another factor is STEC autotransporter mediating biofilm formation (Sab) which was only identified in a subset of non-LEE STEC [79]. Studies examining Sab function found that purified Sab protein binds to epithelial cells, suggesting it may have a role in adherence [86]. STEC autoagglutinating adhesin (saa) has also been identified in non-LEE STEC, including O113:H13 non-LEE STEC that caused a HUS outbreak in Australia [74].

Saa is a plasmid encoded adhesin that is often used as a marker in non-LEE STEC [87]. On the other hand, the subtilase cytotoxin (subAB) which is associated with non-LEE STEC [74] is not involved in adherence but, is a toxin that mimics the characteristics of Stx-induced HUS [88, 89]. SubAB is an AB_5 cytotoxin, the same as Stx; it has an active A subunit and five B subunits that facilitate target cell binding [74, 89]. SubAB cleaves the endoplasmatic heat shock protein in target cells which causes cytotoxic effects in the target [74]. SubAB has also been shown to cause damage to other organs, not just renal tissue [90]. The mechanisms used by non-LEE STEC to cause severe disease are poorly

understood but are most likely due to a combination of factors described above and, those not yet discovered. Future research is needed to fully elucidate the mechanism these STEC use in infection.

1.8 Molecular Methods for STEC Detection

Traditional STEC detection is culture based but with advances in molecular techniques, molecular based methods are often used to complement culture methods [91]. Samples are often screened for specific gene combinations using either conventional or real-time (quantitative) polymerase chain reaction (qPCR) prior to culture isolation to identify samples of interest to focus culture detection to only samples positive for STEC genes (*stx and eae*) and specific O-antigen serogroups to reduce processing time [59]. Collected isolates are fully characterized, which is time consuming and costly. Whole Genome Sequencing (WGS) may have the potential to replace current characterization methods [92]. Advances in qPCR, droplet digital polymerase chain reaction (ddPCR) and WGS in detection of STEC will be discussed further below.

1.8.1 Real-time PCR

Real-time PCR is a system that detects polymerase chain reaction (PCR) products as they are amplified (enumeration) and is rapidly replacing traditional PCR since the starting DNA concentration of the target is determined [93]. Also, since amplification is in a closed tube system, there is no need for post-amplification manipulation of the amplified target, reducing protocol run time and the possibility for contamination. The process is based on dyes that emit fluorescence as the deoxyribonucleic acid (DNA) target is amplified which is converted into enumeration, based on a set of standards.

Over the past years, qPCR has become an important tool for universal STEC screening in clinical, food and environmental samples since, they are rapid to perform [59].

There are two reference methods based on qPCR for detection of the top STEC, one in Europe and one in the USA; they both examine the presence of *stx*$_1$, *stx*$_2$ and *eae* genes and the following serotypes: O157:H7, O26:H11, O145:H28, O111:H8, and O103:H2 and their non-motile variants [59]. The USA also test for O45 and O121 serogroups. Samples positive for *stx*, *eae* and target serogroups are then further processed to isolate the STEC from the sample and after isolation are re-confirmed by qPCR for presence of STEC genes used for screening.

Various studies have used qPCR methodology to identify STEC. Verhaegen et al. (2016) [94] evaluated a method to detect STEC pathogens in food and qPCR identified STEC in 100% of both experimentally inoculated ground beef, lettuce and naturally contaminated carcass swabs and, ~90% of STEC in experimentally inoculated soy bean sprouts. Vallières et al. (2013) [91] compared 3 different STEC detection methods, qPCR, an enzyme immunoassay and culture isolation and, they found that qPCR combined with cultured-based methods increased STEC detection by 320% compared to culture-based assays alone. They also noted that non-O157 STEC would have been missed if only O157 culture methods were used, leading to implementation of qPCR as part of their testing. Balière et al, (2016) [95] used qPCR to evaluate risk assessment of STEC and EPEC by examining virulence genes of these pathogens in shell fish. Based on published research data and official detection standards, qPCR has a wide range of applications and is a valuable tool in STEC detection and is used routinely to study these pathogens.

1.8.2 Droplet Digital PCR

DdPCR is the result of advancement in qPCR technology, and builds on the same principal of quantification using fluorescent dye chemistry [96]. The main difference between the technologies is that the sample is partitioned into individual reaction compartments (droplets) prior to running the PCR. After running the PCR each droplet is assigned as positive or negative, based on fluorescence. Using Poisson statistics, the software calculates the concentration of the DNA template. Contrary, to qPCR which is a relative quantification tool, ddPCR is an absolute quantification tool without the need for standards, which are needed in qPCR. DdPCR can also be used with Evagreen an intercalating dye, similar to SYBR green, removing the need for a probe; therefore any established PCR assay can be quickly adapted to ddPCR technology.

DdPCR has also been added to the STEC detection tool repertoire. McMahon et al. (2017) [97] developed a ddPCR assay designed to identify *eae* and *stx* genes in intact cells. The assay can differentiate between *eae*-positive, *eae*-negative and cells positive for both *eae* and *stx*. QPCR STEC screening has been shown to amplify false positive PCR fragments due to the inability to separate cells that were positive only for one gene. McMahon et al. (2017) [97] was able to develop an assay that removed these false positives. Verhaegen et al. (2016) [98] compared qPCR to ddPCR to quantitate STEC in bovine feces and found that ddPCR quantified target gene fragments in samples equally well compared to qPCR, but in the absence of a standard curve. Furthermore, ddPCR was less affected by inhibitors, suggesting this tool has potential to analyze complex sample matrices such as feces. Yang et al. (2017) [99] used ddPCR to quantitate qPCR standards to enhance qPCR quantification assays, due to the superior accuracy of ddPCR

quantitation over qPCR. Overall, recent studies demonstrate that ddPCR is becoming a promising tool to identify and quantify STEC in a wide range of matrices without the need for internal standards combined with a high tolerance to inhibition.

1.8.3 Whole Genome Sequencing

Advances in Next Generation Sequencing have increased speed and reduced costs of WGS by using benchtop instruments that are easy to operate, leading to changes in how food pathogens are characterized [92, 100, 101]. Traditional methods for STEC isolate characterization are reported to take between 1 -3 weeks to complete, while WGS data is available within days of running an assay [92]. WGS is currently replacing conventional Pulse Field Gel Electrophoresis (PFGE) technology [100]. Often, analysis of PFGE data is unable to pin-point the source of an outbreak due to lack of discriminatory power in distinguishing isolates, while WGS has been shown to overcome these limitations [101]. WGS is also not without limitations, mainly due to the complexity in analyzing the data generated [101]. Currently various USA administrations are working to build a "one-health" WGS database for rapid identification of foodborne pathogens such as STEC to overcome this limitation [100].

Holmes et al. (2018) [102], examined the feasibility of using and standardizing WGS STEC data across different reference laboratories and found that WGS data was highly reproducible, suggesting that standardization is conceivable. The study did highlight that the data produced can be complex and that there is a need to have standard databases with user-friendly bioinformatics for laboratories without this expertise. Another study by Lindsey et al. (2016) [92] used WGS to characterize STEC in a straightforward workflow, from serotyping, resistance and virulence profiles

to streamline standard detection protocols. WGS also had the advantage of providing more information than standard methods; for example, conventional methods in place only detected 5 virulence targets and 9 antimicrobial resistance targets, while WGS detected over 100 genes associated with virulence and resistance [92]. Overall, WGS has the potential to streamline laboratory work, gather more information on STEC virulence genes and help with outbreak detection but there is a need to streamline data analysis for data to be shared across laboratories [103].

1.9 Control of STEC – Preharvest Intervention

In North America, STEC are either considered a top foodborne health burden or a major cause of foodborne illness [16, 104] and they are a substantial foodborne pathogen worldwide [28]. Several postharvest intervention strategies have been applied successfully to reduce farm to fork contamination of STEC but, in order to further secure food supply preharvest interventions are also being investigated [16] and will be briefly discussed below.

Preharvest interventions help break the infectious chain by using a multi-hurdle approach to prevent disease spread. One of the main preharvest interventions for any pathogen is use of good farm and feedlot hygiene practices [16, 105, 106].
Access to clean food, water and, bedding are crucial to help reduce STEC spread between animals. Other factors to consider are biosecurity and cattle grouping [16]. Farms need to minimize the risks for cattle interacting with wild animals and, consider that insects and wild birds may be vectors that can carry STEC from farm to farm. Also, how cattle are grouped together may affect STEC spread and, the age of animals and cattle density, should be considered in management practices. Feed additives and

diet may also have an effect on STEC colonization in cattle; for example, cattle grown on a dry-rolled corn and forage diet shed less O157 than cattle on a grain-based diet [106] and, feed additives such as essential oils have been shown to reduce numbers of STEC O157 in-vitro [105]. Vaccines have also been developed to control STEC but are not widely used [106] and, in some cases have to have shown debatable efficacy [107].

Another area of research within the cattle industry is to use "probiotic/direct-fed microbials (DFM)/competitive exclusion (CE) bacteria" to control STEC and enhance food safety [105]. This approach involves the use of probiotic bacteria to out-compete pathogens such as STEC. Non-pathogenic *E. coli* have been used as a "probiotic, DFM, CE" and studies have shown potential in mitigating STEC [108, 109]. CE is based on the principle that rivals competing for limited resources cannot coexist and, that species with an advantage will eliminate their competitors [110, 111]. Bacterial competition will be further discussed in the following section.

1.10 Bacterial Competition

Competitive interactions between bacteria can happen in any environment where resources and space are limited, [112, 113], including those that harbor *E. coli*. Competition between bacteria can be exploitative or indirect, where one strain outcompetes with another without engaging a competitor [112]. In exploitative interactions, one bacteria restricts access to space and nutrients (metabolizes nutrients quicker or has a shorter replication time), effectively outcompeting the competition. However, excellent exploiters are not always competition winners and weaker bacteria can out-compete a great exploiter using interference-based competition. In interference

or direct based competitions bacteria engage/antagonize their competitors using contact independent and dependent mechanisms [112, 114, 115].

Contact independent mechanisms use diffusible complexes such as quorum sensing molecules which can affect signaling cascades or compounds such as bacteriocins that damage and/or kill competitors (Figure 1.4) [114-117]. Contact-dependent interactions arise through various mechanisms such as contact-dependent growth inhibition (CDI) or secretion systems (like type VI secretion system, T6SS) which require a connection with the competitor to translocate or inject effectors (Figure 1.4). Interference competition mechanisms can vary in their target/specificity range. Bacteriocins and CDI systems usually target only closely related bacteria due to the need of specific target receptors on the competing cells [114, 116, 118]. However, other mechanisms such as the T6SS do not utilize specific receptors and use physical force to inject effectors directly into the competitor [116] and has been shown to target a broad range of bacteria or even eukaryotic cells [114]. Research has shown that *E. coli* possess both contact dependent and independent interference mechanisms [118-120] and potentially strong *E. coli* competitors may out-compete related strains such as STEC using interference strategies.

The idea to utilize non-pathogenic *E. coli* to mitigate pathogens is not novel and in fact over 100 yrs. ago a probiotic *E. coli* (strain Nissle) was recovered from the gut of a German soldier and has since been used to mitigate human intestinal pathogens [121]. Similarly, another study used non-pathogenic *E. coli* to mitigate STEC O157:H7 shedding in cattle [108] and another revealed that non-pathogenic *E. coli* reduced

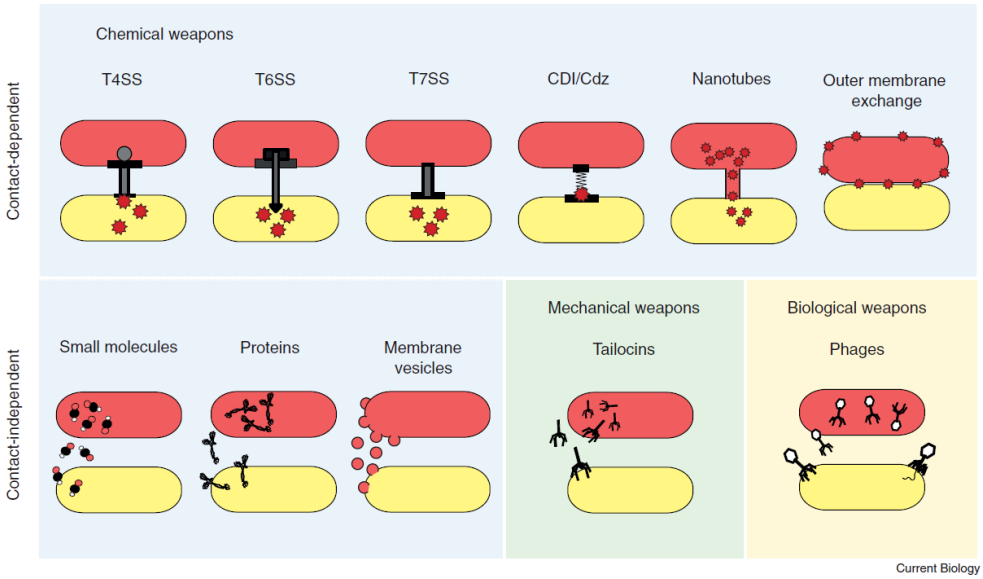


Figure 1.4. Diagram depicting known contact independent and dependent interference mechanisms in bacteria. Type IV secretion systems (T4SS), are mainly recognized to translocate DNA and proteins in Gram-negative bacteria but have also have a role in toxin-targeting during competition. Type VI secretion systems (T6SS) inject toxins in competitors by using a repurposed contractile phage tail. Type VII secretion systems (T7SS) have a role in toxin delivery in Gram-positive bacteria. Contact-dependent growth inhibition (CDI) systems (and similar Cdz systems) use filamentous proteins to attach and translocate toxic molecules into the competitor. Nanotubes bridge the cytoplasm between bacteria to directly transfer toxins and other molecules. Outer membrane exchange (OME) deliver toxic proteins present in the outer membrane by cell to cell contact with competitors. Small molecules are toxins less than 10kDa that are released and disseminated to target cells. On the other hand, proteins are diffusible toxins bigger than 10 kDa. Membrane vesicles can kill competitors by delivering toxins or enzymes to target cells. Tailocins are phages that lack the capsular head and physically puncture the membrane of their target. Phages are released by competitors and kill their targets but not clonemates that carry the phage.

* Both the Figure and caption are adapted from Granato et al, 2019 [115].

shedding of STEC O26 and O111 in cattle [109]. Colicins and microcins are bacteriocins produced by *E. coli* [122] and characterization of probiotic *E. coli* strain Nissle demonstrated that it produces 2 different types of microcins coined H47 and M, which are most likely antagonistic factors making Nissle an effective competitor [121]. In fact, a study examining how Nissle mediates competition in the inflamed mouse intestine demonstrated that the Nissle microcins are essential for this bacterium to out-compete

bacteria under these conditions [123]. Colicins and microcins are an effective tool in *E. coli's* competitive interactions and have potential to be used in STEC mitigation strategies.

1.11 Antimicrobial Molecules: Colicins and Microcins

Competition is one of the driving forces in microbial interactions [124] and interference strategies, such as contact independent bacteriocins have potential to be used to mitigate pathogens [125]. As antimicrobial resistance (AMR) is one of the most pressing health crises currently facing the world, and it presents an immediate threat to socioeconomic aspects globally, novel substances with antimicrobial properties, such as bacteriocins, are being investigated as alternatives to antibiotics. One of the main methods used to screen for production of antimicrobials is the overlay assay [126]. However an innovative method was developed with the added benefit of a physical barrier between competitors to identify production of contact independent antimicrobials such as colicins and microcins [127].

1.11.1 Colicins

Colicins are high molecular weight proteins that range from 25 to 80 kilodaltons (kDa) in size [117] and are lethal to related bacteria [128, 129]. Colicins are plasmid encoded and the colicin operon consists of 3 genes; i.e., the colicin gene, immunity gene and lysis gene [130, 131]. Colicin production has a high cost since release of the colicin results in cell death of the producer but benefits the overall population by removing competitors for space and nutrients [131]. Colicins induce cell death in their competitors via three mechanisms: I) Pore forming, II) Nuclease activity, and III) Peptidoglycanase activity [117]. Pore forming colicins create a pore in the target bacteria's cell membrane,

which results in cytoplasm leakage and loss of electrochemical gradients. Nuclease colicins degrade DNA and ribonucleic acid (RNA) in the target cell, using DNAse, 16S RNAse and tRNAse. Peptidoglycanase colicins digest peptidoglycan precursors, inhibiting peptidoglycan synthesis.

1.11.2 Microcins

Microcins are much smaller than their colicin counterparts and are less than 10 kDa in size [132, 133]. Microcins are also characterized as being highly resistant to proteases, high temperature and extremes in pH [117, 133]. These ribosomally synthesized peptides form a structurally heterogeneous class of bacteriocins but overall share a conserved genetic organization [133, 134]. Typically, the microcin gene clusters found either on plasmids or the chromosome, encode the microcin precursor, secretion factors, immunity proteins and modification enzymes (if needed for microcin processing) [134]. Contrary to colicins, microcins do not encode a lysis protein but use ABC like-transporter systems or efflux pumps encoded by their gene clusters to export from the producer [133, 134]. Furthermore, once exported microcins employ a “Trojan horse” strategy to gain entry into the target cells either by mimicking essential molecules or disguising themselves as a harmless nutrient. Once inside the target cell, microcins induce cell death by affecting vital enzymes in the cytoplasm, targeting DNA/RNA structure and synthesis or interacting with the inner membrane [134].

CHAPTER II: Hypothesis and Objectives

Emerging food-borne pathogens are a global threat. To protect human health and to mitigate virulent bacteria along farm-to-fork supply lines, relevant surveillance data and monitoring systems are mandatory. Currently, monitoring protocols focus on the detection of O-antigen gene fragments or immuno-assays found associated with the 7 most prevalent Shiga-toxin producing *E. coli* (STEC). However, with vast genomic plasticity and extensive global gene transfer, many potential emerging STEC would remain undetected by standard protocols. Furthermore, most of the detection protocols use conventional PCR assays to monitor STEC and it is unknown if: 1) qualitative data and quantitative data follow the same trends in seasonality and 2) if advanced molecular technologies can be utilized to monitor STEC. Additionally there is a lack of efficacy in current STEC mitigation strategies. Alternate mitigation strategies are needed to alleviate STEC along the farm-to-fork continuum or for therapeutics. Use of probiotic bacteria that out-compete STEC has been suggested to help suppress these infections but further research is needed to elucidate the full potential of such strategies, especially with the global emergence of antimicrobial resistance.

The hypothesis of this study is that advanced molecular technologies such as ddPCR could be used as a tool to monitor emerging STEC and that non-pathogenic *E. coli* can be used to mitigate infections by pathogenic *E. coli* in livestock or as human therapeutics.

This study was separated into two parts, the first part focused on gathering data on the proportion of STEC present in Alberta beef cattle and surveillance of *E. coli* O178, an emerging STEC in South America and Europe in Alberta beef cattle using ddPCR. The second part was focused on identifying a strong non-pathogenic *E. coli* able to out-

compete various STEC in a series of competition experiments and to identify the mechanism used by these bacteria to survive. Using a metagenomic DNA sampling pool from approx. 80,000 beef cattle at slaughter as well as a pool of approx. 1000 *E. coli* strains isolated from feces samples collected over two years at two slaughterhouses in Southern Alberta the main objectives were to:

Part I:

I. Determine the prevalence of *E. coli* O178 by developing a conventional PCR assay to detect O178.

II. Develop a ddPCR assay to enumerate total *E. coli* specific gene fragments in order to evaluate the quantity of serogroup and toxin specific gene fragments for O157, O178, stx_1 and stx_2.

III. Determine the distribution and relative abundance of stx_1 and stx_2 in the sampling pool compared to STEC O157.

Part II:

I. Investigate non-pathogenic *E. coli* for their ability to out-compete STEC.

II. Determine if competition is exploitative or interference based.

III. Characterize identified competitive mechanisms to elucidate the potential of using competition interactions to mitigate STEC.

CHAPTER III: Quantitative surveillance of shiga toxins 1 and 2, *Escherichia coli* O178 and O157 in feces of western-Canadian slaughter cattle enumerated by droplet digital PCR with a focus on seasonality and slaughterhouse location

3.1 Introduction

Escherichia coli are commensal organisms found in the gastrointestinal tract of both humans and livestock and a subgroup has been linked to human diseases. Certain pathogenic *E. coli* cause extra intestinal illnesses (e.g. urinary tract infections) and various other groups such as Shiga toxin-producing *E. coli* (STEC) may cause gastrointestinal diseases [24]. All STEC produce at least one Shiga toxin [29] and can cause illness ranging from mild diarrhea to severe infections such as hemolytic uremic syndrome or hemorrhagic colitis [31, 32], and have been linked to both outbreaks and sporadic cases of disease [69].

Cattle are a major reservoir for STEC [135] and are often implicated as the origin of entry into the food chain [35]. Cattle that shed $>10^4$ CFU/g of *E. coli* O157 have been coined "super-shedders" [136] and are deemed to be a major contributor to animal-animal transmissions [36]. A Scottish study found that 9% cattle were "super-shedders" but accounted for >96% of the *E. coli* O157 shed by the group [137]. Identifying "super-shedders" in a herd can be problematic as high levels of shedding can be intermittent with an animal originally negative for *E. coli* O157 becoming an O157 positive super-shedder [138] and identified O157 "super-shedders" no longer super-shedding after 6 days of testing [139]. Ultimately, super-shedding is not limited to O157 as STEC O26 "super-shedders" have been identified in the past [136].

Escherichia coli O157:H7 has been considered the most virulent serotype associated with outbreaks and sporadic STEC-related disease [29], but an increasing

number of non-O157 STEC serogroups have been linked with human diseases [32]. In addition to O157, in 2011, the USDA-FSIS made testing mandatory in beef products for the top six non-O157 serogroups (O26, O45, O103, O111, O121 and O145) [64]. In Argentina, while O157:H7 is often implicated in human disease [32], it has not been as frequently isolated from cattle compared to reports in other countries and non-O157 STEC are more predominant [65]. In both Argentina and Germany, the serogroup O178 isolated from beef cattle has been linked increasingly with human disease [69]. Both *stx*$_1$ and *stx*$_2$ have been found in O178 isolates, although *stx*$_2$ is more common, with O178 part of a group of virulent LEE-negative STEC [65, 69, 140].

Currently, conventional PCR assays are used to monitor non-O157 STEC by amplifying gene fragments to detectable concentrations. Novel technologies, like droplet digital PCR (ddPCR) have been reported to be an improvement compared to real-time PCR assays, offering absolute quantification without the need for internal standards [96].

A previous two-year study by our laboratory characterized the top-7 serogroups present in western Canadian cattle before slaughter [141]. The first objective of the current study was to examine the presence of *E. coli* O178 in Canadian cattle based on our sampling pool. Secondly, to develop a ddPCR assay to enumerate total *E. coli* specific gene fragments to compare serogroup and toxin specific gene fragments for O157, O178, *stx*$_1$ and *stx*$_2$ as a proportion of the total *E. coli* population and thirdly, examine the distribution of *stx*$_1$ and *stx*$_2$ versus O157 to correlate high enumerations of *stx*$_1$, *stx*$_2$ and O157.

3.2 Materials and Methods

3.2.1 Bacterial DNA

Over a two-year period, metagenomic DNA was isolated after enrichment from 1,773 pooled feces samples collected from transport trailers containing up to 45 beef cattle at 2 slaughterhouses (**A** = 928 and **B** = 845) located 200 km apart in Alberta, Canada. In short, fecal samples were mixed by hand and a 15 g subsample was then mixed with 135 mL EC broth (EMD Millipore) using a Seward Model 400 stomacher (Cole-Palmer) at 230 rpm for 1 min. Fecal suspension (10 mL) was then transferred to a sterile culture tube and incubated for 6 h at 37°C. A 1 mL aliquot of the enriched culture was centrifuged at 8,000 X g for 10 min before extraction of DNA from the pellet using the NucleoSpin Tissue Kit (Macherey-Nagel) as described previously [141].

3.2.2 O178 Screening by Conventional PCR

PCR was performed on metagenomic DNA samples from individual enrichments using a multiplex PCR with two standalone primer sets (200nM) targeting the O178 O-antigen gene cluster (Table 3.1). HotStarTaq polymerase (Qiagen) was used with the following cycling conditions: initial denaturation: 95°C– 5 min, 35 cycles: 95°C– 30sec, 59°C– 45sec, 72°C and final extension: 72°C– 5 min. All PCRs were carried out with 2µl template on Bio-Rad cyclers C1000 or T100 and amplicons were visualized with GelRed stain using a Molecular Imager GelDoc-XR+ (Bio-Rad).

3.2.3 Droplet Digital PCR

A selected subset of 168 samples from O178-positive enrichments were screened by ddPCR for absolute quantification of O178, O157, total generic *E. coli* (GEC) using the beta-glucuronidase gene, stx_1 and stx_2. The selection included 21 samples each from

Table 3.1. Primer sequence and amplicon size for O178 conventional PCR screening and for Evagreen droplet digital PCR assays

Primer	Primer Sequence (5'-3')	Size	Reference	Assay
O178 - 3	CCAGAGCTAAACTCAGAGGGG	112 bp	this study	O178 Screening
O178 - 4	GTGTGTTGAGTGTTGGCTCA			
O178 - 5	TCGGACGTATTTGCTGGCGCT	138 bp	this study	O178 Screening and ddPCR
O178 - 6	TCTGGGGGTCATAATTCAACTGGT			
O157 - F2	AGGGGTTGTATGCTCGTTGT	121 bp	[142]	ddPCR
O157 - R2	TGGAACACCTTCAACTTGCTCT			
GEC - uidA	TGGTAATTACCGACGAAAACGGC	147 bp	[143]	ddPCR
GEC - uidA	ACGCGTGGTTACAGTCTTGCG			
stx_1 - TF	CGCAGTCTGTGGCAAGAGCGAT	260 bp	this Study	ddPCR
stx_1 - TR	TGCCACGCTTCCCAGAATTGCAT			
stx_2 - F	ACTGTCTGAAACTGCTCCTGTG	307 bp	[142]	ddPCR
stx_2 - R	CGCTGCAGCTGTATTACTTTCC			

Note: GEC denotes: Generic *Escherichia coli* and *stx* denotes: shiga toxin

Summer I (June, July and August 2013), Winter I (December 2013, January and February 2014), Summer II (June, July and August 2014) and Winter II (December 2014, January and February 2015) for both slaughterhouses. The ddPCR used the EvaGreen assay as per ddPCR Applications Guide (Bio-Rad) using 2µl of template and the following PCR (QX200 EvaGreen ddPCR Supermix, Bio-Rad) conditions: Enzyme activation: 95°C–5 min, 40 cycles: 95°C–30sec, 60°C–60sec, signal stabilization: 4°C–5 min, 90°C–5 min. The primer concentration was 100nM (Table 3.1). The amplicons were read on the droplet reader (QX200, Bio-Rad) and analyzed using Quantasoft software (Bio-Rad).

3.2.4 Droplet Digital PCR analysis

Proportion (in %) was calculated based on the total number of positive amplicons for O178, O157, *stx*$_1$ and *stx*$_2$ compared to total GEC amplicons (Appendix II, Figure A2.1.). *Stx*$_1$, *stx*$_2$ and O157 amplicon distribution was determined by plotting *stx*$_1$, *stx*$_2$ enumerations versus O157. For *stx*-related contrast analyses, the total amount of O157 was reduced based on a recent finding that 23% of O157 isolates in the sampling pool lacked *stx* genes (Stanford—unpublished data).

3.2.5 Statistical Analysis

Conventional PCR results for *E. coli* O178 were examined for seasonal prevalence and difference between slaughterhouses, as determined by a generalized linear mixed model (Proc Glimmix, SAS 9.3) using a binomial distribution. Numerical data generated by ddPCR for GEC, O178, O157, *stx*$_1$ and *stx*$_2$ were examined for normal distribution and the data were log transformed prior to analyses. Seasonality, slaughterhouse, year, and interactions were determined for GEC, O178, O157, *stx*$_1$ and *stx*$_2$ using a mixed linear model (Proc Mixed, SAS 9.4). *P* values < 0.05 were considered significant.

3.3 Results

3.3.1 Conventional PCR Screening of Metagenomic DNA for O178

Screening DNA from 1,773 enrichments identified 873 (50%) samples positive for both O178-specific PCR fragments compared to previously reported 1,378 (79%; n = 1,749) *E. coli* O157 PCR positives within the same sampling pool [141]. Primers specific for the O178 O-antigen gene cluster showed no cross-reaction with the top 7 STEC (data not shown). O178 was identified across all seasons at both sampling sites

in Alberta (Table 3.2) and differed in seasonal prevalence ($P = 0.0001$) with O178 presence higher in winter compared to summer. Prevalence of O178 also differed between the two sampling locations and was lower at slaughterhouse **A** than **B**, ($P = 0.0004$). Based on these results, 21 samples from each sampling location (slaughterhouse) and season (summer and winter) were selected for subsequent ddPCR analysis.

3.3.2 Droplet Digital PCR

3.3.2.1 Total Numbers of GEC, O178, O157, *stx*$_1$ and *stx*$_2$.

Fragments representative of GEC were enumerated in the original DNA samples and analyzed using Quantasoft (Figure 3.1). On average, GEC numbers were 2-times higher at slaughterhouse **B** (Figure 3.1) compared to **A** ($P < 0.05$). The O178 amplicons were on average 1.5-times higher at site **A** compared to **B** ($P < 0.05$), which is inconsistent with the findings of the conventional O178 PCR screening (Table 3.2). O157 ddPCR quantification revealed higher numbers at slaughterhouse **A** compared to **B** ($P < 0.05$) (Figure 3.1). Either *stx*$_1$ and/or *stx*$_2$ gene fragments were detected in all samples (n = 168) except four winter samples. Across all samples, *stx*$_1$ and *stx*$_2$ were 7- and 2.8-times higher at site **B** compared with **A**, respectively (Figure 3.1; $P < 0.05$).

Table 3.2. Percentage of conventional PCR *Escherichia coli* O178 positives by season and slaughterhouse from 1,773 enrichments.

		Mean (% Positives)	Std. Error	Probability	Total Positives
Season	**Summer**	35.6[a]	0.024	**0.0001***	181
	Fall	33.0[a]	0.023		150
	Winter	71.5[b]	0.020		276
	Spring	50.5[c]	0.028		266
Slaughterhouse	**A**	43.1[a]	0.019	**0.0004***	374
	B	52.4[b]	0.018		499

Note: [a, b, c] Means with different superscripts within category differ by probabilities shown.

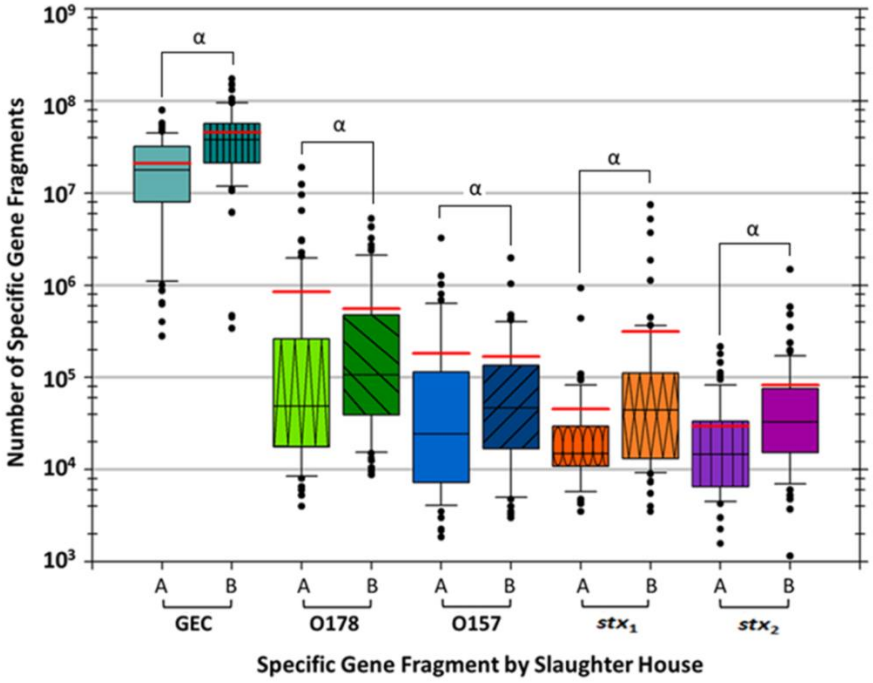


Figure 3.1. Total number of Generic *E. coli* (GEC), O178, O157, shiga toxin (*stx*) 1 and 2 specific gene fragments in samples from Slaughterhouse **A** or **B** (calculated using droplet digital PCR).
In each comparison between slaughterhouse **A** and **B** there were significant differences in enumeration of gene fragments, denoted by symbol α.
Note: *The red line is the average number of GEC, O178, O157, stx_1 and stx_2 at each slaughterhouse and the black line in the boxes is the median.

3.3.2.2 Proportion of O178, O157, *stx*$_1$ and *stx*$_2$.

The individual O178, O157, *stx*$_1$ and *stx*$_2$ numerical data were used to calculate the proportion of gene fragments of O178, O157, *stx*$_1$ and *stx*$_2$ in the original DNA sample and compared to GEC (Figure 3.2). The proportion of O178 did not differ between slaughterhouses and ranged from ≤1% to 44% at the **A** site and ≤1% to 27% at the **B** site. In contrast, O157 proportion of GEC was higher at **A** ($P < 0.05$), ranging from ≤1% to 12% at **A** compared with ≤1% to 7% at **B**. The average proportion of O178 (2.8%) was greater ($P < 0.05$) than O157 (0.6%) (Figure 3.2) and varied by slaughterhouse and season (Figure 3.3). The *stx*$_1$ proportion did not differ between slaughterhouses and ranged from ≤1% to 23% at **A** and ≤1% to 28% at **B** (Figure 3.2). Likewise, *stx*$_2$ proportions were similar ($P > 0.05$) at **A** (≤1% to 13%) and at **B** (≤1% to 9%). In total, the average *stx*$_1$ proportion in all samples was 1.4%, compared to 0.5% for *stx*$_2$ (Figure 3.2).

The proportion of O178 did not differ significantly ($P > 0.05$) between seasons (Figure 3.3). In contrast, proportions of O157 versus GEC were higher ($P < 0.05$) in winter compared to summer at both sites (Figure 3.3). The proportions of *stx*$_1$ were also higher ($P < 0.05$) in winter compared to summer for both slaughterhouses while the proportion of *stx*$_2$ did not differ significantly ($P > 0.05$) across seasons (Figure 3.3).

3.3.2.3 Distribution of *stx*$_1$ and *stx*$_2$.

Examining the *stx*$_1$ and *stx*$_2$ enumerations versus the reduced (minus 23% non-STEC O157) O157 enumerations identified 67% samples where either one or both toxins had higher enumerations than could be accounted for by O157 (Figure 3.4).

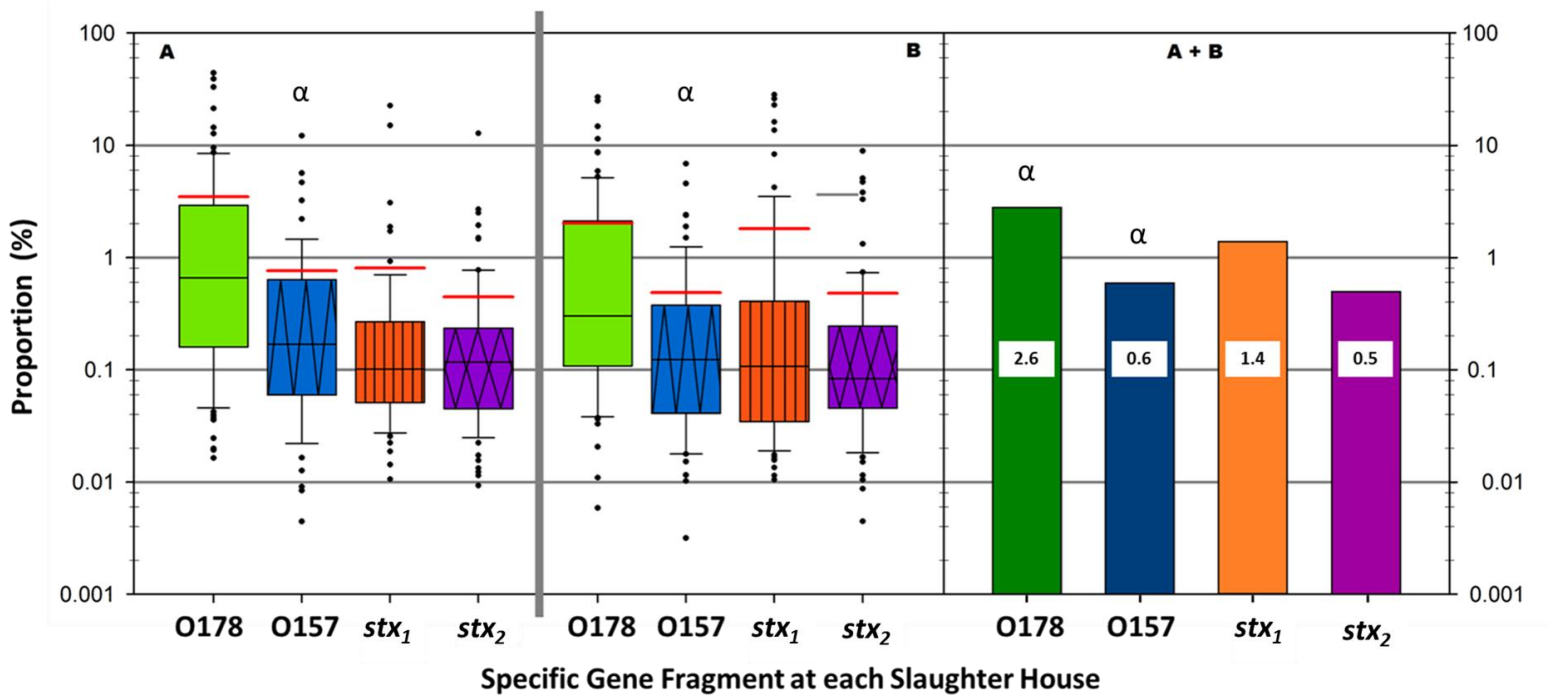


Figure 3.2. Proportion of O157, O178, shiga toxin (*stx*) 1 and shiga toxin 2 specific gene fragments in samples for **A** and **B** slaughterhouses calculated using droplet digital PCR enumerations and comparison of the average proportion of the target gene compared to generic *Escherichia coli* (GEC) in the samples.
Note: *Proportions are based on the total *E. coli* counts. **Red line is the average proportion for the group and black line in the boxes is the median. *** Symbol: α denotes a significant difference between O157 at site A and B and between the average proportion of O178 and O157 ($P < 0.05$).

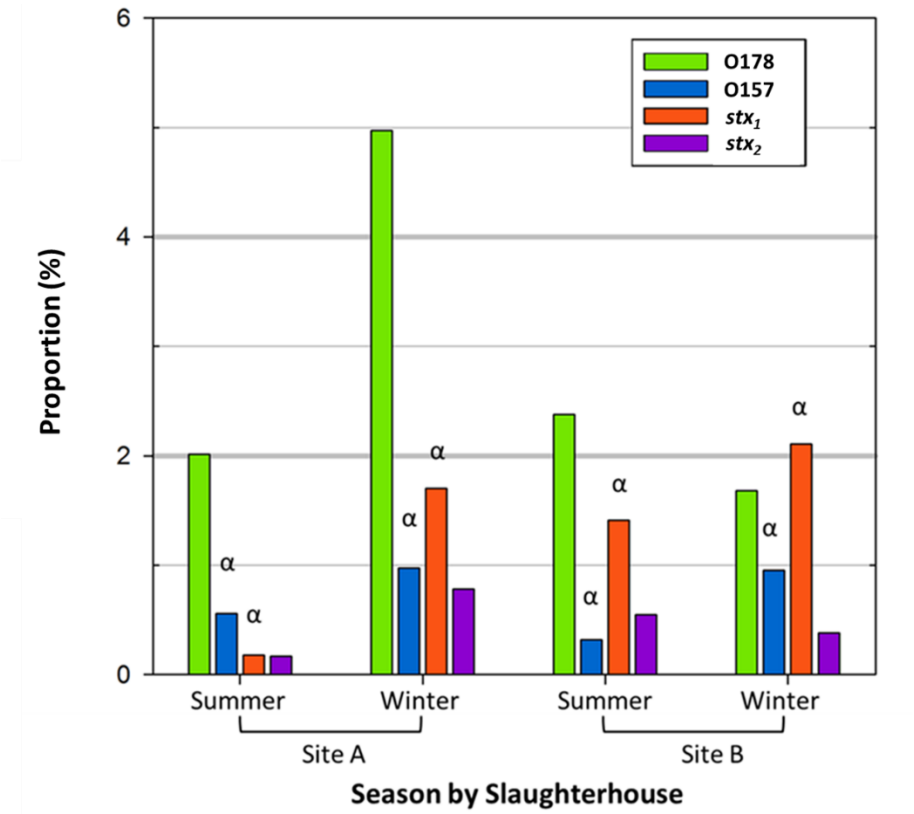


Figure 3.3. Comparison of the average proportion for O178, O157, shiga toxin (*stx*) 1 and 2 for the two sampling sites, **A** and **B** for each season based on the total generic *E. coli* count.
Note: * Symbol: α above specific gene fragment denotes a significant difference in enumerations between summer and winter for that target ($P < 0.05$).

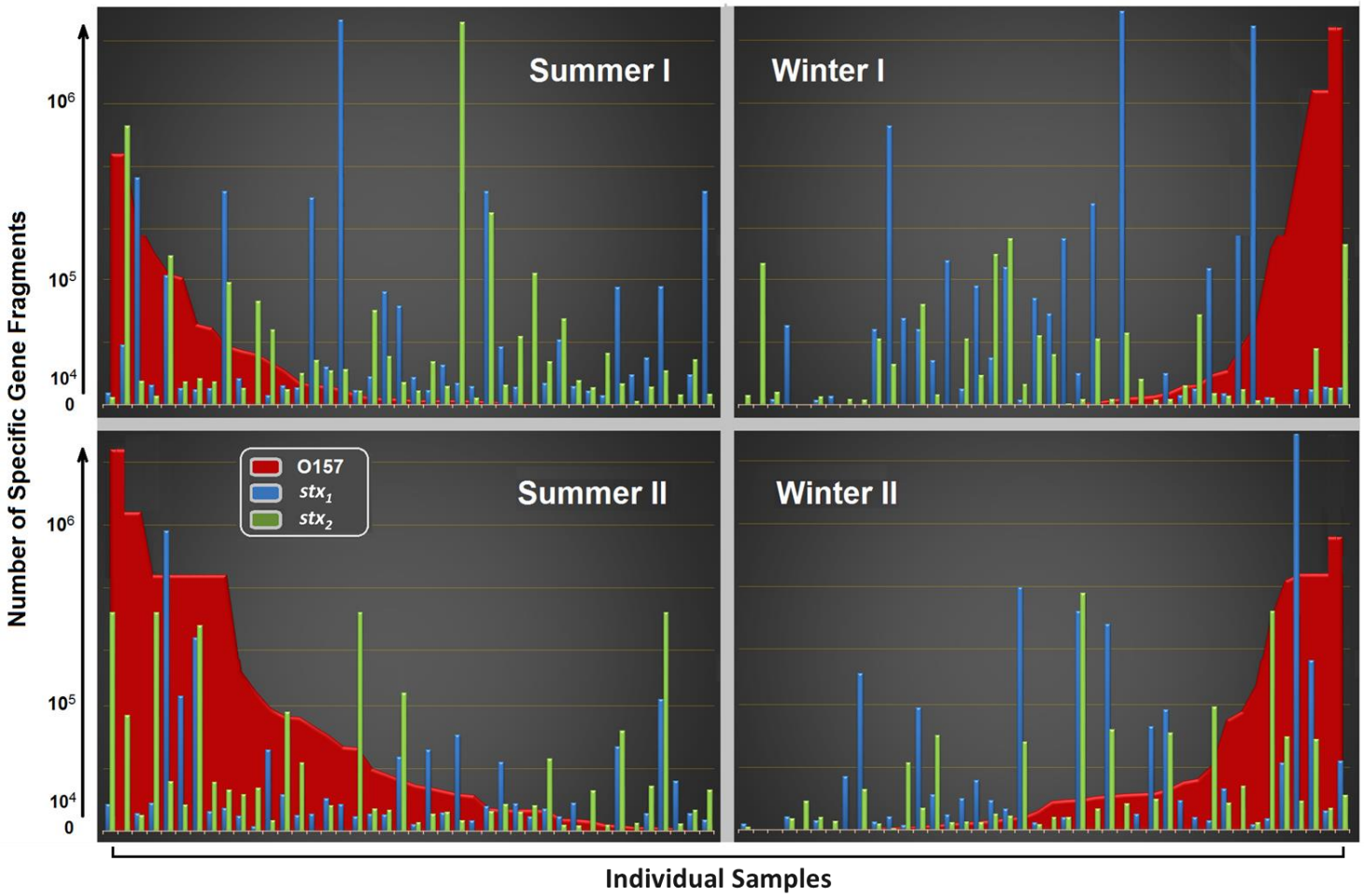


Figure 3.4. Total number of shiga toxin (*stx*) 1 and 2 specific gene fragments versus reduced O157 enumerations (-23% for non-STEC O157) for summer and winter across the sampling period.
Each peak denotes a sample and the color (red = O157, blue = stx_1 and green = stx_2) indicates the target.

3.4 Discussion

3.4.1 Conventional PCR Screening of Metagenomic DNA for O178

In the pool of samples analyzed, *E. coli* O178 was identified in approximately half of samples (Table 3.2) as compared to O157 detection in 79% of samples. In contrast, only 7% of bovine rectum contents were positive for O178 in Argentina [144]. Masana et al. [145] revealed STEC O178:H19 as the most prevalent serotype (10.9%) among 35 different non-STEC serotypes from cattle feces and carcasses in beef slaughterhouses in Argentina. Pooling the feces from up to 45 cattle in our study might be one explanation for detecting higher prevalence compared to samples from individual animals. However, diagnostic limits may have underestimated detectable cell numbers in previous studies and/or the conditions in Alberta may favour increased O178 proliferation.

The presence of O178 in Canadian cattle was not unexpected as O178 has been isolated from cattle in other countries including Argentina [144], Spain [30] and Germany [69]. A study examining food sources in Germany, Switzerland and France identified O178:H19 in 80% of beef products, 10% dairy products and 10% goats and/or goat products, suggesting cattle are a major reservoir for this serotype [146].

In the present study, the occurrence of O178 showed significant differences between slaughterhouses **A** and **B**, and between the summer and winter seasons, suggesting that O178 prevalence is affected by animal origin (location) and seasonality. Consequently, we sought to determine whether the prevalence of O178 among the entire *E. coli* population was shifting across seasons and locations. Therefore, a representative subset of samples were further analyzed to calculate the proportion of O178, O157, stx_1 and stx_2 versus total GEC populations by ddPCR and the distribution of stx_1 and stx_2 compared to O157.

3.4.2 Droplet Digital PCR

3.4.2.1 Total Numbers of GEC, O178, O157, *stx*$_1$ and *stx*$_2$.

We quantified and compared the total GEC in pooled cattle feces samples to the number of gene fragments from serogroups O178, O157 and *stx*$_1$ and *stx*$_2$ genes. Enumerations by slaughterhouse revealed that on average O178 and O157 were more numerous at **A** site, while GEC and *stx*$_1$ and *stx*$_2$ was greater at **B** site (Figure 3.1). Studies examining outbreaks [147] and infection [148] incidence of *E. coli* O157 in the United States identified a geographic trend with a greater occurrence in northern states compared to southern states. Contrary to Heiman et al. [147] and Sodha et al. [148] our data for feedlot origin at both slaughterhouses showed no discernable geographic differences between the animals at both locations but revealed divergences in feedlots shipping cattle to each slaughterhouse [141]. Ultimately, individual on-farm management factors may have a greater impact on the identified *E. coli* subtypes than does geographic location, although locational differences in prevalence of *E. coli* serogroups has been reported [149].

3.4.2.2 Proportion of O178, O157, *stx*$_1$ and *stx*$_2$.

Further analysis of the bacterial enumerations was used to compare the proportion of *E. coli* O178, O157, *stx*$_1$ and *stx*$_2$ to total GEC. Overall, the average proportion of O178 was 4-times higher compared to O157 across all samples (Figure 3.2). These results are in accordance with Tanaro et al. [144] and Masana et al. [145] which found O178 to be either the most prevalent or among the most predominant serogroups in cattle in Argentina. Our data indicate that in Alberta, populations of O178 are also more numerous than those of O157. Miko et al. [69] reasoned that cattle feedlot management

practices in Argentina may cause the emergence of O178 strains compared to grazing fed cattle. The greater proportion of O178 at both slaughterhouses compared to O157 might demonstrate a similar relationship with national standards in animal management selecting for specific microflora and/or suggesting feedlot animals are predisposed to greater O178 carriage compared to grazing animals. A review by Karmali [61], discussed that in North America infections related to STEC O157:H7 have decreased over the last two decades, mainly due to enhancements in food safety. However, Karmali [61] noted that despite these improvements, severe diseases associated with non-O157 STEC and emerging hybrid STEC strains are increasing. Ultimately, O178 may have always been relatively abundant, but were not noticed in earlier investigations.

More than 400 serotypes of STEC have been identified in cattle [30] and, both *E. coli* serogroups (O157 and O178) accounted for 3.4% of the total *E. coli* cells while the remaining 96.6% *E. coli* present were not tested for O-serogroup assignments (Figure 3.2). High throughput genome sequencing technologies [92] may substitute for current sub-typing gaps in the future.

The average proportion of stx_1 was 3-times greater than stx_2 across all samples (Figure 3.2) and reflects data on stx_1 being more frequently isolated from patients in Alberta [150]. Conversely, other studies [151-153], found stx_2 to be more prevalent in cattle samples, in Iran, Korea and the United States, respectively. Differences in proportion for stx_1 versus stx_2 may be geographically based or may be due to the sample size in this study as animals from different feedlots have dissimilar Shiga-toxin profiles [107].

Approximately 1.9% of the total *E. coli* population in our study carried either stx_1 and/or stx_2 (Figure 3.2). This leaves a reasonable cause for concern as an infectious dose for O157:H7 of 100 colony forming units (CFU) [154] is considerably lower than the STEC numbers identified in our samples. As virulence genes may be transferred to a previously *stx*-negative population [62] 98.1% of the GEC in our study were potentially available as hosts. A "new" STEC was recently seen in Germany when an enteroaggregative O104:H4 acquired the stx_2 gene resulting in a particularly virulent strain [63] and in France with the emergence of an O80:H2 hybrid [155].

Comparing the average proportion of *E. coli* O178 by season at each slaughterhouse showed no significant differences (Figure 3.3). A previous study [144] examined the prevalence of non-O157 in feces across different seasons and reported the highest prevalence of non-O157 STEC in winter (52.7%) compared to summer (36.1%). The proportion of O178 at slaughterhouse **A** followed a similar trend as the previous report [144] with greater proportions in winter compared to summer. The opposite trend was revealed at site **B**, although overall differences in seasonality were not identified. Food safety concerns for consumers are considered high during summer months due to increased O157 prevalence [156, 157] but our O178 numerical data revealed equal year-round numbers even though prevalence based on conventional PCR was higher for O178 in the winter than in the summer.

In comparison to O178, *E. coli* O157 proportion at both slaughterhouses was lower in summer versus winter (Figure 3.3). Our data contrasts with earlier studies [156, 157] which reported increased seasonal shedding of *E. coli* O157 during summer compared to winter which is thought to be correlated with increased O157 infections in

summer [148]. Our finding of increased O157 proportions during winter months do not support the elevated numbers of infections in summer which might also be attributed to factors such as warm ambient temperatures or food handling practices [148], and/or an increased consumption of barbequed/grilled meat products [158]. However, the previous reports determined presence/absence and did not enumerate the total O157 *E. coli* population. The discrepancies between qualitative and quantitative data require further attention to evaluate the potential of food safety risks.

Comparing the average *stx*$_1$ versus *stx*$_2$ proportion among the two sampling sites by season demonstrated that seasonality was associated with *stx*$_1$ (higher in winter, lower in summer) but not for *stx*$_2$ (Figure 3.3). Both of our results are contradictory to Dewsbury et al. [159] findings that *stx*$_1$ and *stx*$_2$ were only present in the summer while we found *stx*$_1$ and *stx*$_2$ were present in both seasons with *stx*$_1$ having significantly higher proportions in winter compared to summer. Dewsbury et al. [159] screened isolates found in feces after immunomagnetic separation, different from our study which examined the total DNA from all bacteria present in the collected feces. Comparing methodologies, Dewsbury et al. [159] supplemented media with antibiotics which may have altered the microflora detected in feces [160, 161].

3.4.2.3 Distribution of *stx*$_1$ and *stx*$_2$.

Examining the distribution of *stx*$_1$ and *stx*$_2$ compared to O157 demonstrated several high-*stx*-events across seasons which cannot be attributed to O157 (Figure 3.4) and likely belong in part to the non-O157 STEC identified by Stanford et al. [141]. Cattle termed “super-shedders” defecate >10^4 CFU/g of O157 [136] and are considered the main source of *E. coli* O157 contamination [162]. Data reviewed by Chase-Topping

et al. [162] estimated that 8–9% of cattle are "super-shedders" but account for ≥96% of the bacteria shed. The sampling days where toxin numbers exceeded O157 numbers suggests that virulent but unidentified O-groups are being shed at high concentrations (Figure 3.4). Enumeration data also suggests that O178 may be super-shed by individual cattle based on O178 being more prevalent at site **B** but in higher numbers at **A** site (Table 3.2 & Figure 3.1). "Super-shedders" are thought to have a disproportionate impact on transmission along the farm-to-fork continuum and super-shedding is not limited to O157 as STEC O26 "super-shedders" have also been identified [136]. The discovery of peaks of *stx* amplicons not attributed to O157 suggests that it may be worthwhile to identify STEC "super-shedders" using virulence markers instead of serogroups as super-shedding events with uncommon STEC may be missed.

Droplet digital PCR technology was used in this study to quantify the total bacterial populations as well as individual strains by absolute quantification of genetic markers. An earlier study [98] found that ddPCR provided absolute quantification of STEC without the need for standards and that ddPCR is robust to inhibition which is often a concern screening environmental samples and/or using qualitative assays. Another study [99] used ddPCR to measure the qPCR standards used to screen pathogen loads to enhance their quantification and stated that ddPCR had superior accuracy than qPCR. Here, ddPCR successfully quantified the total *E. coli* population as well as specific *E. coli* O-serogroups, stx_1 and stx_2 in feces samples. The individual proportions generated by ddPCR identified various differences in location and seasonality and demonstrated a different trend in seasonality for O157 other than previously reported and contradicted the prevalence data for O178 at Alberta slaughterhouses. DdPCR data also

identified peaks in *stx*$_1$ and *stx*$_2$ not associated with O157 that may indicate "super-shedders" of STEC other than O157. Data from ddPCR showed insight into the *E. coli* population pattern as part of the cattle microbiome composition. Future studies using ddPCR may further elucidate differences in trends between quantitative and qualitative data and help guide mitigation strategies for STEC by identifying periods of heightened virulence.

3.5 Conclusions

Our data show that O178 is a subset of the total *E. coli* population present in Alberta cattle feces. Both, *stx*$_1$ and/or *stx*$_2$ were present in almost all cattle feces tested. Our data suggest that besides virulent O157, STEC "super-shedders", not exclusively attributed to O157 exist. Overall, the data illustrate that food safety surveillance should focus on monitoring virulence factors instead of serogroup screening. Emerging pathogens are a global threat to the food industry, challenging current food safety protocols and taxing healthcare resources due to outbreaks and/or particularly virulent strains of *E. coli*. Pathogens are evolving by continuous host and environmental adaptation. Further understanding regarding their emergence using novel technologies may help elucidate approaches to reduce health risks along the farm-to-fork continuum.

CHAPTER IV: Competition among *Escherichia coli* strains for space and resources

4.1 Introduction

In 1934 the Russian biologist, Georgii Gause conceived that rivals for limiting resources cannot coexist and that one competitor would become "prey" and the other "predator", demonstrating this concept using yeast and protozoa [110]. This became known as the "Competitive Exclusion Principle" [111], which states "Complete competitors cannot coexist". Accordingly, in an environment where one species has an advantage such as increased growth rate, the species with the advantage (predator) will dominate over the long term and eliminate the weaker competitor (prey).

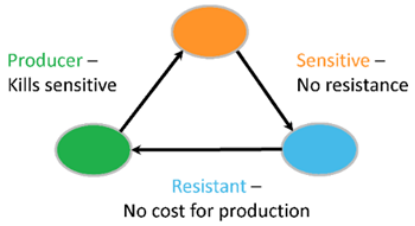
Environments populated by *Escherichia coli* are subject to competition pressures due to limited availability of space and resources. Bacteria use various weapons to ensure their survival [163] and ability to compete which can take place in any natural environment [112, 113]. Competition can be exploitative where a predator restricts nutrients and starves the prey [112]. Effective exploiters can prevail in competitions, but weak exploiters can also out-compete a vigorous exploiter using interference competition. Interference-based competition uses antagonistic factors produced by competitors such as toxins to prevent or remove competitors from their environment [112]. The competitive outcome depends on the dynamic of the competitive interactions, which is often referred to as the "rock-paper-scissors" game (Figure 4.1) [164].

Producers generate molecules such as antibiotics, specialized enzymes, and colicins that can kill, prevent growth and/or disrupt signal cascades [112]. However, there is a high cost to producing molecules such as colicins and in a two-strain system, removal of the sensitive strain provides an advantage to the producer (Figure 4.1 A,B) [165].

A

Competition	Winner	Loser
Producer vs Sensitive	Producer	Sensitive
Producer vs Resistant	Resistant	Producer
Sensitive vs Resistant	Sensitive	Resistant

B



C

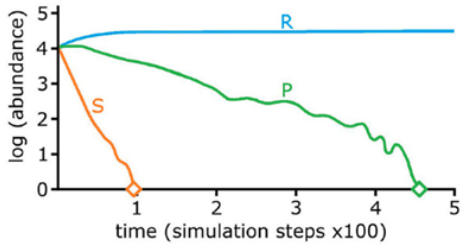


Figure 4.1. "Rock-Paper-Scissor" game of competition dynamics. **A** – Describes competition winners in two strain dynamic. **B** – Describes basis for winning or losing. **C** – Describes the competition winner in a three strain dynamic. Note: R = resistant, P = producer and S = Sensitive. * Adapted from ([164] (A), [166] (B) and [165] (C)).

In contrast, sensitive strains do not have any metabolic cost for either producing or resisting, providing a metabolic advantage in competitions with resistant strains, while succumbing in the presence of producers. Resistant strains, avoid metabolic costs associated with molecule production, but there are costs associated with resistance, which are less than production but higher compared to sensitive strains. In a three-strain dynamic with no spatial structure (well-mixed conditions, such as an aquatic environment) competitive advantage shifts to resistant strains (Figure 4.1 C) [165, 167].

Interference based competition can be divided into independent and contact-dependent methods [116]. Contact independent mechanisms rely on diffusible compounds such as bacteriocins and antibiotics to damage and/or kill competing bacteria

or signals such as quorum-sensing molecules to facilitate interactions between bacteria. Direct-contact interactions also occur with contact-dependent growth inhibition (CDI), which requires specific receptors on competing cells or type VI secretion systems (T6SS), which do not need specific targets [116, 118]. *E. coli* has been shown to possess all three types of interference-based competitive mechanisms [118-120].

Escherichia coli is a commensal bacterium and part of the gastrointestinal microbiota in humans and livestock. While many *E. coli* are considered harmless or beneficial, others such as Shiga-toxin producing *E. coli* (STEC) are virulent causing gastrointestinal diseases [24]. STEC are classified by the ability to produce at least one Shiga toxin (Stx) [29] and can cause severe infections such as hemolytic uremic syndrome or hemorrhagic colitis [31, 32].

Ruminants, especially cattle, are a major host of STEC and many technologies to control STEC in livestock have been evaluated including vaccines, direct-fed microbials, and tannins [107, 168, 169]. Ultimately, none of these has shown consistent efficacy. As well, treatment options for humans infected with STEC are limited as some antibiotics may increase Shiga toxin production and/or release by inducing the bacterial SOS response [170]. As dependable methods to control STEC in cattle and humans are not yet available, use of non-pathogenic *E. coli* predator strains to out-compete and control pathogenic STEC strains may have potential. Almost 100 years ago, *E. coli* strain Nissle was recovered from the gut of a soldier and has been subsequently used as a probiotic to mitigate intestinal infections in humans [121]. Similarly, another study reported *E. coli* O157:H7 mitigation in cattle after the use of a direct-fed microbial consisting of non-pathogenic *E. coli* strains [108].

Our primary objectives were to investigate competitive non-pathogenic *E. coli* strains isolated from cattle feces with the potential to control STEC such as O157:H7 and investigate the competitive mechanisms utilized by these predator strains and potentially identify if these strains were producers and/or resistant.

4.2 Materials and Methods

4.2.1. Bacterial Strains: Cultures, Media and Culture Conditions

All strains used in this study were isolated from feces collected from transport trailers of slaughter cattle at two Alberta slaughter plants [141] (Table 4.1). *E. coli* were streaked from glycerol stocks onto MacConkey Agar (MAC, BD, Sparks, NV, USA). Plates were incubated overnight (16–18 h) at 37 °C. A single colony was selected from each plate and inoculated into 10 mL *E. coli* broth (EC) (EMD Millipore, Etobicoke, ON, Canada) and incubated overnight at 37 °C statically (liquid competition) or with shaking at 150 rpm (other competitions).

4.2.2. Competition Experiments

Various *E. coli* strains were examined for competitive fitness using tests to identify strong candidates that prevented or modified growth of STEC.

4.2.2.1. Liquid Competition

The liquid competition was adapted from that previously described [171]. Overnight cultures of each strain were diluted to a starting cell density of ~1000 cells based on optical density (OD) measured at a wavelength of 600 nm. Both competitors (100 µL each) were added to EC for a final starting volume of 10 mL and grown 24 h at 37 °C, statically. A pure culture control (100 µL in EC) of each competitor was also prepared and grown under the same conditions as the competition cultures. After 24 h,

Table 4.1. *Escherichia coli* strains utilized in this study

	Serogroup		H-type	Toxin	eae	hlyA	Liquid	Omelette	Plug n' Prey	Discussed in Manuscript
Strongest	O103F		NM	—	—	—	✓	✓	✓	✓
Strong	O26E		H9	—	—	—	✓	✓		✓
	O178A		H7	—	**NT**	**NT**		✓		✓
	O178B		NT	—	—	—		✓		
Weak	O26	A	NT	—	—	**NT**	✓			
		B	NT	—	+	**NT**	✓			
		C	H18	—	—	—	✓			
		D	H11	***stx 1***	+	+	✓	✓		
		F	NM	—	+	—	✓			
	O45	A	H4	—	—	—		✓		
		B	NT	***stx 1***	+	**NT**		✓		
		C	NT	—	—	**NT**		✓		
	O51	A	NM	—	—	—		✓		
	O103	A	NM	—	+	+	✓			
		B	NM	—	+	+	✓			
		C	NM	—	+	+	✓			
		D	H38	—	—	—	✓			
		E	NM	—	+	+	✓	✓		
	O111	A	NM	***stx 1***	+	+	✓	✓		
		B	NM	***stx 1***	+	+	✓			
		C	H8	***stx 1***	+	+	✓			
		D	H8	***stx 1***	+	+	✓			
		E	NM	***stx 1***	+	+	✓			
		F	NM	***stx 1***	+	+	✓	✓		✓
	O145	A	NM	***stx 1***	+	+		✓		
		B	H25	—	+	+		✓		
	O157	A	H7	***stx 1 & 2***	+	+	✓	✓		✓
		B	NT	***stx 1 & 2***	+	**NT**	✓			
		C	NT	***stx 1 & 2***	+	**NT**	✓			
		D	H7	***stx 1 & 2***	+	+	✓			
		E	NT	***stx 2***	+	**NT**	✓	✓	✓	✓
		F	NT	***stx 1 & 2***	+	**NT**	✓			

Note: NT = Not tested, Symbol: − = tested and not present, + = tested and present. Multiple representative strains of each serogroup were used in this study. (√) checkmark symbol identifies which strains were used in each test and which strains are discussed in the manuscript. STEC (Shiga toxin-producing *Escherichia coli*) were defined as *Escherichia coli* strains positive for at least one shiga toxin gene by PCR

100 µL from each competition and control were inoculated into 9.9 mL of fresh EC and grown for another 24 h, with this repeated for a total of 14 days. Samples were removed (100 µL of culture) at time = 0, 3, 7, and 14 days for quantitative real-time PCR (qPCR) monitoring of copy numbers of O-serogroup specific gene sequence to extrapolate cell density of a particular strain in the culture. Primers, probes, and PCR conditions were performed, as previously described [171].

4.2.2.2. Omelette Method

After overnight incubation, one competitor was streaked across a 4 mm MAC plate using a cotton swab. The hereinafter bottom strain was then incubated for 24 h at 37 °C. After 24 h, the agar was flipped and 3 strains were streaked perpendicular to the bottom strain and separated from the bottom strain by the thickness of the agar (Figure 4.2). Plates were then incubated for an additional 24 h at 37 °C and examined for zones of no growth directly over the bottom strain and graded using a scale of 1-to-10, with 1 being no growth over the bottom strain and 10 being full growth. Plates were incubated for an additional 6 days (7 days total) at 37 °C to monitor changes in zones of growth inhibition. A second trial was performed, as described above, with the following change: Agar thickness of MAC plates was increased to 7 mm and 10 mm and compared to the original 4 mm thickness.

4.2.2.3. Plug ‘n’ Prey

The competition assay was performed in 2 mL tubes prepared with slants of 200 µL MAC agar. Slants were overlaid with 800 µL saline buffer (0.9% NaCl). Overnight cultures for both trials were prepared as previously described and were diluted to an OD 600 nm of 0.100 and grown to an OD 600 nm of 0.3–0.4 at exponential cell growth.

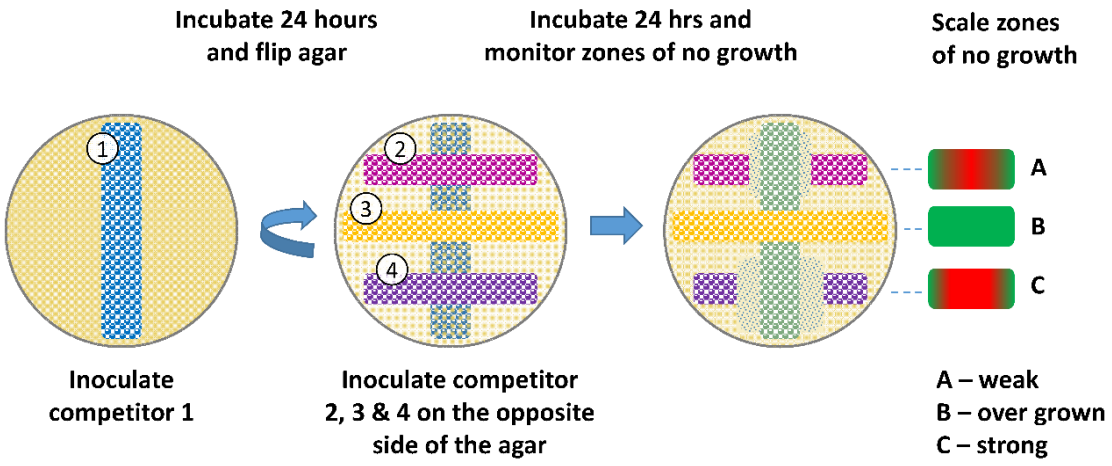


Figure 4.2. Schematic of Omelette method.
Competitor strains were streaked on both surfaces of a 4 mm MAC plate. The bottom strain was streaked and grown for 24 h at 37 °C. After 24 h, the agar was flipped and three strains were streaked perpendicular to the bottom strain, separated by the thickness of the agar. Plates were then incubated for an additional 24 h and examined for zones of no growth graded by a scale of 1-to-10. Plates were incubated for another 6 days (7 days total) to monitor changes in zones of no growth. Agar thickness of 4, 7, and 10 mm were evaluated.

Actively growing cells were diluted to a total starting cell density of ~1000 cells. In the first trial 100 µL of each competitor was inoculated into the 2 mL tubes at the same time (Figure 4.3). Samples were taken at time points 0, 2, 4, and 6 days. Tube contents were divided into supernatant and slant and individually analyzed by qPCR targeting O-serogroup specific gene to monitor the copy numbers. The second trial set-up was equivalent to the first except one strain was inoculated into the 2 mL tube 3 h prior to the competitor.

4.2.3. Statistical Analysis

Numerical data generated by qPCR for the liquid competition and plug ‘n’ prey were examined for normal distribution and the data were log transformed prior to analyses. Serogroup, competition, control, and interactions were determined for both liquid and plug ‘n’ prey using a mixed linear model (Proc Mixed, SAS 9.4, SAS Institute Inc., Cary, NC, USA). Given P values < 0.05 were considered significant.

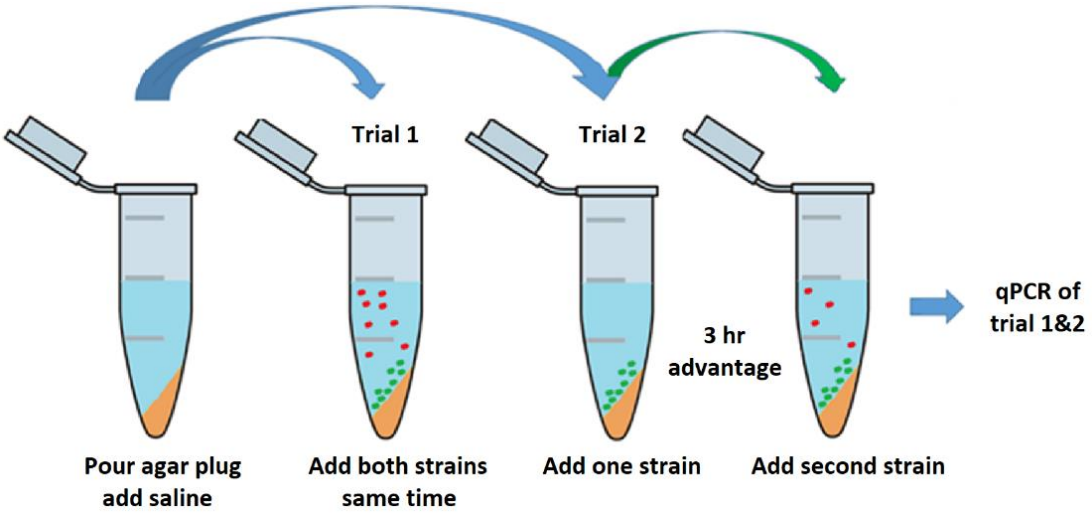


Figure 4.3. Schematic of Plug 'n' Prey.
A 2 mL tube prepared with slants of 200 µL MAC agar slants were overlaid with 800 µL saline buffer (0.9%). In the first trial, overnight cultures were diluted to an OD 600 nm of 0.100 and grown to an OD 600 nm of 0.3–0.4 to ensure actively growing cells. Actively growing cells were diluted to a starting concentration of ~1000 cells and 100 µL of each competitor was inoculated at same time. The second trial was performed, as described, with the following change: One strain was inoculated 3 h prior to the competitor. Samples for both trials were taken at time points 0, 2, 4, and 6 days. Tube contents were divided into supernatant and slant and individually analyzed by qPCR monitoring copy numbers of O-serogroup specific gene fragment amplification.

4.3. Results

4.3.1. Liquid Competition

The qPCR enumerations from 72 competitions of either O103 (six non-pathogenic strains tested) vs O157 (six STEC tested) or O26 (five non-pathogenic strains and one STEC) vs O111 (six STEC) identified strong non-pathogenic *E. coli* competitors that were able to eliminate O157 and O111 (Figure 4.4). The O103 vs. O157 competitions identified a strong strain, O103F that eliminated all six opponent strains of O157 tested to levels undetectable by qPCR by day 14. Similarly, a strong non-pathogenic O26, O26E, eliminated five of six pathogenic O111 with only O111B remaining after 14 days and

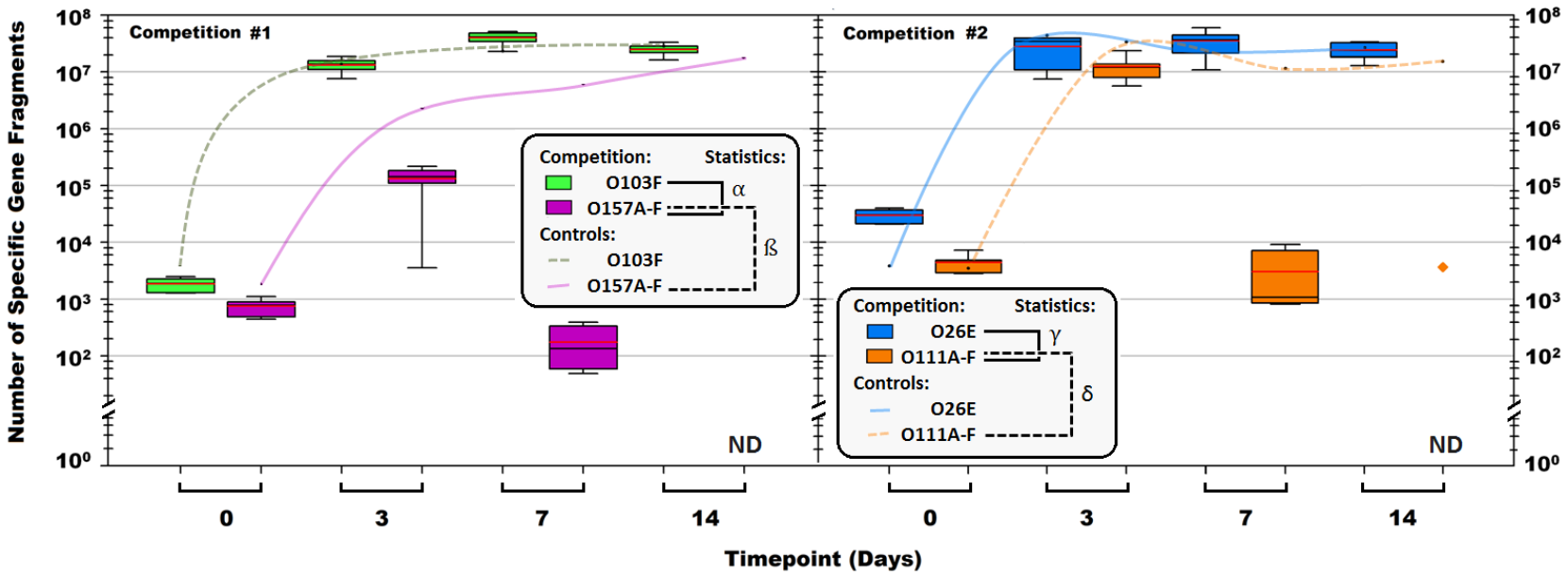


Figure 4.4. Total number of *Escherichia coli* O103F, O157A-F, O26E and O111A-F specific gene fragments in each liquid competition and corresponding pure culture controls calculated using qPCR.
The boxes are serogroup specific enumerations for competition cultures and the lines are the pure culture controls.
Note: ND = not detected. Red line in the boxes is the average proportion for the group and black line in the boxes is the median. The ◆ symbol denotes the O111B strain that was still present after 14 days. Symbols: α, β, γ, and δ denote a significant difference between: O103F and O157A-F in competition, O157A-F in competition and O157A-F controls, O26E and O111A-F in competition and O111A-F in competition and O111A-F controls, respectively ($P < 0.05$).

present in lower numbers (10^3) compared to O26E (10^7). Growth of 103F was greater than each O157 tested and growth of O26E exceeded that of each O111 ($P < 0.05$). Growth of pure culture controls for each competition set O103F, O157A-F, and O26E, O111A-F did not differ ($P > 0.05$) and all were present over the 14 days (Figure 4.4).

4.3.2. Omelette Method

The semi-quantitative data from the first trial (4 mm MAC plates) with various strains also identified the O103F from the liquid competition as a strong competitor (Appendix III, Figure A3.1). O103F had a strong zone of no growth at 24 h and maintained the zone for seven days, while the O157 initially had a less pronounced zone of no growth at 24 h and at day seven the strong O103F was grown over the original no growth zone of O157A. From the 4 mm thickness trial, another strong competitor was identified, *E. coli* O178A, which maintained a strong zone of growth inhibition over seven days against the O103F and O26E strains from the liquid competition (Appendix III, Figure A3.2a). Furthermore, both the O103F and O178A strains maintained stronger zones of no growth compared to O26E, which had a less prominent zone of no growth (Appendix III, Figure A3.2a and b).

The 24 h semi-quantitative data from the second trial (four, seven, and 10 mm MAC plates) with O103F, O178A, O111F, and O157A demonstrated that at four mm agar thickness, all four strains had a strong zone of no growth and as agar thickness increased the size of the zones of growth inhibition varied by strain (Figure 4.5). O157A did not maintain a zone of no growth at seven mm and all three competing strains (O103F, O178A, and O111F) had solid growth when O157A was the bottom competitor.

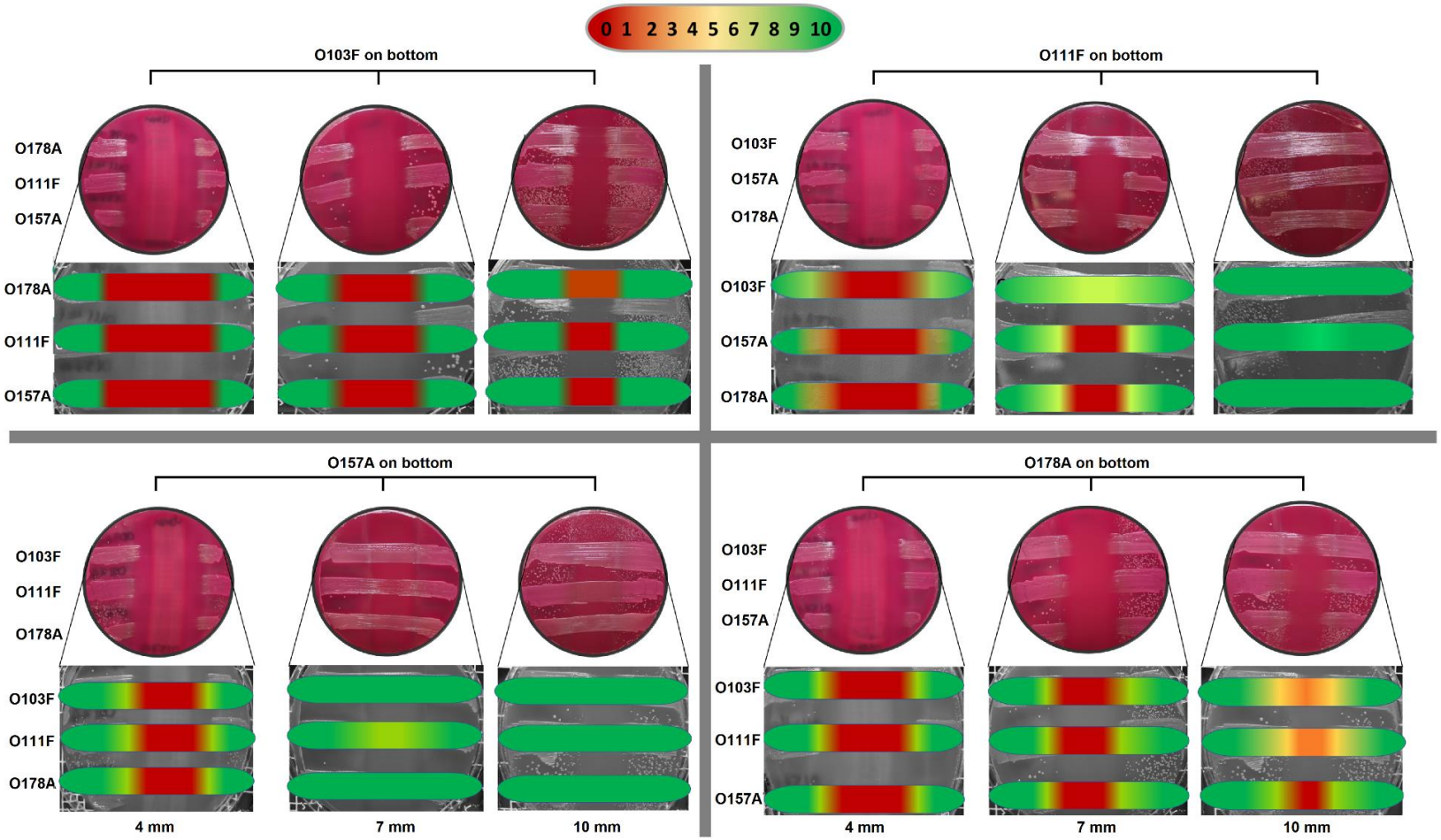


Figure 4.5. Omelette method results for *Escherichia coli* O103F, O111F, O157A, and O178A with varying plate thickness (4, 7, and 10 mm) against each other examining zones of no growth.
Growth zones are graded beneath each plate with red (0) representing no growth to green (10) representing no inhibition of growth.

In comparison, O111F at 7 mm maintained the zone of no growth only for O157A and O178A, but not O103F. Both O178A and O103F maintained a narrowed zone of no growth at seven mm but at 10 mm thickness, O103F still maintained a zone of no growth for O111F and O157A and very limited growth for O178A. In contrast, O178A at 10 mm maintained the zone of no growth only for O157A with both O111F and O103F growing over the bottom O178A strain.

4.3.3. Plug 'n' Prey

The qPCR enumerations for plug 'n' prey demonstrated the ability of O103F to out-compete O157E when nutrients were limited (Figure 4.6). The first trial (I) with O103F and O157E in competition demonstrated that by day six, O103F was 10 times more numerous in both matrices, slant and supernatant, but differences in growth between O103F and O157E were found only for the supernatant ($P < 0.05$). The second experiment (IIa) with O157E having a three h advantage demonstrated that O103F overcame the disadvantage by day two and had 10 times higher concentrations of cells by day six, although overall growth did not differ ($P > 0.05$) between O103F and O157E. When O103F had the three h advantage (trial IIb) it won by 30 times higher concentrations in comparison to the O157E for the slant environment and was 40 times higher in the supernatant ($P < 0.05$). Comparing the O157 pure culture controls to O157 across all three competitions showed higher growth ($P < 0.05$) for controls of O157E, as compared to competition for trial IIb. In contrast, even in competition, O103F grew similar to the O103F control in all trials ($P > 0.05$).

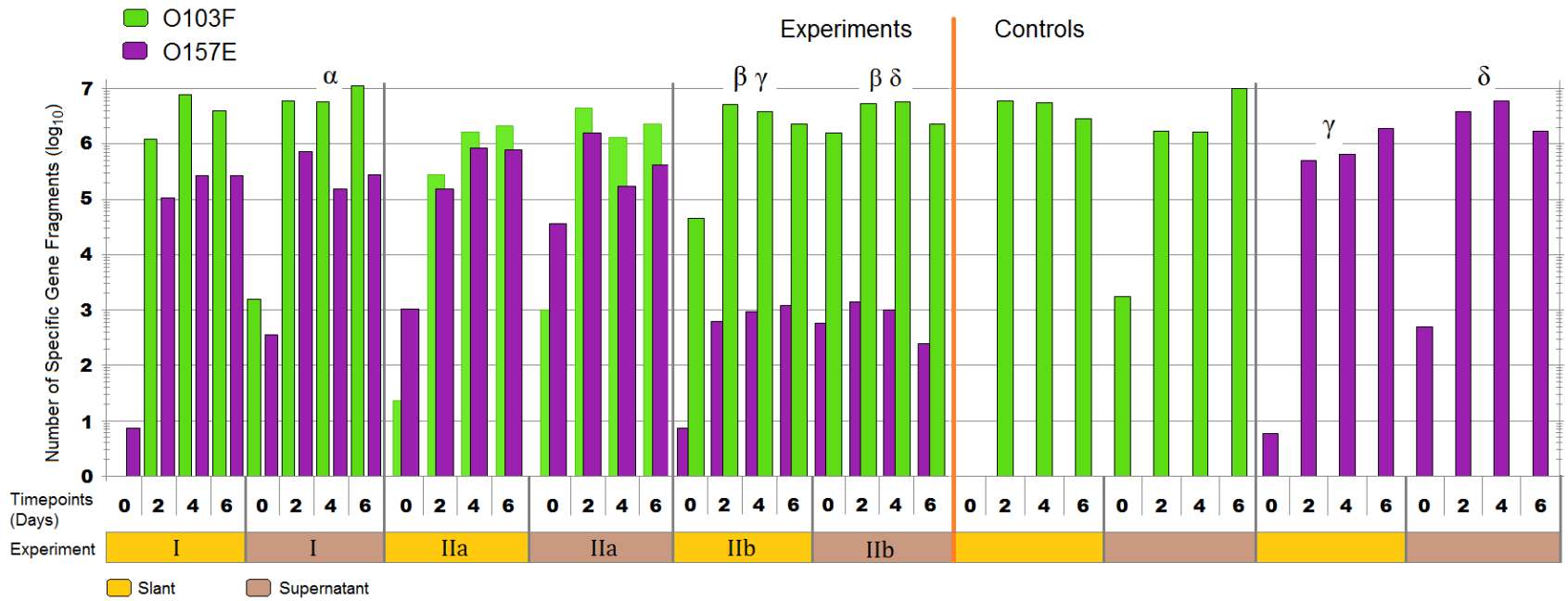


Figure 4.6. The total number of *Escherichia coli* O103F and O157E specific gene fragments in the supernatant and on the agar slant, across three plug 'n' prey experiments in comparison to the controls at each time point.

Note: I = O103F and O157E added at the same time (bars side by side), IIa = O157E 3 h advantage over O103F (O157E bar on top), IIb = O103F 3 h advantage over O157E (O103F bar on top). Time point 0 is first time point after addition of both competitors. Symbols: α, β, γ, and δ denote a significant difference between: O103F and O157E in experiment I—supernatant, O103F and O157E in experiment IIb—slant and supernatant, O157E in experiment IIb and O157E control—slant and O157E in experiment IIb and O157E control—supernatant, respectively ($P < 0.05$).

4.4. Discussion

In three different settings, the competition of *E. coli* strains for resources and/or space was monitored. Using liquid media permitted cell-to-cell contact, while solid media created a physical barrier between opposing strains. Previous research reported that interference-based competitions can be contact-dependent or contact independent [116], although when using a physical barrier only a producing predator would win competitions for space. During liquid competitions, both distant and close combat tactics were applicable. However, within the homogeneous distribution of cells and nutrients in liquid, it cannot be determined if coordinated strategies by strains took place or if one competitor conquered due to an advantage in metabolism and/or proliferation.

4.4.1. Liquid Competition

Using liquid competition, we identified two non-pathogenic *E. coli* strains (belonging to serogroups O103 and O26), which significantly reduced opponent strains to below limits of detection or resulted in a 4-log10 reduction of six different strains each of O157 (vs. O103F) and O111 (vs. O26E) ($P < 0.05$). This finding, that non-pathogenic *E. coli* have the ability to outcompete pathogenic *E. coli* strains, is in accordance with a previous study reporting that calves treated with probiotic *E. coli* shed significantly less O26:H11 and O111:NM STEC compared to untreated calves [109]. Furthermore, strong competitors within our *E. coli* strains were previously identified [171]. However, O26C, a strong competitor previously reported [171] was weaker compared to O26E identified in this study.

Competition can be exploitative or interference based [112] and it is possible that the O103F and O26E identified in the liquid competition are great exploiters and simply

out-competed their competitors for resources. However, the qPCR enumerations for all the pure-culture controls suggest otherwise since all strains proliferated to similar concentrations in EC without competition ($P > 0.05$), suggesting that the liquid competition results are due to interference and not exploitation.

E. coli is known to have both contact independent and dependent interference-based systems and have been shown to produce various bacteriocins known as colicins [119], may possess a CDI system [118] and/or harbor a T6SS system [120]. In a pure culture of planktonic bacteriocin producing *E. coli*, 0.5–3% of the population express bacteriocin spontaneously [172]. Possibly, O103F and O26E expressed bacteriocins that out-competed the O157 and O111 strains tested. CDI systems in *E. coli* have also been shown to be active in liquid culture as *E. coli* EC93 inhibited *E. coli* K-12 cells [173]. Moreover, CDI systems require specific receptors on the competing bacterial cells that are often found on closely related strains or within the same species, suggesting the CDI mechanism could be employed during competition. Contrary to CDI systems, the T6SS targets cells non-specifically by using physical forces to deliver effectors [116]. Here, T6SS was an unlikely mode of action, since a highly-active T6SS competitor was unable to target sensitive strains in liquid medium in contrast to a solid medium [174], which suggests that, in liquid competitions, bacteria do not use T6SS, but rely on other mechanisms.

4.4.2. Omelette Method

The results from the semi-quantitative omelette assay revealed the production of diffusible toxins or noxious products since the competitor strains were separated by a physical barrier preventing contact-dependent competition. Agar plates are often used

to visualize competitive interactions due to the manifestation of visible phenotypes that identify competition winners [167]. Similar to the results of the liquid competition, O103F produced a stronger diffusible substance than the competitors. Here, we also identified another strong contact–independent strain, (O178A) with zones of no growth similar in size to those of O103F. In comparison, the O26E identified in the liquid competition did not have as strong of a zone of no growth as O103F, suggesting the presence of a weaker diffusible substance. The second trial further demonstrated the strength of the O103F diffusible substance through distance. O103F maintained the strongest zone of no growth as agar thickness increased for all strains tested including O178A from the first trial. Overall, while O103F appeared to have the strongest diffusible substance and best resistance, all strains tested showed an initial zone of no growth at 24 h, suggesting that they all produced a detrimental substance capable of preventing growth of other *E. coli*, which conforms to previous reports that *E. coli* produces diffusible toxins [119, 122]. Furthermore, various studies have examined *E. coli* strains for production of bacteriocins and the percentage of strains that produce bacteriocins can vary from 10% to 70% depending on the environment where they were isolated [175].

Bet-hedging is a survival tactic used by bacteria, where they express phenotypes randomly instead of in reaction to environmental cues [131]. Bacteriocin production has been shown to occur at a low frequency in growing producer populations [172] and a recent study has shown that *E. coli* colicin producers use bet-hedging as a survival strategy [131]. A previous study suggested that such a low-level production could be considered a pre-emptive attack against sensitive strains [176]. Possibly, the diffusible

molecule produced by O103F and others was spontaneously produced during the first 24 h of the omelette study providing an advantage to the bottom strain prior to addition of the competitors. Furthermore, low-level producers in an established colony can signal sister cells to mount a collective attack against invading cells [176]. Potentially, if some O103F cells in the established colony were already producing bacteriocins, these cells could have sensed the competitors and signaled sister O103F cells, mounting stronger attacks against the "invading cells" indicated by wider zones of no growth. On the other hand, bacteriocins have been suggested to act first as a signaling and repelling molecule rather than being lethal and possibly the competitors of O103F, were repelled by the molecule produced by O103F [177].

Based on the classification of competitors in a bacterial warfare as either producers, resistant or sensitive [164] (Figure 4.1), all strains tested in this study appear to be producers. However, revealing the characteristics of a producer, strain O103F can also grow over other strains over time, implying that O103F is both a strong producer and a resistant strain. Bacteriocin production is based on the translation of three genes encoding toxin, immunity, and lysis [131]. Therefore, production confers immunity and likely any diffusible molecule will be paired with the production of immunity molecules to prevent the killing of sister cells. *E. coli* strains have been shown to produce more than one colicin and microcin [122] and possibly O103F is resistant to some bacteriocins and/or diffusible(s) from other *E. coli* strains because O103F produces a similar diffusible, which confers immunity. On the other hand, immunity can also be conferred by mutations that either alter receptors or the translocation system for the bacteriocin [122, 164] and O103F may be resistant to diffusible(s) of other *E. coli* strains due to these

types of mutations. Future research on the specific O103F diffusible(s) may determine if (**I**) the diffusible is a bacteriocin, (**II**) the diffusible is killing or signaling competitors and (**III**) the diffusible immunity is conferred by production of the diffusible or due to mutations that grant immunity, which may further elucidate the strength of the O103F strain as a competitor.

4.4.3. Plug 'n' Prey

This study was designed to examine the effect of limiting nutrients on competition outcome between the identified strong O103F and the previously tested O157E strain. Limiting nutrients did not change the overall outcome in all the trials and O103F won all the competitions, including when inoculated with a three h disadvantage into vials containing the nutrients but growth between O103F and O157E was only different ($P < 0.05$) for trial I—supernatant and trial IIb—both matrices. Perhaps, the lack of nutrients in plug 'n' prey affected the ability of O103F to compete effectively once nutrients were depleted. Previous research has shown that bacteriocin production in a lactic acid bacterium was modified when the carbon source changed and bacteriocin production increased or decreased depending on the carbon source [178]. Conceivably, the same modification of bacteriocin production may be seen by limiting the carbon source.

Ultimately, O103F won both competitions having either an advantaged or disadvantaged access to limited nutrients. It overcame the O157E advantage to win overall and prevented O157E from growing past the inoculation density of 1000 cells when O103F had the advantage. Having the advantage, O103F won all competitions, which is in accordance with a previous study that reported that an "established" colony is

more successful mounting attacks against invading competitors [176]. With a three hour advantage, O103F suppressed the growth of O157E over six days by preventing O157E from growing beyond 1000 cells. However, the "established" O157E was not able to prevent O103F from growing, which is contrary to previous reports [176]. In the end, after a three h advantage O103F was able to maintain a competitive advantage, while O157E was not able to fortify the nutrient source during this time, further demonstrating the predatory strength of O103F compared to O157E. Future studies with different nutrients and different advantage times may further elucidate if and how the competitive mechanisms of O103F are affected by nutrition.

Bacteria, such as *E. coli* are found in almost every habitat on earth and face fierce competition for space and resources [113, 116, 179]. In order to ensure their survival, bacteria use various competitive mechanisms which can be exploitative and/or interference based [112, 116]. Among others, habitats for virulent *E. coli* are humans and their food sources. During medical treatment and along the food production chain, control of proliferation, and/or colonization by virulent *E. coli* remains a challenge.

STEC are a significant food borne pathogen [180] and are classified by the ability to produce at least one Stx [29]. Cattle are considered the main reservoir for STEC and STEC carriage in cattle is asymptomatic due to a lack of receptors for Stx [34]. On the other hand, Stx is considered a main virulence trait to cause human disease [181] since Stx binds to globotriaosylceramide (Gb3) present on endothelial cells [180]. Benefiting from natural competitiveness, *E. coli* champions may offer mechanisms to mitigate STEC as protective culture and/or additive within the food chain or as a probiotic treatment option for human infections.

It should be noted that highly-competitive non-pathogenic *E. coli* may have the potential to become STEC if infected with a Stx bacteriophage. However, a study examining the ability of various stx_2-phages to infect *E. coli* from different pathotypes found that while all strains could be infected with Stx-phages, not all Stx-phages infected every *E. coli* and phage integration was rarely stable [182]. Other studies examining Stx-phage infection on various food sources found that for Stx-phage infection to occur both the donor and recipient need to be present in high concentrations not typically found in food samples [183, 184]. Together, these studies suggest that while Stx-phage infection is possible: 1) it is unlikely to occur and 2) rarely is phage integration stable. Unless there is stable integration, the phage DNA is removed, rendering *E. coli* a non-STEC. Furthermore, identification of the diffusible(s) produced by these highly competitive non-pathogenic *E. coli* would mitigate the risk by removing the need to use the bacteria and instead only use their products.

This study identified a strong O103 competitor based on three different experimental settings against various strains including STEC O111 and STEC O157. The exact mode of action used by O103F to out-compete other *E. coli* strains remains unknown but O103F likely produces at least one diffusible substance that affects the viability of other *E. coli*. Diffusible molecules produced by *E. coli* can be colicins, antibiotics, or quorum sensing molecules [112], and are potential alternatives to antibiotics [114]. Future evaluation of *E. coli* O103F may identify the effective diffusible substance(s) produced by this strain and may provide an alternative STEC mitigation strategy as therapeutic treatment or protective culture in the food industry.

4.5. Conclusions

Among living organisms, fierce battles exist to secure habitats and natural resources or even for survival. Between bacterial competitors, predators, and prey have developed several strategies to protect their existence and survival combat interactions that might ultimately be correlated to energy conversion efficiencies and the capacity to proliferate. Our observations revealed a number of highly competitive *E. coli* strains, but ended with one exclusive champion. Over 100 years ago, a champion (*E. coli* Nissle) from a human host was discovered to battle virulent bacteria and has been successfully marketed as a probiotic, mitigating human infections since then. Novel emerging pathogens are a global concern and new approaches for mitigation strategies require further evaluation. Here, numerous Shiga-toxin producing *E. coli* strains were outcompeted by a non-pathogenic *E. coli* strain that was isolated from cattle feces. This non-pathogenic strain shows the potential to be used to control pathogenic *E. coli* that compromise health and/or food safety.

CHAPTER V: Properties of an antimicrobial molecule produced by an *Escherichia coli* champion

5.1 Introduction

At a certain point in time, humans triggered an evolutionary Big Bang by shaping a new microbial multiverse. Ancestral humans were “hunter-gatherers”, living in small nomadic communities foraging for food. Later those communities transitioned to settle permanently in one place, cultivating food and resources. This was the origin of establishing areas with dense populations of humans and livestock [5, 185]. Simultaneously, microbial communities adapted, competed, evolved and proliferated within those close interactions across agriculture and humans alike. Commensal or virulent *Escherichia coli* were some of the species among them. The emergence of lethal diseases (e.g. as early as pandemic reports of the Justinian Plague or Black Death) carried by host adapted pathogens could only be sustained in areas of dense human populace [185]. These environments are also prime spaces to foster bacterial competition for existence often coined as “survival of the fittest” [126, 186-188].

Competition can be categorized as exploitative or interference based [112]. Exploitative interactions are those where one bacterium is using nutrients more efficiently, effectively starving a competitor. However, exploiter champions can be out-competed by a weaker exploiter using interference competition. Interference competition is based on the production of antagonistic factors that eliminate competitors using contact dependent (growth inhibition and Type VI secretion systems) or contact independent means (bacteriocins and other diffusibles, such as antibiotics) [116, 186].

Bacteriocins are molecules (proteins and peptides) produced by both Gram positive and Gram negative bacteria [117] and are most often inhibitory to close relatives

only [189, 190]. However, some Gram positive bacteriocins have been shown to combat Gram negative bacteria [191]. Gram negative bacteriocins have been studied and identified in *E. coli* and are coined colicins and microcins [134]. Colicins are high-molecular mass proteins (30-80 kDa) [191] and tightly controlled by the bacterial SOS system [187, 192]. In contrast, microcins, peptides with molecular masses below 10 kDa, are protease and temperature resistant, and are reported to resist pH extremes [132, 191]. These characteristics are often associated with bacteriocins of lactic acid bacteria [191].

Bacteriocins are being considered as potential agents to replace antibiotics and have been suggested for use in humans and in livestock as a method to mitigate pathogens [193]. Bacteriocins are considered "agents of competition" [189] and represent a microbial strategy to out-compete their rivals [187, 191]. Bacteriocins are unique compared to traditional antibiotics, as they harbor a restricted killing spectrum, targeting specific bacteria or species [189, 193]. The targeted killing of specific bacteria makes producers of these bacteriocins an ideal probiotic. In fact, discovered 100 years ago, Mutaflor® is a commercially available probiotic that contains the strain *E. coli* Nissle which produces two microcins, which are thought to be crucial in the ability of this strain to antagonize *E. coli* and *Salmonella* pathogens [121, 123, 194].

Among bacterial pathogens, Shiga toxin-producing *E. coli* (STEC) produce a potent toxin (Shiga toxin) which in conjunction with other factors causes severe, often foodborne, infections in humans [29, 31]. Various mitigation strategies for this pathogen have been considered, including vaccines [107], direct-fed microbials [168] and tannins [169] but none have consistent efficacy. Another mitigation strategy being considered to control STEC is using probiotic bacteria to competitively eliminate the pathogens [195],

as demonstrated by the effective use of *E. coli* Nissle to alleviate intestinal infections in humans [121]. Likewise, a colicin-producing *E. coli* isolated from sheep fecal samples was shown to inhibit STEC O157:H7 [195].

In a previous study in our laboratory, we demonstrated that various *E. coli* strains isolated from beef cattle feces produce diffusible molecules capable of affecting competitor growth, when separated by a 4 to 10 mm barrier of agarose [127]. However, strength of these molecules varied among strains which led to the identification of a strong competitive non-pathogenic *E. coli* champion that produces a diffusible molecule(s) capable of outcompeting 31 different *E. coli* strains including STEC O26, O111 and O157 [127].

The objective of this project is to further characterize those diffusible molecule(s) produced by this *E. coli* champion, while investigating the physiochemical and biological properties of the molecule as well as its antimicrobial potential.

5.2. Materials and Methods:

5.2.1. Bacterial Strains: Cultures, Media and Culture Conditions

Both *E. coli* strains used in this study, O157A and O103F, were described previously [127]. Briefly, *E. coli* strains were streaked from glycerol stocks onto MacConkey Agar (MAC, BD, Sparks, NV, USA). Plates were incubated overnight (16–18 h) at 37°C. A single colony was selected from each plate and inoculated into *E. coli* broth (EC, EMD Millipore, Etobicoke, ON, Canada) and incubated overnight at 37°C with shaking at 150 rpm. Overnight cultures of O157A (STEC) and O103F (no detected virulence genes) were diluted to an optical density (OD) of 0.1 measured at a wavelength of 600 nm in fresh EC and grown for 3 h. The 3 h culture was then used as inoculation for the treatments.

5.2.2. Molecule Isolation Assay:

Molecule isolation assay was adapted from Kulp and Kuehn, 2010 [196] by following the isolation protocol for natural outer membrane vesicles up to and including step 2. O103F molecule is the cell free supernatant collected after 12 h of growth of O103F in EC, containing **A**nti**M**icrobial **Mo**lecule(s) and will subsequently be referred to as AMMO. O157A molecule is the cell free supernatant collected after 12 h growth of O157A in EC and will be subsequently referred to as SPENT. After isolation, AMMO and SPENT underwent various treatments.

Both *E. coli* (O103F and O157A) cultures were grown individually for 12 h and centrifuged (10,000 x g) for 10 minutes to prepare the supernatant (Figure 5.1). The supernatant was then filter-sterilized using a 0.22 µm filter (Pall Life Sciences, Ann Arbor, MI, USA) to remove all bacterial cells. The O103F supernatant (AMMO) was added to fresh EC to test the growth of O157A cells during the inhibition assay. Each experiment had a complete set of four controls to measure the effect of AMMO. The first control, O157A was diluted to a starting OD of 0.1 in a final volume of 5 ml of fresh EC, to demonstrate regular/healthy growth of *E. coli* O157A in fresh media. The second control, O157A was diluted to a starting of OD 0.1 in a total of 5 ml (3.75 ml SPENT and 1.25 ml culture and fresh EC) to take into account the effect of depletion of nutrients and metabolic end products in the isolated supernatants on O157A growth. The third and fourth controls, 3.75 ml of the supernatants (AMMO or SPENT) were each individually added to 1.25 ml fresh EC to ensure that filter-sterilization was successful and all live cells were removed from the supernatants (AMMO and SPENT).

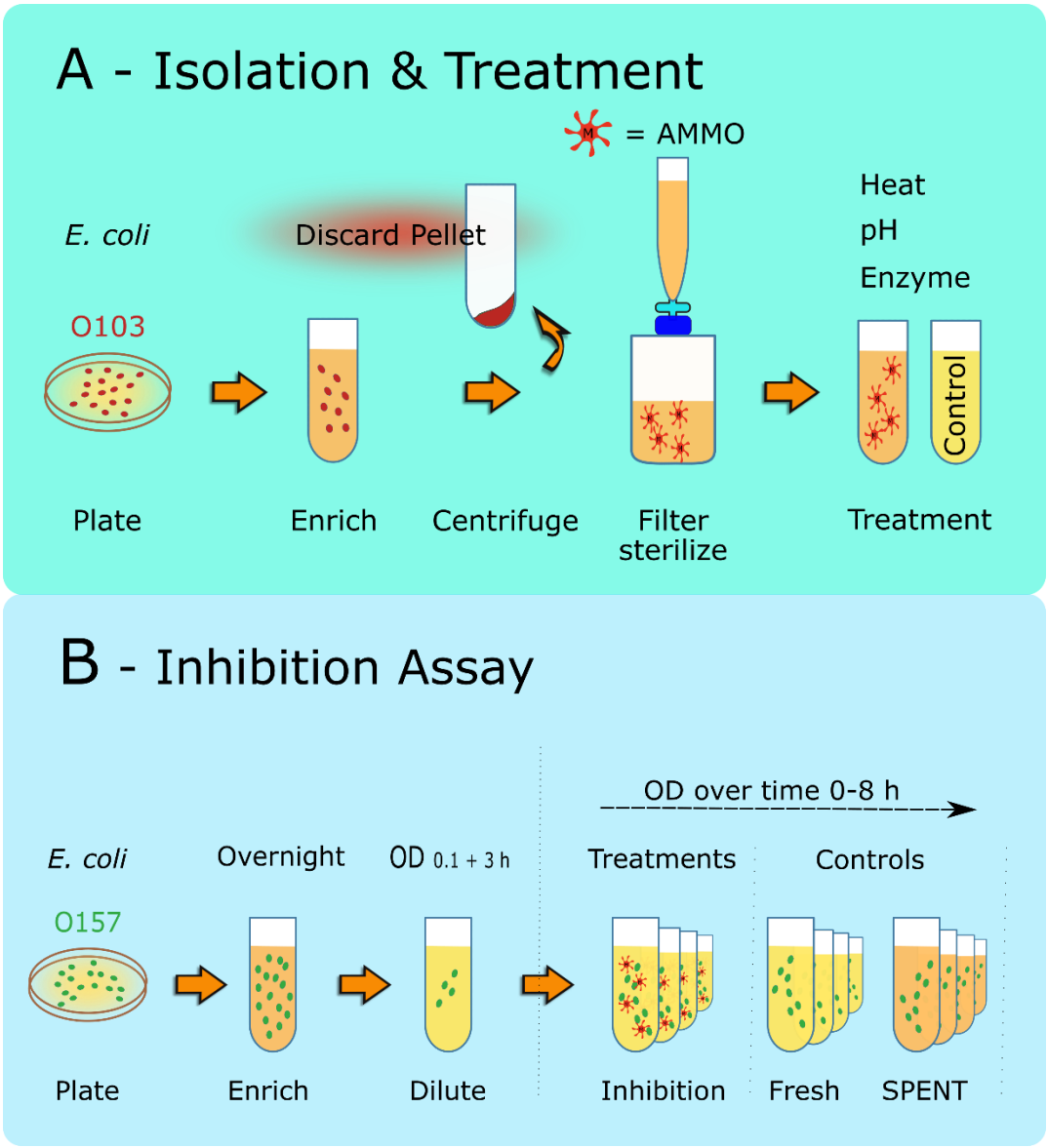


Figure 5.1. Schematic of the Molecule Isolation Protocol and Inhibition Assay. **A: Isolation and Treatment.** *E. coli* O103F was grown overnight on a MAC plate and a single colony was grown in EC for 12 h. Cells were pelleted at 10,000 x g for 10 minutes. The cell pellet was discarded and the supernatant was filter-sterilized using a 0.22 µm filter. The sterilized supernatant was then treated to examine, heat, pH or protease digestion. Note: SPENT was also prepared using the same protocol. **B: Inhibition Assay.** O157A was grown overnight for 12 h in EC. The cells were then diluted to an OD_{600nm} of 0.1 and grown for 3 h. This culture was then used to inoculate the AMMO and the controls. OD_{600nm} measurements were taken at 0, 2, 4, 6 and 8 h.

Note: 1) O103F was also prepared using the same protocol when utilized in the experiment. 2) AMMO is the cell free supernatant collected after 12 h *E. coli* O103F growth. SPENT is the cell free supernatant collected after 12 h *E. coli* O157A growth.

5.2.2.1. Isolation Confirmation:

A culture of either O157A or O103F was diluted to a starting OD of 0.1 in a final volume of 5 ml (3.75 ml of AMMO or SPENT and 1.25 ml culture and fresh EC). A second control was prepared with O157A or O103F diluted into fresh EC. Subsequently, all cell preparations were incubated at 37°C at 150 rpm for 24 h. OD measurements were taken at 0, 2, 4, 6, 8 and 24 h to extrapolate cell densities using an initially determined strain specific growth curve data (slope equation: O157A → $y = -6^{x}10^{6}x^{2} + 4^{x}10^{7}x - 2^{x}10^{6}$ and O103F → $y = -2^{x}10^{7}x^{2} + 7^{x}10^{7}x - 3^{x}10^{6}$). The growth curve data were analyzed by comparing strain specific OD values versus CFU plate counts in parallel at the time point 0, 2, 4, 6, 8 and 24 h (data not shown). All experiments with AMMO and SPENT supernatants were conducted likewise, following the treatments as described below. All experiments were replicated on alternate days with fresh cultures, AMMO and SPENT.

5.2.2.2. pH Treatment

To examine the effect of pH on AMMO, hydrochloric acid (HCl) or sodium hydroxide (NaOH) was added to the supernatants containing the isolated AMMO or SPENT to lower or increase the pH of the solutions to 3 or 11, respectively. The supernatants were then incubated at either pH for 3 h. After the incubation, the supernatants (AMMO and SPENT) pH were neutralized to the pH pre-treatment by titrating either HCl or NaOH solutions and subsequently used for the inhibition assay (Figure 5.1B).

5.2.2.3. Autoclave Treatment

To examine the effect of heat and pressure treatment, the prepared AMMO and SPENT supernatants were autoclaved for 20 minutes at 121°C and 18 psi. After cooling to room temperature, the supernatants were subsequently used for the inhibition assay (Figure 5.1B).

5.2.2.4. Trypsin Treatment

To examine the effect of trypsin (Calbiochem, La Jolla, CA, USA) digestion, 5 µl of a prepared trypsin solution (7.5 units/µl) was added to 20 ml of the prepared AMMO and SPENT supernatants and incubated for 3 h at 37°C. After the incubation, trypsin digestion was stopped by heating the mixture for 10 minutes at 95°C. After cooling to room temperature, AMMO and SPENT supernatants were used for the inhibition assay (Figure 5.1B).

5.2.2.5. Chymotrypsin Treatment

To examine the effect of chymotrypsin (Sigma-Aldrich, Oakville, ON, Canada) digestion, 4 µl or 40 µl (1 unit/µl) of prepared chymotrypsin solution was added to 20 ml of the prepared AMMO and SPENT supernatants. The supernatants were then incubated for 3 h at room temperature. After the incubation, chymotrypsin digestion was stopped by heating the mixture for 10 minutes at 80°C. After cooling to room temperature, AMMO and SPENT supernatants were used in the inhibition assay (Figure 5.1B).

5.2.3. Analysis of Cell Densities

Extrapolated cell densities of O157A grown in either AMMO or SPENT were examined for the difference in cell numbers between the two supernatants by subtracting the number of cells in the AMMO from the SPENT.

5.2.4. Statistical Analysis

Numerical OD data measured for each experiment was examined for normality and subsequently used for analyses. Time, treatment, control, and interactions were determined for all the experiments using a mixed linear model (Proc Mixed, SAS 9.4, SAS Institute Inc., Cary, NC, USA). *P* values < 0.05 were considered significant. Calculated standard deviations from each experiment are shown as bars within the Figures.

5.3. Results:

5.3.1. AMMO Isolation Protocol Confirmation

Preliminary studies for both strains determined that OD measurement corresponded to plate counts in the provided growth curve in section 5.2.2.1. OD based measurements were subsequently used to determine cell densities.

Comparison of OD measurements of O157A grown in a mixture of EC and AMMO to O157A grown in a mixture of EC and SPENT, demonstrated that O157A inoculated into AMMO had significantly lower growth at 4, 6 and 8 h ($P < 0.05$) (Figure 5.2B). Furthermore, the difference in cell density increased over time and the *E. coli* O157A in SPENT had ~ 6 x 10^7 CFU/ml more cells than O157A in AMMO at 8 h (Figure 5.2A). In contrast, the OD of O103F grown in a mixture of EC and SPENT compared to O103F grown in a mixture of EC and AMMO, demonstrated that the O103F cell proliferation was significantly greater ($P < 0.05$) in the presence of SPENT than in AMMO (Figure 5.2B). Concurrently, the difference in cell density demonstrated that O103F in SPENT had ~1.0 x 10^8 CFU/ml more O103F cells than O103F in AMMO at 8 h (Figure 5.2A). Furthermore, comparing the growth of O157A in SPENT to O103F

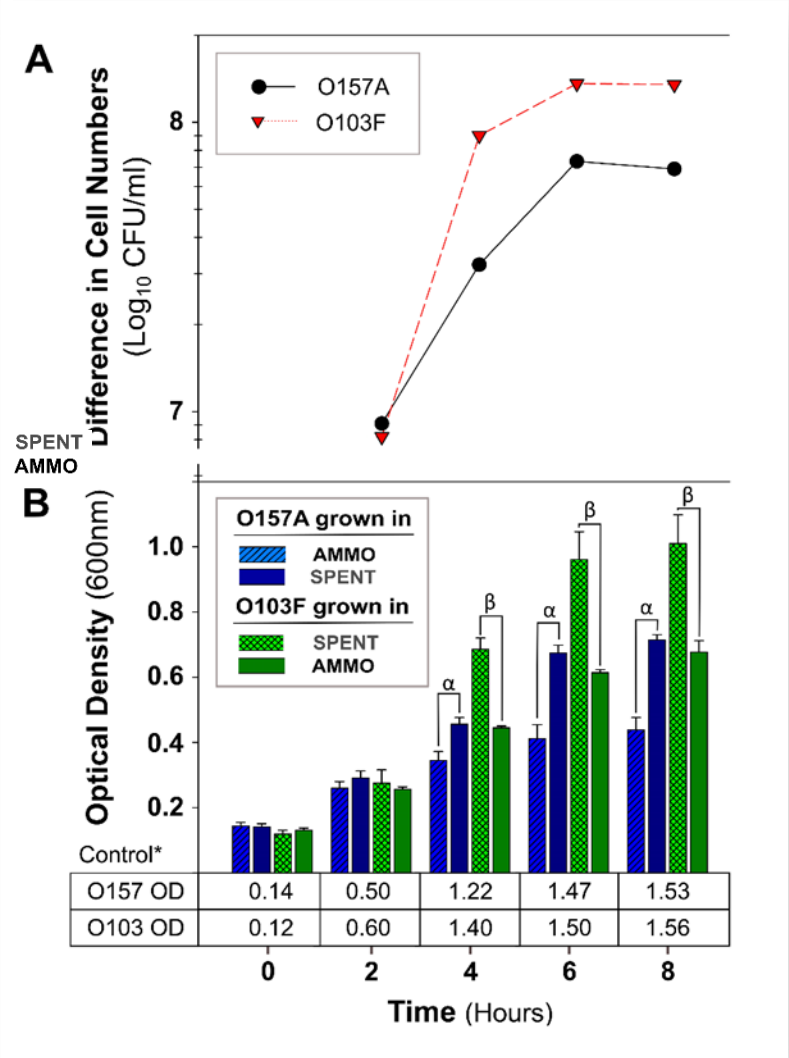


Figure 5.2. The molecule isolation protocol confirmation results for *E. coli* O157A grown in AMMO and SPENT in comparison to *E. coli* O103F grown in SPENT and AMMO.
A: The difference between the cell numbers for either O157A or O103F in the SPENT and in AMMO. **B:** The OD_{600nm} data for O157A in AMMO, O157A in SPENT, O103F in SPENT and O103F in AMMO and *control in fresh EC as numerical value below the bars. Symbols: α and β denote a significant difference between growth in AMMO and SPENT for O157A and O103F, respectively ($P < 0.05$). Comparison of O103F growth in AMMO to O157A growth in SPENT revealed they are not significantly different.
Note: 1) AMMO is the cell free supernatant collected after 12 h *E. coli* O103F growth. SPENT is the cell free supernatant collected after 12 h *E. coli* O157A growth. 2) Bars are the calculated standard deviation for AMMO and SPENT in each experiment (O157A and O103F) at each time point.

in AMMO did not identify a significant difference in growth ($P > 0.05$). Expectedly, all controls of O103F or O157A in fresh EC grew to a higher turbidity than O103F and O157A grown in SPENT (Figure 5.2B).

5.3.2. Investigation of AMMO Properties

Preliminary studies revealed that between 8 and 24 h, AMMO inhibition was diminishing and at 24 h no longer detectable (data not shown). Data are reported for the period up to 8 h.

5.3.2.1. The Effect of pH, Autoclaving, Trypsin and Chymotrypsin Digestion

The OD of O157A grown in a mixture of EC and AMMO after treatment (pH, Autoclaving, Trypsin and Chymotrypsin) was not significantly different from O157A grown in EC with untreated AMMO (Figure 5.3B, 5.4B, 5.5B and 5.6B). In contrast, comparison of O157A grown in EC and AMMO (treated or untreated) to the O157A grown in EC and SPENT (treated or untreated) revealed a significant inhibition of O157A growth at 4, 6 and 8 h ($P < 0.05$). Additionally, the difference in cell density for treated or untreated AMMO and SPENT increased over time (Figure 5.3A, 5.4A, 5.5A, 5.6A) with the largest difference in cell density at 8 h (Table 5.1). The pure culture control of O157A in fresh EC grew to a higher turbidity than O157A grown in SPENT in each experiment (Figure 5.3B, 5.4B, 5.5B, 5.6B).

* Note: In all the tests, the OD_{600nm} of the 12 h culture was measured prior to preparing the supernatant and in each case both O103F and O157A had similar growth densities at 12 h.

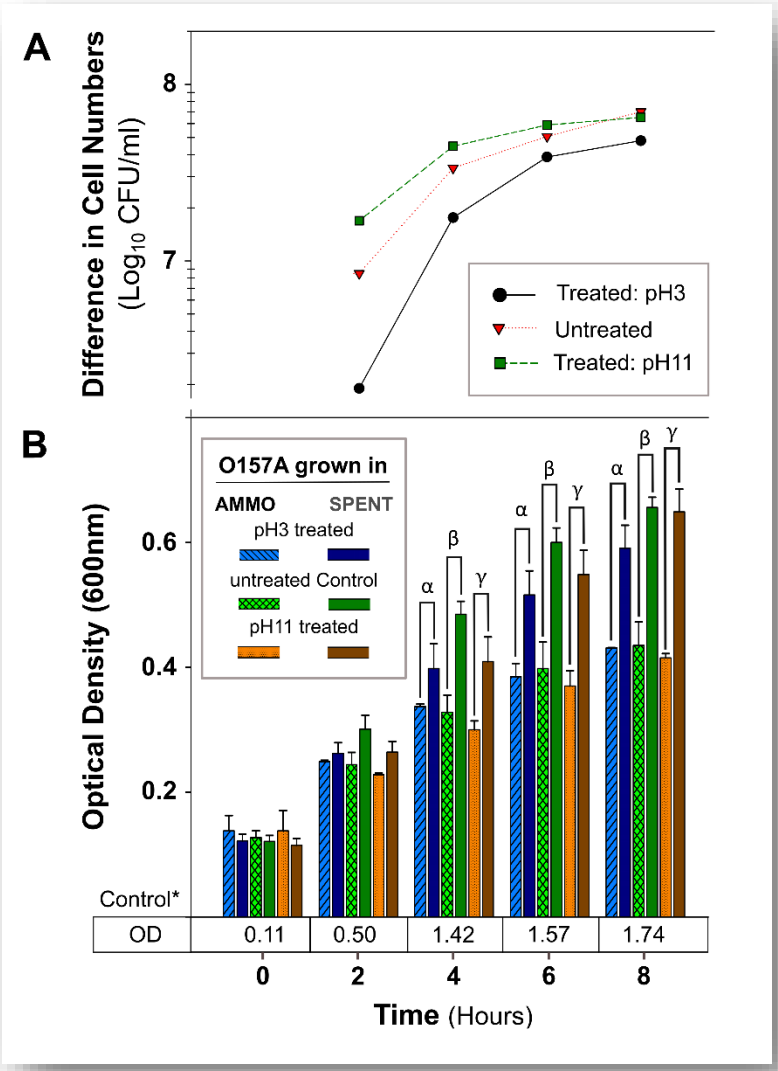


Figure 5.3. The effect of pH treated AMMO on growth inhibition of *E. coli* O157A at two pHs 3 and 11.
A: The difference between the cell numbers for O157A in SPENT and in AMMO at pH 3, pH 11 and untreated supernatant. **B:** The OD_{600nm} data for O157A grown in AMMO, in SPENT (pH 3, pH 11 and untreated) and *control in fresh EC as numerical value below the bars. Symbols: α, β and ɣ denote a significant difference between: O157A grown in AMMO and in the SPENT for pH 3, untreated and pH 11 supernatants, respectively ($P < 0.05$).
Note: 1) AMMO is the cell free supernatant collected after 12 h *E. coli* O103F growth. SPENT is the cell free supernatant collected after 12 h *E. coli* O157A growth. 2) Bars are the calculated standard deviation for the treated or untreated AMMO and SPENT at each time point.

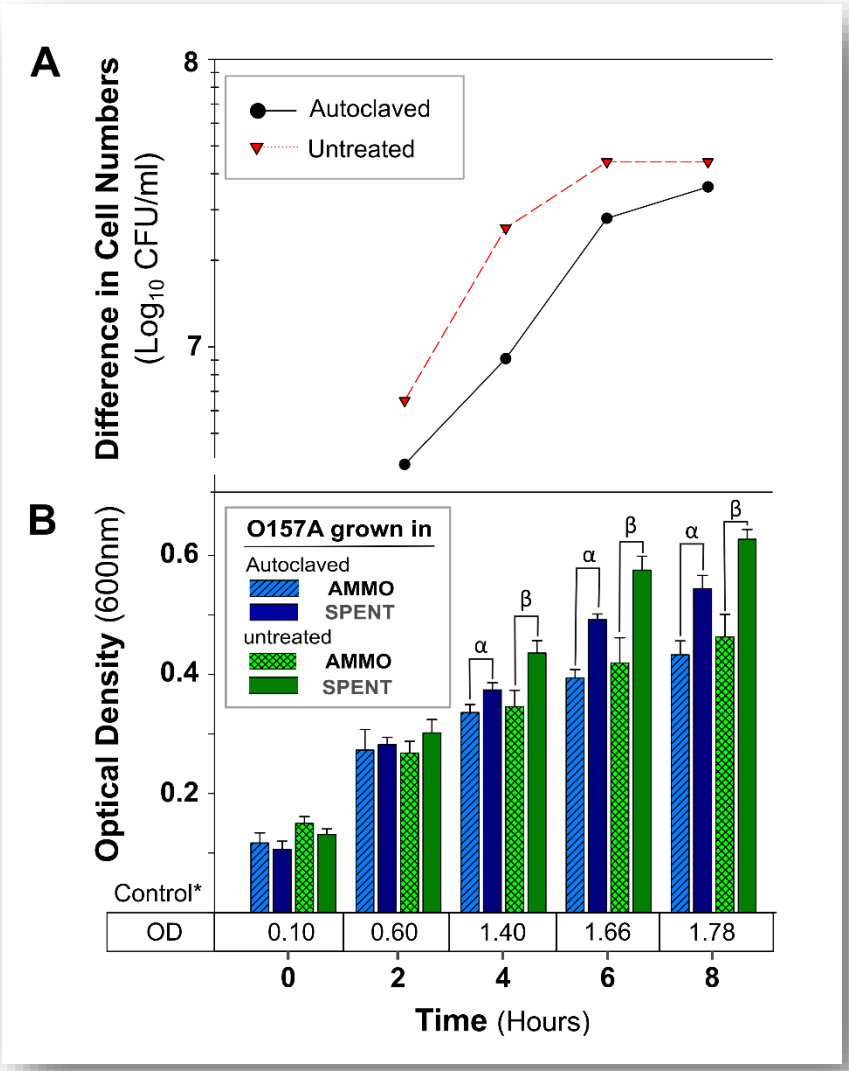


Figure 5.4. The effect of autoclaving AMMO on growth inhibition of *E. coli* O157A.
A: The difference between the cell numbers for O157A in SPENT and in AMMO with treated and untreated supernatant. **B:** The OD_{600nm} data for O157A grown in AMMO, in SPENT (treated and untreated) and *control in fresh EC as numerical value below the bars. Symbols: α and β, denote a significant difference between: O157A grown in AMMO and in SPENT for treated and untreated supernatants, respectively ($P < 0.05$).
Note: 1) AMMO is the cell free supernatant collected after 12 h *E. coli* O103F growth. SPENT is the cell free supernatant collected after 12 h *E. coli* O157A growth. 2) Bars are the calculated standard deviation for the treated or untreated AMMO and SPENT at each time point.

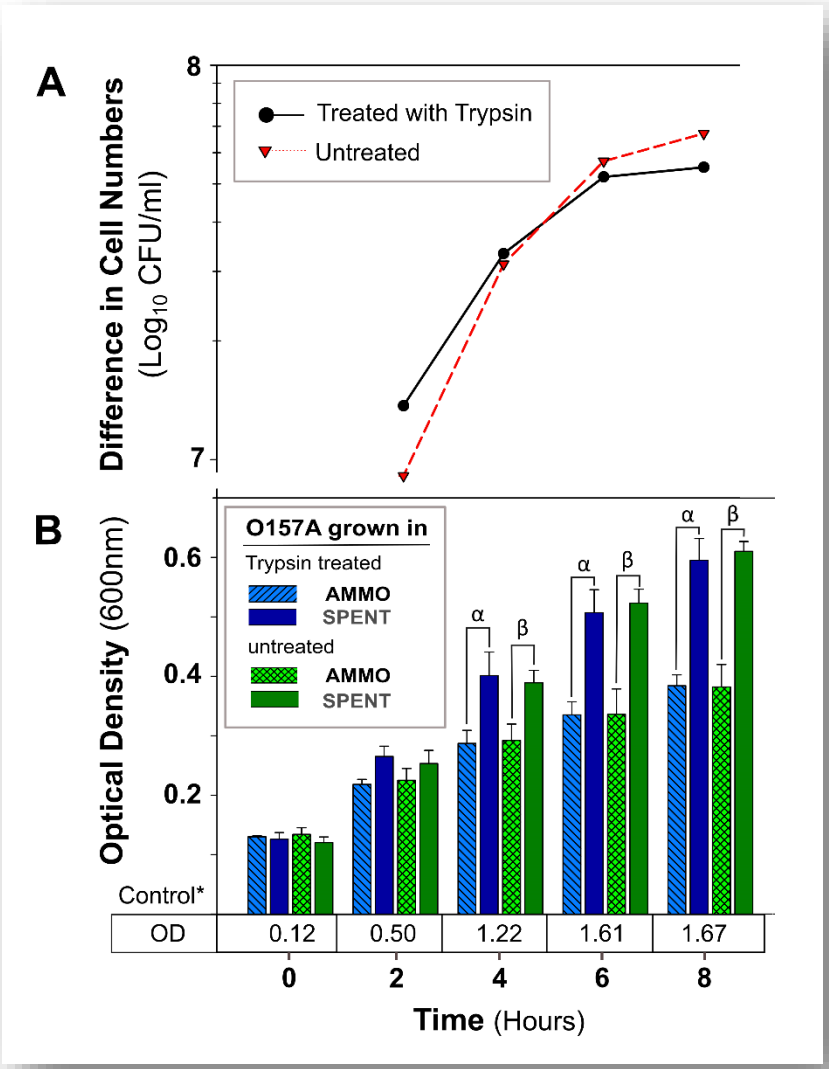


Figure 5.5. The effect of trypsin protease digestion of AMMO on growth inhibition of *E. coli* O157A over time.
A: The difference between the cell numbers for O157A in SPENT and in AMMO treated and untreated supernatant. B: The OD_{600nm} data for O157A grown in AMMO, in SPENT (treated and untreated) and *control in fresh EC as numerical value below the bars. Symbols: α and β, denote a significant difference between: O157A in AMMO and in SPENT for treated and untreated supernatants, respectively ($P < 0.05$).
Note: 1) AMMO is the cell free supernatant collected after 12 h *E. coli* O103F growth. SPENT is the cell free supernatant collected after 12 h *E. coli* O157A growth. 2) Bars are the calculated standard deviation for the treated or untreated AMMO and SPENT at each time point.

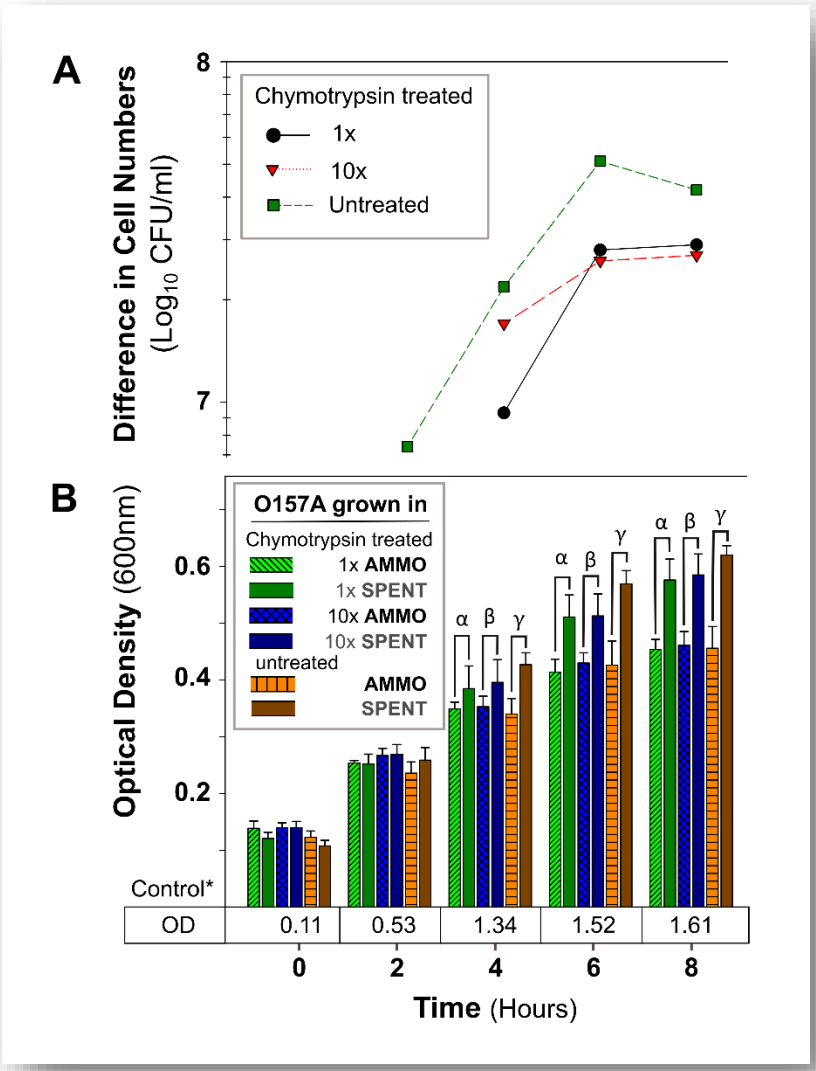


Figure 5.6. The effect of chymotrypsin protease digestion of AMMO on growth inhibition of *E. coli* O157A over time.
A: The difference between the cell numbers for O157A in SPENT and in AMMO with treated and untreated supernatants. **B:** The OD_{600nm} data for O157A grown in AMMO, in SPENT (treated and untreated) and *control in fresh EC as numerical value below the bars. Symbols: α, β and ɣ, denote a significant difference between: O157A in AMMO and in SPENT for 1X, 10X and untreated supernatants, respectively ($P < 0.05$). Time point = 2 difference in cell number data not shown for chymotrypsin treated AMMO and SPENT (1X and 10X) due to parallel OD_{600nm} data, and difference is ~zero.
Note: 1) AMMO is the cell free supernatant collected after 12 h *E. coli* O103F growth. SPENT is the cell free supernatant collected after 12 h *E. coli* O157A growth. 2) Bars are the calculated standard deviation for the treated or untreated AMMO and SPENT at each time point.

Table 5.1. Difference in cell density between O157A grown in SPENT versus O157A grown in AMMO at 8 h.

Treated	Supernatant Trials (Cell numbers in $\times 10^7$)					
	pH 3	**pH 11**	**Autoclaved**	**Trypsin**	**C-Trypsin 1x**	**C-Trypsin 10x**
SPENT minus AMMO*	5	6	3	5	3	3
Untreated Control	**pH**		**Autoclaved**	**Trypsin**	**Chymotrypsin**	
SPENT minus AMMO*	6		4	6	4	

*Differences in cell density.

Note: 1) Difference in cell density is approximate and calculated using previously generated growth curve numerical data for O157A (see 5.3.1). 2) AMMO is the cell free supernatant collected after 12 h *E. coli* O103F growth. SPENT is the cell free supernatant collected after 12 h *E. coli* O157A growth. 3) C-Trypsin = Chymotrypsin

5.3.2.2. Comparison of Inhibition Activity

The comparison of the average inhibition activity of AMMO across the different treatments (pH, autoclaving, trypsin and chymotrypsin) at 4, 6 and 8 h to untreated AMMO further revealed no effect of treatment on inhibition ($P > 0.05$) (Figure 5.7). Variability within the treated and untreated AMMO is minor as demonstrated by the standard deviation, shown as bars on the graph.

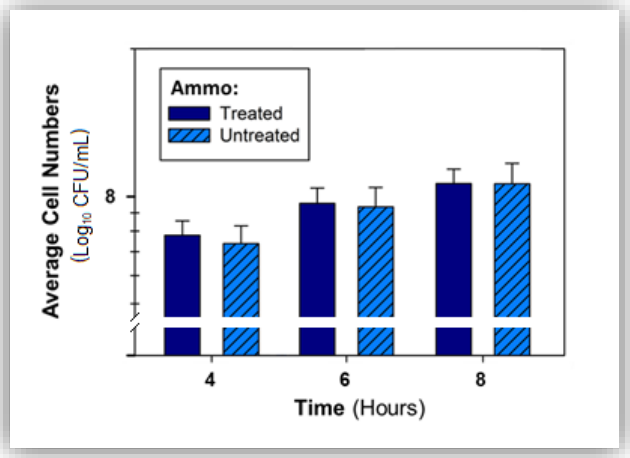


Figure 5.7. Comparison of average inhibition activity against *E.coli* O157 of treated AMMO across all treatments versus untreated AMMO (control) at time point 4, 6 and 8 hours.

Note: 1) Bars are the calculated standard deviation for the treated or untreated AMMO, respectively at each time point. 2) AMMO is the cell free supernatant collected after 12 h *E. coli* O103F growth.

5.4. Discussion

Interference competition is either contact dependent or contact independent. Due to the removal of viable bacterial cells in our study, the inhibition of the competitor growth was solely based on a contact independent mechanism. Contact independent inhibition occurs by one of the following known mechanisms: release of small molecules (less than 10kDa), proteins (larger than 10 kDa), membrane vesicles, tailocins and bacteriophages [115]. Across these mechanisms, a striking difference is the sensitivity of the active substances to physiochemical and biological treatments. Except for microcins none of those known inhibitory agents are reported to persist the combination of extreme heat and pressure (autoclaving). In addition to heat resistance, microcins excreted by Gram-negative bacteria have been reported to be resistant to extreme pH and are resistant to proteolysis [197].

5.4.1. AMMO Isolation Protocol Confirmation

A previous study in our laboratory [127], revealed a competitive O103F champion producing strong diffusible molecule (AMMO), which inhibited the growth of a wide range of STEC isolates including an STEC runner-up (O157A). Here we isolated and further investigated the AMMO[1] properties. The trials after isolation confirmed that O157A grown in AMMO was significantly inhibited compared to the O157A grown in SPENT[2] ($P < 0.05$). Additionally, the cell density was greater in SPENT than in AMMO and the difference in cell density between them increased over time. Furthermore, our data demonstrate that SPENT did not inhibit O103F growth, further confirming our

[1] **AMMO:** cell free supernatant collected after 12 h of growth of O103F in EC, containing **A**nti**M**icrobial **Mo**lecule(s).
[2] **SPENT:** the cell free supernatant collected after 12 h growth of O157A in EC.

previous results [127] and in fact O103F grown in SPENT had a higher cell density than O103F grown in AMMO. Feasibly, O103F has a metabolic advantage and is able to utilize nutrients not used by O157A as the metabolic pathways of different *E. coli* strains have been shown to vary in their ability to utilize different carbon sources [198]. Likely, O103F utilized a remaining nutrient source still present in SPENT. Comparison of O103F grown in AMMO to O157A grown in SPENT did not identify a significant difference in growth in the nutrient depleted media, providing evidence that the inhibition effect on O157A growth in AMMO is due to presence of an antimicrobial compound(s) and not due to a lack of nutrients in the media. In addition, grown in fresh media, O157A and O103F had a higher turbidity, demonstrating that inhibition (O157A) or no inhibition (O103F) was due to an external factor (AMMO synthesis) and not due to a strain fitness effect. In confirmation with our previous data, which demonstrated the production of a diffusible antimicrobial through an agarose barrier (no contact between competitors), the isolation of a diffusible and inhibitory AMMO occurred as *E. coli* has been reported to produce colicins and microcins targeting other *E. coli* and close relatives [128, 134]. Furthermore, our results revealed that AMMO is effective in the absence of live bacterial cells, which may alleviate regulatory hurdles probiotic bacteria encounter when being considered as a drug to treat human disease [199]. In accordance with previous studies, AMMO inhibited the STEC O157A and similar effects have been reported for the microcin producing probiotic *E. coli* Nissle which displaced pathogens from an inflamed gut [123] or reduced the number of STEC O157:H7 and O104:H4 in vitro [200].

Colicin and microcin producers target various systems in their prey and kill them using several mechanisms from forming cell wall channels to the corruption of the

intracellular machinery but are themselves resistant to this activity [201, 202]. Production of either microcins or colicins is always coupled with resistance genes and antidote synthesis. O157A succumbed to at least one AMMO produced by O103F, suggesting a lack of resistance. In contrast and in accordance with our previous results [127], O103F was not inhibited by any antimicrobial produced by O157A, suggesting O103F is resistant to any antimicrobials produced by O157A. Resistance to colicins and microcins can occur through production of these antimicrobials since production is always paired with synthesis of resistance proteins or through mutations in receptors or uptake mechanisms for the bacteriocin [201]. *E. coli* has been shown to produce more than one colicin and/or microcin [122]. Plasmids encoding bacteriocins are stably maintained in microbial populations most likely due to the lethal disadvantage of lost resistance [187]. Here, O103F may either have resistance to any O157A antimicrobial because it can produce the antimicrobial or has mutations in the receptors for the O157A antimicrobial as previous research revealed that *E. coli* can acquire resistance to bacteriocins in competition assays [124].

5.4.2. Investigation of AMMO Properties

The results across all trials revealed that AMMO was not affected by any of the treatments. Neither pH, heat (pressure) nor protease digestion affected the inhibitory properties of AMMO. In an applied scenario, the stability of the molecule would offer a wide range of technical treatment options for the implementation of purifications within an industrial-scale setting.

In each trial, there were no significant differences in O157A growth between treated and untreated AMMO and comparison of the inhibition activity of only the treated

AMMO revealed a similar inhibition pattern across all treatments, further demonstrating that treatment had no effect. Additionally, O157A growth was significantly inhibited ($P < 0.05$) when grown in presence of AMMO compared to SPENT, regardless of treatment, showing treatment did not affect the ability of AMMO to inhibit O157A growth. Concurrently, this effect was further demonstrated when examining cell numbers of O157A, which were more numerous in treated or untreated SPENT compared to treated or untreated AMMO in each experiment. Additionally, pure culture controls of O157A grew to higher cell numbers compared to O157A grown in SPENT, demonstrating that the inhibitory effect was due to the presence of AMMO and not due to any variation of viability. The evidence of physiochemical and enzymatic resistant characteristics of AMMO suggests that at least one microcin is produced by O103F.

Microcins are small antimicrobial peptides and have been mainly discovered in *E. coli* (one in *Klebsiella*) with a molecular weight of less than 10 kDa [203]. Microcins have a narrow killing range primarily targeting *E. coli* and their close relatives. Remarkably, despite being these "killing machines" the mode of action of many microcins is unknown, including microcin M, one of the microcins produced by *E. coli* Nissle, a probiotic that has been used for over 100 years to mitigate intestinal pathogens [197]. Microcin properties of extreme pH, protease and heat resistance [133], are commonly shared with bacteriocins from lactic acid bacteria, [134, 203]. Resistance to extreme pH or proteases varies among microcins. Microcin E492 is resistant to low pH [204], while J25 is resistant to both low and high pH extremes [205]. Others are resistant to the protease trypsin but not to chymotrypsin digestion, vice versa, or resistant to both [204]. Heat resistance is shared among microcins [204] and microcin J25 has been shown

to resist autoclaving (15 minutes at 121°C) [205]. The AMMO produced by O103F has analogous characteristics and plausibly is a molecule of similar design. Furthermore, our data demonstrated that the AMMO effect was no longer detectable after 24 h, an indicator for a "single-use" effect. Microcin C enters the host cells by mimicking nutrient properties and after cell uptake is cleaved by the host intracellular machinery to create the active compound [133]. Plausibly, this cleavage of microcin C is irreversible and AMMO undergoes a similar transformation.

Aside from those properties discussed above, microcins can be further differentiated from colicins because they are not induced by the bacterial SOS system and secreted microcins are not lethal to the producing cells [201]. To date 14 microcins have been identified [191] but only 8 have been structurally characterized [197].
Some microcins, such as H47 and I47 or C7 and C51 have similar microcin gene clusters and only differ by 3 or 1 additional genes, respectively [132]. Ultimately, AMMO produced by O103F appears to be a microcin with a change in the microcin gene cluster for an enhanced killing potential. Research comparing the microcin gene cluster of O103F with sequences of known microcins may elucidate such a probability.

The production of microcins by *E. coli* [134] is a tactic used to out-compete their competitors [187]. Microcins are deemed a potential replacement for antibiotics to mitigate pathogens both in human medicine and in the farm-to-fork continuum [193]. Antibiotic resistance is a global challenge evoked by their over use in the livestock industry and human medicine, which led to emergence of resistant pathogens.
Contrary to traditional broadband antibiotics, microcins have a narrow, species specific killing range [189, 193]. Since his discovery about 100 years ago in the battlefields of

WWI, the *E. coli* Nissle strain has been effectively used to treat human intestinal infections and the specific Nissle microcins are considered the active antimicrobial substance [121, 123].

As a food borne pathogen, STEC cause severe intestinal infections in humans [29, 180] and effective mitigation strategies are lacking [107, 168, 169]. Use of probiotic bacteria that can out-compete STEC is being considered as an approach to eliminate this pathogen and colicin producers isolated from sheep fecal samples have been shown to inhibit STEC O157:H7 growth [195].

A previous study in our laboratory showed a diverse STEC growth inhibition by AMMO produced by O103F [127] and the results from this study strongly suggest that O103F produces at least one very effective microcin. Microcins are considered part of the killing repertoire of the probiotic *E. coli* Nissle strain. The type of microcins produced by *E. coli* O103F holds the potential to be used as a STEC mitigation strategy. In logical stepwise approaches we aim to further evaluate this molecule and gain more knowledge on the antimicrobial properties produced by O103F. Future studies examining the genome and/or plasmid sequences of O103F for microcins, the biochemical structure of AMMO and/or physiological properties including metabolic pathways are required to elucidate the therapeutic potential of this strain and the antimicrobial it produces.

5.5. Conclusion

Agriculture, as an evolutionary Big Bang triggered a new microbial multiverse and changed the dynamics of human and microbe (pathogen) interactions. Most recent global transformation including biological technologies (and the use of antimicrobial substances) have fundamentally altered the way, size, speed and scope of how we

produce and consume food, but in addition promoted the emergence of virulent microbes with resistance to antibiotics. Microcin molecules are regarded as a potential alternative to antibiotics. Therapeutic microcin properties may warrant the use as a next generation control strategy in livestock production systems or to mitigate pathogens after human infections. Ultimately, the microcin we tested here inhibited the growth of *E. coli* pathogens after a range of physiochemical and enzymatic inactivation treatments. The antimicrobial properties suggest that this type of isolated molecule could be an antibiotic candidate even in absence of the viable *E. coli* producer. The resistance to treatments (pH, autoclaving, trypsin and chymotrypsin) makes the molecule an ideal candidate for industrial-scale isolation and purification technologies.

CHAPTER VI: General Discussion

6.1 Background

Emerging pathogens, in particular those with resistance to antibiotics, are a major global concern, testing current food safety protocols and strain healthcare resources due to outbreaks in the presence of extremely virulent strains. The World Health Organization (WHO) identified that over half of the deaths worldwide in 2016 were traced to 10 main causes. Among those are respiratory and diarrheal diseases which together were responsible for approximately 4.4 million deaths [206]. Another study examining emerging infectious diseases (EID) found that 54% of EID's are caused by bacteria [207]. Both of the above mentioned studies highlight that despite advances in health technologies, pathogens causing human disease are still a grave concern. The focus of this study was to use novel technologies to identify virulent human pathogenic strains and to examine an alternate strategy to combat either human infections or protect the farm to fork continuum. The data of the current study indicate that we need to change our approach to food safety monitoring to take into account emerging diseases and that alternate mitigation strategies exist and are potential alternatives to combat these emerging pathogens in order to account for the continual evolving relationship between humans and agriculture (population evolution), the effect of environmental factors and bacterial adaptations on emerging diseases.

6.2 Population Evolution

As humans evolved from living in small nomadic communities to settle permanently in stationary settlements they triggered an evolutionary "Big Bang" by provoking changes in corresponding microbial communities. The shift from nomadic

hunter-gatherers to settled human communities living in one place and the advent of agriculture changed how humans and animals interacted [185]. Agriculture altered how humans produced food and created areas with dense populations of humans and livestock, such as cattle (Figure 6.1) [5, 185]. Farming increased the amount of food readily available to humans and consequently populations flourished. This has led to the intensification of agriculture using newly developed technologies [208] to meet growing demands, thus creating a cycle of increasing human populations and the need to intensify agricultural operations to meet the physiological need for food. In fact, the world population is projected to grow to ~9 billion people by the year 2050 and in order to feed this growing population, food production is projected to increase approximately 70% to meet this demand between 2007 to 2050 and respond to the current conundrum surrounding food waste [209]. It is estimated that in developing countries food supply will need to double to meet this increasing need. Ultimately, intensification and a growing population will further cluster humans and animals in condensed areas.

Agglomerating humans and livestock also created an optimal ecosystem for microbial communities to adapt, compete and proliferate. A sedentary lifestyle ensured that humans and animals were present in one area for prolonged periods of time, leading to increased bacterial contamination of human living areas [210] and increasing potential number of hosts for zoonotic pathogens, many asymptomatically carried by livestock [5]. The increase in both human and animal populations also led to an increase in waste production and improper segregation of drinking water, waste and unsanitary living conditions were linked to the spread of disease. Improving sanitary conditions in the 19th

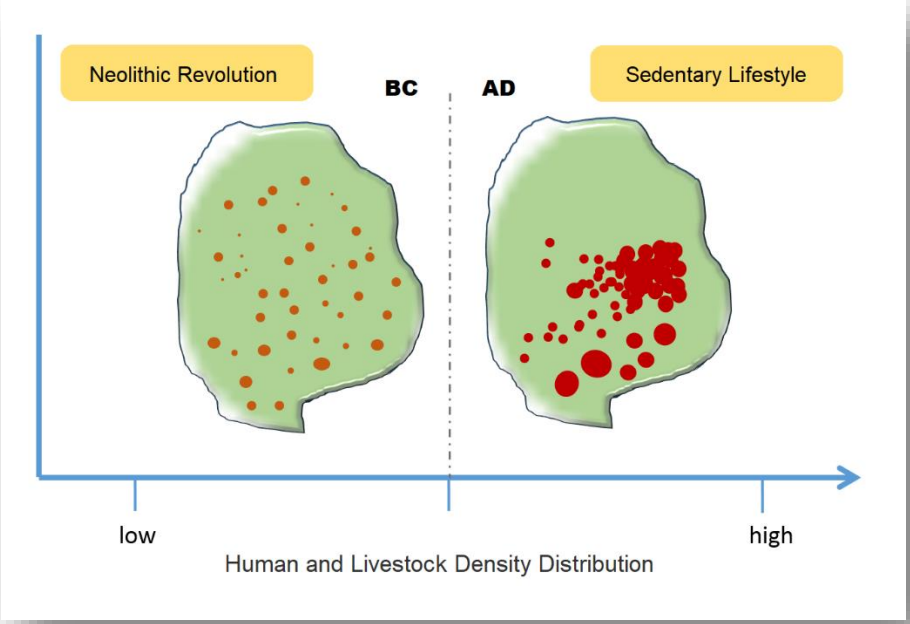


Figure 6.1. Human and animal density extrapolation from the beginning of the Neolithic revolution to intensification of agriculture today.

century had health benefits, reduced the rate of child mortality and prolonged human life spans [211]. Increasing waste also compounds the potential to contaminate waterways and land and increase pathogen dissemination across large populations [208]. While a sedentary lifestyle changed the interaction between human and animals, it has also changed the interaction between humans.

With the Neolithic revolution, humans settled in towns and cities, people interacted and still interact with each other on a daily basis, from engaging in social activities to business transactions. This increase in interacting humans in one area has also led to increases in diseases [210]. Diseases have always affected humans but the types of disease encountered have changed as humans evolved. Hunter-gatherers had different disease challenges than current humans [210] as ancestral humans could not support epidemic diseases due to small populations [185]. Epidemic diseases are

considered “new world” diseases that can only be sustained in large populations created by the rise of agriculture and many emerging disease challenges are coined “crowd” diseases due to this necessity [185]. Anywhere in our daily human interactions, individuals could potentially be ill or an asymptomatic carrier of a disease, spreading disease through a population. Asymptomatic carriers are infected with a disease but are symptom-free, spreading disease while remaining undetected which can undermine control strategies for disease prevention/spread [212]. Further aggravating disease control strategies is the continual emergence of new technologies that permit diseases to “travel the world” in a short amount of time.

Speed for hunter-gatherers to travel long distances was limited and one can say that “old world” diseases travelled at “horse speed”. As technology advanced, “horse speed” has changed to “airplane speed”; where horse-drawn stagecoach could travel on average 60 to 70 miles a day [213], airplanes can travel 9500 miles in under 19 h [214]. Infected humans can travel to any destination world-wide prior to exhibiting symptoms of that disease [211]. Microbes are being dispersed globally either through human travel or through commodities, such as food or primary agricultural products due to the globalization of trade [215]. A study examining horizontal gene transfer (HGT) in the human microbiome identified that recent HGT events can be identified across the different continents demonstrating microbe “travel” activities [216]. Outbreaks are no longer confined to a remote and/or isolated areas or originating countries, as demonstrated with the 2014-2016 Ebola outbreak. The Ebola outbreak started in Guinea but quickly spread to neighboring countries, eventually further to 7 other countries including the USA [217]. As populations continue to increase in size, both human and

livestock, so will the corresponding microbial communities leading to increased disease dissemination and the emergence of new pathogens that can only be sustained in large populations.

6.3 Environmental Factors: Climate Change

One of the biggest challenges for all living creatures including microbes are changes within the Earth's ecosphere and/or climate system. Recent changes in temperature profiles are starting to shift ecosystems and affect agriculture production systems [218]. The reduction of carbon emissions are being discussed as one central point to limit climate change and while agriculture is severely threatened by climate change it is also one of the biggest industries contributing to emissions [219]. Agriculture industries are responsible to a certain extent of the world's greenhouse gas (GHG) emissions [208] demonstrating farming is contributing to the issue while being adversely affected by global warming [218, 219].

As the human population continues to grow agriculture intensification will be needed to meet the demand of food supply world-wide. However, change in climate and environmental conditions necessitate that agriculture will need to adapt to the new conditions to protect food security [218]. Climate-adapted crops will be needed to survive periods of extreme weather [218] and farmers will need to change current practices to mitigate the effect of climate and environmental changes [219].

As mentioned, climate change affects all living organisms and climate changes will also affect microbial communities [220]. A shift of climate with increases in temperature can lead to shifting bacterial growth by allowing those bacteria suited to growth in high temperatures to flourish. Possibly, increases in carbon dioxide may favor

bacteria adapted to growth in such conditions and if these bacteria are pathogenic, this could potentially lead to increases in human disease. For example, *E. coli* pathogens have larger genomes than their non-pathogenic counterparts [18] and, possibly may have adaptations that are better suited to the environments created by the changing climate conditions. Also, bacteria can acquire new genetic content using mechanisms such as HGT [216] and altering environmental conditions may promote HGT events across the globe. Climate change has also lead to periods of heavy rain and flooding which can displace bacteria to new habitats, affect water quality and change the interaction between pathogens and humans [220]. Contaminated water is one of the ways that pathogens infect humans and waterborne outbreaks can affect large geographical areas [49].

Climate change will also change farming practices. Currently, irrigation of crops accounts for ~90% of world-wide consumption of water, mainly supplied by ground water leading to decreased availability of ground water globally [221]. Changes in temperature induced by climate change could increase the amount of water used for irrigation which taxes water systems and lowers the amount of water remaining for other uses [220]. The process of irrigation itself is also thought to increase GHG emissions through various mechanisms, from using fossil-fuel derived pumping systems to encouraging soil microbial processes such as denitrification, which produces a more potent GHG which may potentially lead to more global warming [221]. Intensification of agricultural processes also increases irrigation needs, depleting already stressed water supplies but also increases the potential for polluting waterways and food with microbes, increasing microbial dissemination [208].

Embedded in the earth biosphere, agriculture, human population growth and the environment are engaged in a never-ending cycle where the anthropogenic solution to one problem potentially creates another (*action and reaction*). Increases in population also decreases available farmland requiring condensation and intensification of farming operations and, destroys ecosystems to increase available land, all while agglomerating animals, humans and microbes, increasing waste production and potential for disease outbreaks. Agriculture intensification is needed for future food security but current practices are altering our ecosystems and need to change in order to protect the environment and prevent the potential disasters associated with climate change.

6.4 Bacterial Adaptation and Antimicrobial Resistance

Increasing human and livestock populations has also increased and diversified microbial populations. To put it in perspective the microbes in our own microbiome out-number human cells by at least 10-fold in each human [3] and for example ruminants with a stomach volume of 100L or more are filled with a diverse array of microorganisms [222], multiply that by each individual equals an incomparable number of bacteria. As both of these populations increase, bacteria, commensal and pathogenic, flourish and have a continual supply of hosts. Furthermore, these bacteria are easily spread through interactions among humans and animals [5], food supply lines and through waste [208] exposing them to new habitats, new microbes and new hosts.

Bacteria are constantly adapting to their environments and exchanging genetic information with each other through processes such as HGT and continually evolving [216]. They gain genes that confer an advantage and lose those that are no longer needed, such as those for antibiotic resistance. Previously, bacterial disease was thought

to have one cause (1 bacteria = disease) but this is not the case and variants of a bacterium can cause the same disease [211], variants "created" through processes such as HGT events. Mingling of bacteria in environments such as cattle and human microbiomes allows for the exchange of genetic information with related and unrelated bacteria which can lead to the emergence of novel pathogens, such as STEAEC, a combination of STEC and EAEC. Many pathogens have evolved from non-pathogenic counterparts that are part of the normal gut flora of humans and livestock [5]. Zoonotic pathogens are often carried by livestock such as cattle and transferred to humans through interactions between humans and agricultural products.

An example of how interactions between humans and agriculture have shaped pathogens was demonstrated with a study revealing that the human *E. coli* pathogenic STEC strains have most likely emerged from bovine commensal *E. coli* [223]. The study demonstrated that important virulence genes such as LEE are present in bovine commensal *E. coli* and that there is selection pressure to maintain these strains in the bovine environment. However many variants of STEC exist and examining STEC recovered from HUS patients using multilocus sequence typing (MLST) revealed that they do not cluster together suggesting that different strains have emerged various times over the years with unique sets of genes that cluster together as a group [224]. Examining how STEC strains evolved and relate to one another suggests that they are all most likely linked by an ancestral strain and as they continue to evolve potentially novel STEC will emerge (Figure 7.2). The question remains, what is the next step in their evolution? Will the next cluster be a novel emerging strain or a group of STEC, such as the German STEAEC?

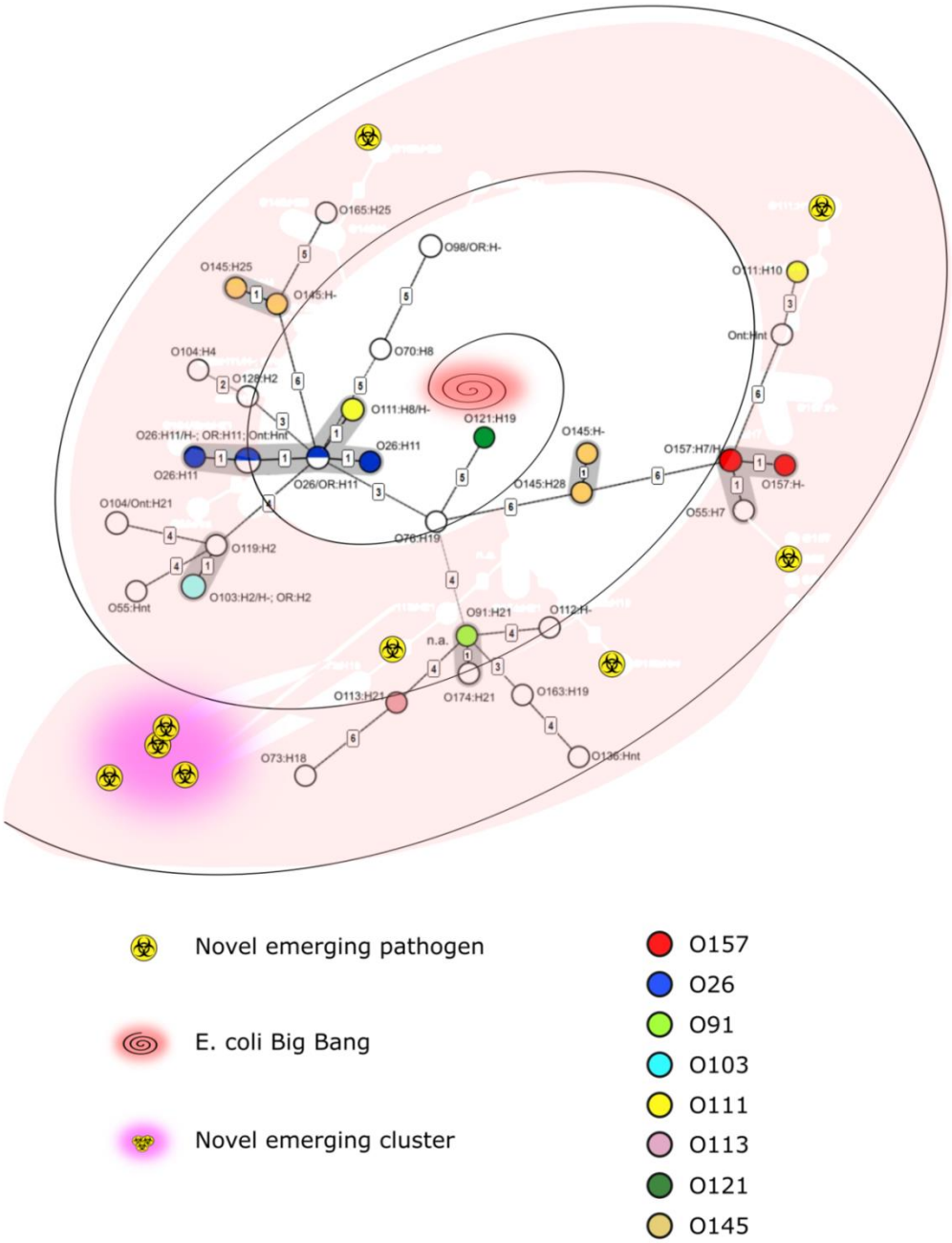


Figure 6.2. Schematic merging of current knowledge linking various STEC into a tree based on multilocus sequence typing with the idea that all STEC are related by an ancestral strain (*E. coli* Big Bang) and as they evolve novel strains will most likely emerge.
*MLST correlation of *E. coli* O-type correlation adapted from: Mellmann et al.[224]
Note: The number identified in each square is the number of shared alleles between sequence types.

Cattle are considered a main reservoir for STEC [8] and, without the interaction between humans and livestock and their products, human pathogens such as STEC, would most likely not be dispersed to humans, preventing infection altogether. Over 400 STEC serotypes have been identified in cattle [30] and any one of these strains can exchange genetic content with the myriad of microbes identified in the rumen to create an especially virulent STEC. However, current STEC identification protocols for food samples in the USA focus only on the "top 7" STEC, O26, O45, O103, O111, O121, O145 and O157 [64]. My study highlights this vulnerability showing that potentially virulent bacterial strains can be detected using novel molecular technologies that would be missed using standard testing protocols. Furthermore, this strain (*E. coli* O178) was 4 times more numerous than serogroup O157 in my study, demonstrating that current food safety protocols need to be updated to take into account the potential for emerging pathogens. While this study focused on STEC, novel emerging pathogens are a global concern [207] demonstrating that disease identification and food safety protocols need to adapt to changing microbial populations. Intensification of livestock operations to meet food demand due to increasing human population is likely to increase the amount of microbes present and the potential for emerging pathogens.

As bacteria adapt to their environments and exchange genetic information they also adapt to current mitigation strategies used in human medicine and along the farm to fork continuum. The discovery of antibiotics led to the idea that disease treatment was going to be revolutionized due to these "wonder drugs" [211] and good hygiene was no longer going to be needed as the main mitigation strategy against human pathogens [225]. However, as diverse bacteria acquired antibiotic resistant genes it has led to the rise of

antimicrobial resistance (AMR) to antibiotics. AMR has been linked to overuse and improper use of antibiotics, especially when used in livestock operations. The dense rumen microbial habitat [222] has been shown to rapidly share AMR genes across close and distant relatives through processes such as HGT [225].

However, AMR is not only an issue in agricultural operations and it is thought that improper disposal of human and livestock waste into the environment have severely compounded the issue [225]. Many human pathogens are resistant to antibiotics and very few effective antibiotics now remain and are quickly becoming obsolete as AMR increases; there is a need for alternative treatment strategies or discovery of new antimicrobials to combat human infections. Microbes such as those that reside in our intestines face intense competition due to limited space and resources and have evolved various warfare strategies to ensure their survival [115]. One of these strategies is the production of antagonistic factors such as toxins to eliminate competing bacteria and this mechanism has potential to be used as alternative mitigation strategy against pathogens. My study identified a strong non-pathogenic *E. coli* competitor that produces a diffusible antimicrobial that has potential to be used as a therapeutic to mitigate STEC. Furthermore, the results from my study suggest this antimicrobial may be unique and warrants further research. The study focused on STEC due to possible adverse effects of antibiotics to mitigate this pathogen but the general concept can be adapted to a wide range of old as well as novel emerging pathogens.

The progression of AMR in various environments such as soil, livestock and human microbiomes, strongly suggests that emerging pathogens are likely to be resistant or to become resistant to current antibiotics. Alternate mitigation strategies will be

required before our only mitigation strategy against all pathogens becomes good hygiene practice [225]. Potentially, the combat strategies employed by bacteria to out-compete rivals could be used for pathogen mitigation as bacteria have evolved to be combat specialists generating various weapons to ensure their survival and, these mechanisms could be adapted to alleviate disease [115]. However, it is important to note that novel mitigation strategies and the search for new antimicrobials will always be a continual process due to bacterial evolution. Resistance should always be viewed as "if resistance is biochemically possible, it will occur" [225].

6.5 Overall Conclusion

Many factors affect the threat of emerging pathogens. Interactions between humans and agriculture, how advancement in agriculture is contributing to environmental changes and, how bacteria adapt to these changing conditions through resilience and genetic plasticity, demonstrate a "vicious continual cycle" where one affects the other and vice-versa. The reality is that, the pathogen of today may not be the pathogen of tomorrow and "prehistoric" safety policies focusing on specific strains of a pathogen require adaptation to be more encompassing of the possibilities for novel emerging pathogens with constant review of how pathogens are evolving. Equally essential is the need to develop alternative strategies to mitigate pathogens to help protect food supply lines and to alleviate human infections. Pathogens will continue to evolve and our response must to be equally adaptable.

CHAPTER VII: Future Outlook

Novel technologies, such as ddPCR are considered an advancement compared to real-time PCR assays by offering absolute quantification without the need for internal standards. Furthermore, ddPCR is more robust to inhibition which is often a concern when screening complex matrices and/or using qualitative assays. This study utilized ddPCR technology to quantify generic *E. coli*, specific serogroups of *E. coli* O178 and O157 as well as *stx*$_1$ and *stx*$_2$ genes in enriched samples. Overall the results identified that qualitative and quantitative data do not follow the same trends in seasonality currently accepted for O157 and identified that high-*stx* events occur that do not correspond to O157, suggesting other STEC are present. The results revealed that ddPCR is a valuable tool in pathogen monitoring and can highlight potential deficiencies in current monitoring protocols but future studies are needed to fully elucidate the potential of this technology.

Studies examining:

- characterization of other virulent STEC strains would allow us to identify high shedding events or seasonality in bacterial contamination,
- test other virulence genes associated with STEC and seasonal trends in infection,
- hypothesize potential use of virulence profiles to identify STEC along the farm-to-fork continuum instead of serogroup monitoring,
- potential of adopting ddPCR as a "gold standard" for STEC monitoring to improve surveillance and mitigation strategies.

Mitigation strategies for STEC along the farm to fork chain have variable efficacy and under certain circumstances use of antibiotics for medical intervention is considered controversial. Alternate mechanisms such as use of competitive interactions are being considered to mitigate STEC and protect food supply lines and/or alleviate human infections. Using various competition settings, a strong non-pathogenic *E. coli* O103F was identified. A physical barrier between opponent bacteria revealed that this champion produced a diffusible antimicrobial molecule which impaired the growth of competitors. Further evaluation of this antimicrobial revealed that the molecule is most likely a microcin. The antimicrobial produced by *E. coli* O103F has potential to mitigate STEC but future studies are needed prior to adapting this antimicrobial for use along the farm-to-fork continuum or in human or animal therapeutics.

Future studies should examine:

- the genome and/or plasmid sequences found in O103F to allow identification of microcins in the bacterium,
- purify the antimicrobial molecule and characterize its structure,
- develop an antagonistic spectrum of the molecule produced by O103F for enteric pathogens other than STEC,
- test the antimicrobial molecule found in O103F on food samples to determine potential applications.

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APPENDIX I
Protocol on the development and validation of O178 specific primers

A1.1 Primer Design:

Escherichia coli O178 O-serogroup primers were designed with available sequence data of *Escherichia coli* genes for O-antigen biosynthetic locus of AB812075 and KJ778799 from the NCBI Genbank using Geneious software (Geneious 9.x). Three different primer sets were generated and tested to identify the optimum primer sets for the surveillance screening and ddPCR assays (Table A1.1). All 6 primer sequence data were compared against an internal Geneious data base comprising 196 designated O-groups[3] to be specific for O178. All primer sets were tested against a sequenced O178 positive and examined for optimal PCR conditions and specificity.

A1.2 O178 Positive Control Enrichment and DNA Extraction

The *E. coli* O178 positive control was streaked from glycerol stocks onto MacConkey Agar (MAC, BD, Sparks, NV, USA). Plates were incubated overnight (16–18 h) at 37 °C. A single colony was selected from each plate and inoculated into 10 mL *E. coli* broth (EC) (EMD Millipore, Etobicoke, ON, Canada) and incubated overnight at 37 °C with shaking at 150 rpm. A 1 mL aliquot of the enriched culture was centrifuged at 8,000 X g for 10 min before extraction of DNA from the pellet using the NucleoSpin Tissue Kit (Macherey-Nagel). The extracted O178 positive control DNA was subsequently used for PCR validation of the primers.

[3] DebRoy C, Fratamico PM, Yan X, Baranzoni G, Liu Y, Needleman DS, et al.: **Comparison of O-antigen gene clusters of all O-serogroups of *Escherichia coli* and proposal for adopting a new nomenclature for O-typing**. *PLoS ONE*. 2016; 11(1): e0147434

Table A1.1. Primer sequence and amplicon size for O178 primer validation PCR screening

Primer Label	Primer Sequence (5'-3')	Amplicon Size
O178 - 1	GCATTCGGTAACGGCTTTGC	715 bp
O178 - 2	CTCTGGTTAACCCCAGCCAA	
O178 - 3	CCAGAGCTAAACTCAGAGGGG	112 bp
O178 - 4	GTGTGTTGAGTGTTGGCTCA	
O178 - 5	TCGGACGTATTTGCTGGCGCT	138 bp
O178 - 6	TCTGGGGGTCATAATTCAACTGGT	

A1.3 Optimization of PCR Conditions:

All 3 primer sets (final concentration 200nM) targeting the O178 O-antigen gene cluster were subjected to a PCR gradient with following annealing temperatures: 65°C, 63.3°C, 59°C and 55.7°C. HotStarTaq polymerase (Qiagen) was used with the following cycling conditions: initial denaturation: 95°C– 5 min, 35 cycles: 95°C– 30sec, specific annealing temperature (see above)– 45sec, 72°C– 10sec and final extension: 72°C– 5 min. All PCRs were carried out with 2µl DNA template on Bio-Rad cyclers C1000 or T100 and amplicons were visualized with GelRed (Biotium) stain using a Molecular Imager GelDoc-XR+ (Bio-Rad). The following gel electrophoresis conditions were used for all agarose gels made with sodium borate (SB) buffer[4]: 2% gel, ran at 150 volts for 45 minutes and post-stained for 30 minutes in 3X Gel red. Results determined that all temperatures produced equally strong bands of the correct size for each of the primer sets (Figure A1.1).

[4] Brody, J.R. and Kern, S.E.: **History and principles of conductive media for standard DNA electrophoresis**. *Analytical Biochemistry*. 2004; 333: 1-13

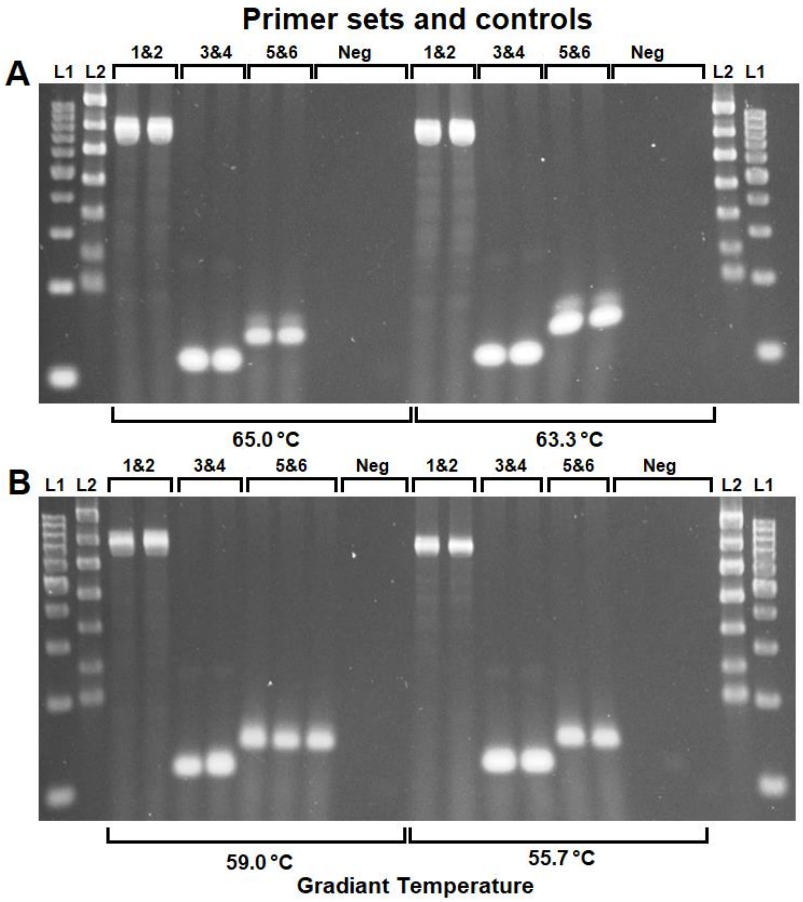

Figure A1.1- O178 positive control gradient PCR agarose gel results with all 3 primer sets (1&2, 3&4 and 5&6) at 4 different temperatures (65.0°C, 63.3°C, 59.0°C and 55.7°C). **A** – The gradient results for 65.0°C and 63.3°C. **B** – The gradient results for 59.0°C and 55.7°C. Note: 1) The gel conditions are as follows: 2% gel ran at 150 volts for 45 minutes and post-stained in 3X Gel Red for 30 minutes. 2) L1 is the 100bp ladder and L2 is the 7-plex ladder[5].

[5] Conrad CC, Stanford K, McAllister TA, Thomas J, Reuter T: **Further development of sample preparation and detection methods for O157 and the top 6 non-O157 STEC serogroups in cattle feces**. *Journal of Microbiological Methods*. 2014; **105**(Supplement C):22-30.

A1.4 Primer Specificity

All three primer sets were first examined for specificity by comparing the primer sequences with genome sequence data from the NCBI Genbank. Using PCR, specificity was further tested against DNA from the top 7 STEC (O26, O45, O103, O111, O121, O145 and O157) (Figure A1.2) with all 3 primer sets (200nM) and HotStar Taq polymerase with the following cycling conditions: initial denaturation: 95°C– 5 min, 35 cycles: 95°C– 30sec, 59°C 45sec, 72°C– 10sec and final extension: 72°C– 5 min. Primer set O178 1&2 (715bp) revealed cross activity with another non-O178 *E. coli* strain and was subsequently excluded (data not shown). Primer sets (O178 3&4 and O178 5&6) were target specific for O178 using both tests and were used in a multiplex PCR for surveillance screening.

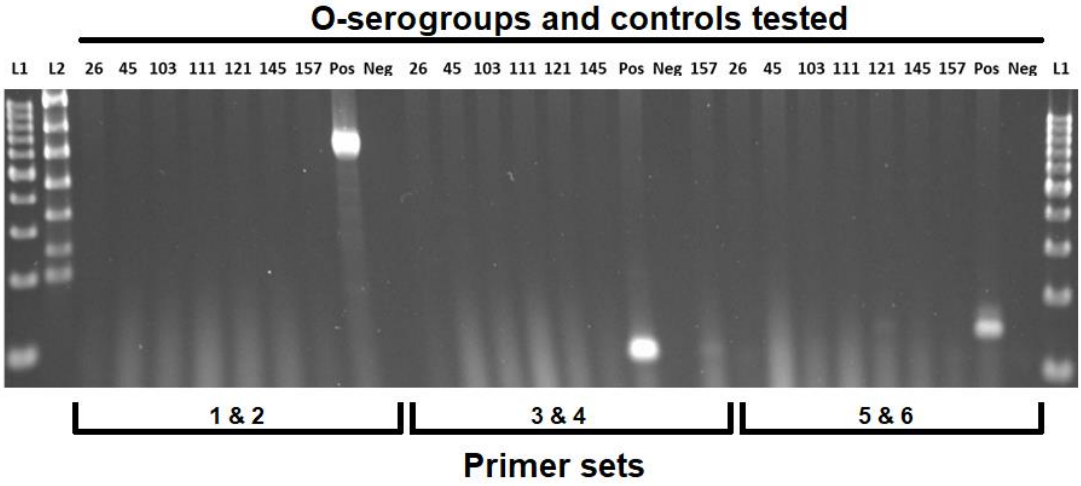

Figure A1.2 - O178 primer specificity PCR trial agarose gel result for all 3 primer sets 1&2, 3&4 and 5&6) against DNA from O26, O45, O103, O111, O121, O145and O178. Note: 1) The gel conditions are as follows: 2% gel ran at 150 volts and post-stained in 3X Gel Red for 30 minutes. 2) L1 is the 100bp ladder and L2 is the 7-plex ladder.

APPENDIX II
Supplemental figure as submitted for Chapter III

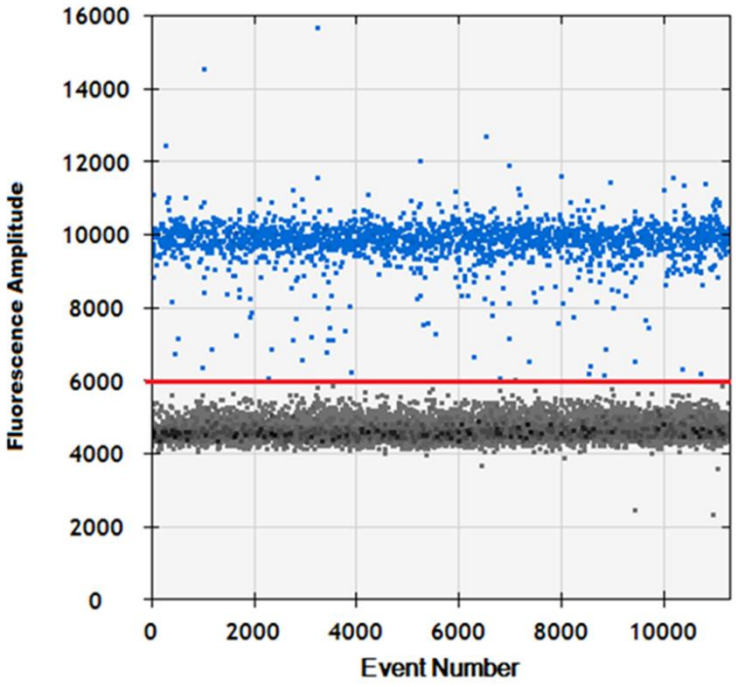

Figure A2.1. One-Dimensional snapshot of one ddPCR data set detecting O178 indicating the event number (droplets) versus the fluorescence amplitude shows the separation between positive droplets (blue) and the negative droplets (black).
Droplets above the red threshold line (calculated by Quantasoft) are positive and those below negative.

APPENDIX III
Supplemental figures as submitted for Chapter IV

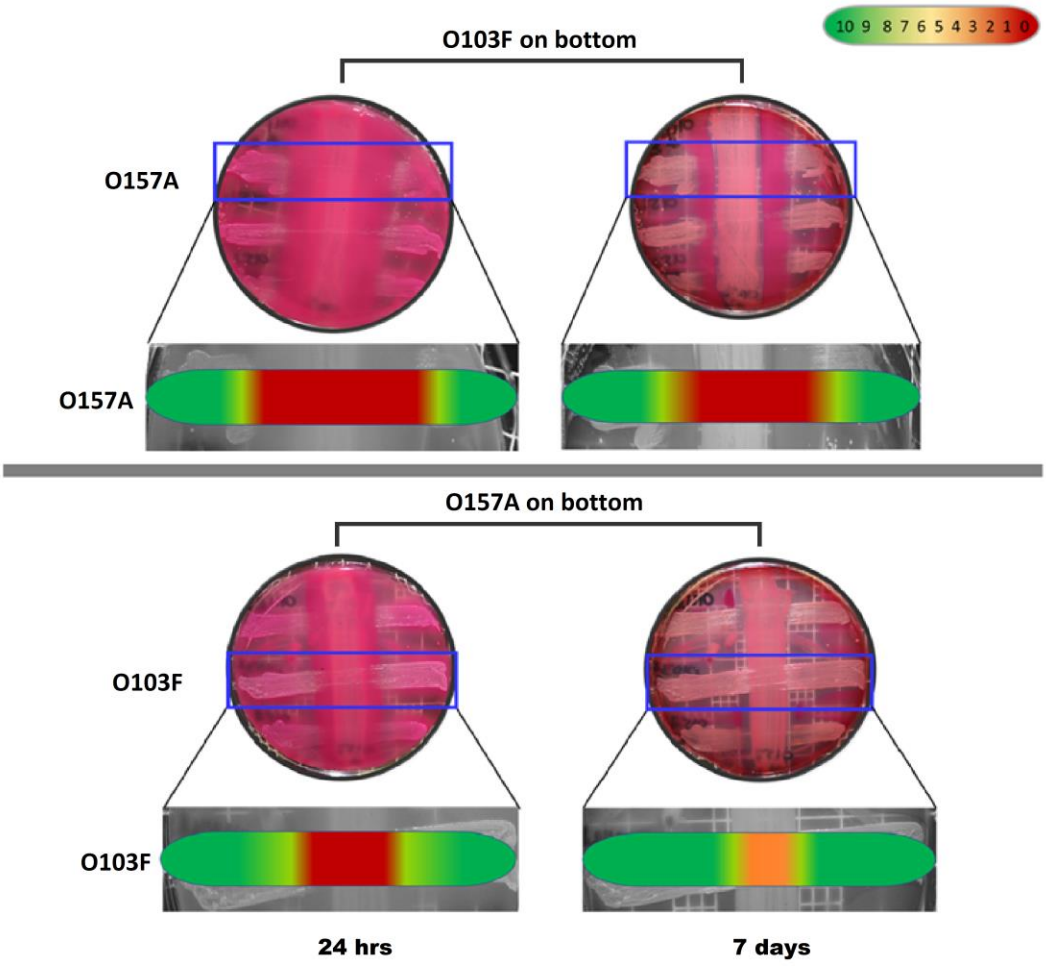

Figure A3.1. Omelette method results for *Escherichia coli* O103F and O157A at 24 h and 7 days against each other examining zones of no growth. Zone of no growth strength is graded with green (10) being thick growth and red (0) being no growth on plates. Note: Selection corresponds to either O103F or O157A, other weak competitors were also grown on the plates but they are not discussed within the paper.

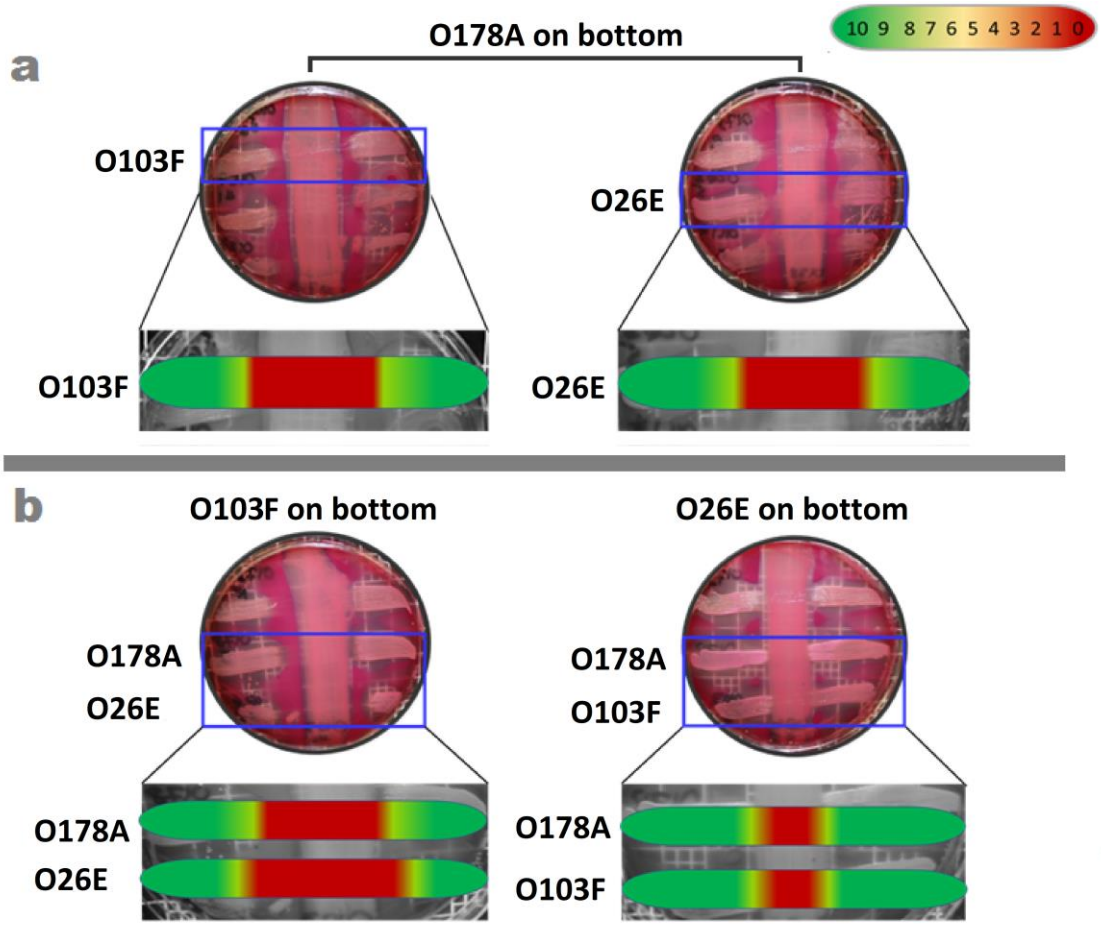

Figure A3.2. Omelette method results for *Escherichia coli* O26E, O103F and O178A against each other examining zones of no growth at day 7. Zone of no growth strength is graded with green (10) being thick growth and red (0) being no growth on plates. Note: Selection corresponds to O26E, O103F or O178A, other weak competitors were also grown on the plates but they are not discussed within the paper.

APPENDIX IV
Escherichia coli: using physiological cues to turn on antimicrobial molecule production

A4.1 Introduction

Physiological adaptations are defined as "metabolic or physiologic adjustment within the cell/tissues of an organism in response to an environmental stimulus resulting in an altered ability of that organism to cope with its changing environment" [226][6]. When bacteria encounter environmental changes, they trigger modifications in gene expression allowing for physiological adaptive responses [227].

E. coli has developed unique/specific adaptations to survive and persist in the intestinal tract of warm-blooded animals such as humans. In fact, a comparison of genomes from environmental and enteric *E. coli* identified genes unique to each biotope [228]. Along the gastro intestinal tract (GIT), *E. coli* encounters stressors, including the low pH of the stomach, bile in the small intestine (a bacteriocidal) and competing microflora [11, 229, 230].

A previously described strong *E. coli* O103 competitor [127] has been shown to produce a diffusible antimicrobial molecule with pronounced microcin characteristics [231]. However, it is unknown if molecule production in *E. coli* O103F is affected by physiological properties. *E. coli* O157 has been shown to upregulate/downregulate specific genes in response to: **I**) physiological changes along the GIT [232] and **II**) carbon nutrients [233]. The objectives of this study were to use in vitro experiments to examine the effect of carbon source, bile salts and growth time on *E. coli* O103F

[6] All references in Appendix IV are part of the numbered references list in the reference section.

antimicrobial production and to investigate molecule specificity to define the target range of the antimicrobial.

A4.2 Materials and Methods

A4.2.1. Bacterial Strains: Cultures, Media and Culture Conditions

Both *E. coli* strains (O103F and O157A) used in this study were described and grown as described previously [127]. *Klebsiella* and *Salmonella* isolates were streaked from glycerol stocks onto tryptic soy agar (TSA) and incubated overnight (16–18 h) at 37°C. A single colony was selected from each plate and inoculated into tryptic soy broth (TSB) incubated overnight at 37 °C with shaking at 150 rpm.

A4.2.2 Effect of Carbon Source on Molecule Production

Various media were used to examine *E. coli* O103F molecule production to determine the effect of various nutrients and selected components on the ability of O103F to inhibit *E. coli* O157A growth. Liquid media: TSB, Luria-Bertani (LB), TSB+lactose (TSB with 2.5g of lactose added) or EC – used for the molecule isolation assay (described: Paquette et al, 2019 [231 - Chapter V]) and solid media Luria-Bertani agar (LBA), TSA, nutrient agar, Mueller-Hinton II (MH) agar or MacConkey agar (MAC) – used for the omelette method (described: Paquette et al, 2018 [127 - Chapter IV]).

A4.2.3. Effect of Bile Salts on Molecule Production

The effect of bile salts was examined using the molecule isolation assay as previously described [231] with TSB (no bile salts – 0% bile salts), 50:50 mix of TSB and EC (0.75g/L bile salts in liquid media – 50% bile salts) and EC (1.5g/L bile salts in liquid media – 100% bile salts) as growth medium. In short, both O103F and O157A were separately grown for 12 h in the above media and subsequently filter-sterilized to

remove all the bacterial cells. The filtered supernatant was then used as inoculation media for O157A (inhibition assay) and differences in growth between the supernatants was monitored using optical density (OD_{600nm})

A4.2.4 Effect of *E. coli* O103F Growth Time on Molecule Production

To test the effect of *E. coli* O103F growth time, a continuous culture of *E. coli* O103F was grown for 4, 6, 8, 10 and 12 h in EC, a sample was removed at each time point for the molecule isolation assay. *E. coli* O157A was grown to match the OD_{600nm} measurement of O103F to ensure equal nutrient deletion in both isolated supernatants.

A4.2.5 *E. coli* O103F Antimicrobial Specificity

The previously described omelette method [127] was used to assess the specificity of the O103F antimicrobial against *Salmonella* or *Klebsiella* as the competitor. In short, the O103F competitor was inoculated on one side of a MAC agar plate and incubated for 24 h. After the incubation the agar plate was flipped and either *Klebsiella* or *Salmonella* was inoculated perpendicular on the other side of the agar. The plate was incubated and monitored for presence of zones of no growth every 24 h for 72 h.

A4.2.6. Statistical Analysis

Numerical OD data measured for each liquid culture experiment was examined for normality and subsequently used for analyses. Time, media, control, and interactions were determined for all the experiments using a mixed linear model (Proc Mixed, SAS 9.4, SAS Institute Inc., Cary, NC, USA). P values < 0.05 were considered significant.

A4.3 Results and Discussion

A4.3.1 Effect of Carbon Source on Molecule Production

Previous results have shown that a strong *E. coli* O103F competitor produces a diffusible antimicrobial molecule in EC [231] and on MAC [127] which inhibited the growth of *E. coli* O157A. However, when examining the effect of other media, *E. coli* proliferated in all the media tested, but the molecule was only produced when grown on/in EC and MAC. Both, EC and MAC, contain lactose and bile salts. The type of metabolized carbon source has been described to effect gene expression by turning genes on in presence of a stimulating carbon source such as glucose and repressing other genes. This phenomenon is known as carbon catabolite repression (CCR) [234-236]. Microcin production has been suggested to be under CCR regulation [205]. The results from this study revealed that the tested nutrients did impact the molecule production for *E. coli* O103F. Production of microcins is thought to be induced by stress conditions such as nutrient depletion [132]. However, our results suggest that certain macromolecules have an effect on production and should be considered when designing experiments.

A4.3.2 Effect of Bile Salts on Molecule Production

Examining the effect of glucose and lactose demonstrated that both carbon sources did not turn on molecule production in *E. coli* O103F and since both did not turn on molecule production, it suggests that the bile salts in EC and MAC may be responsible for this activity. Examining the effect of bile salts demonstrated that a 50:50 mixture of TSB and EC was sufficient to turn on molecule production in *E. coli* O103F compared to TSB only but did not significantly inhibit O157A growth. Bile salts are present in the small intestine of humans and are considered bactericidal [232, 237] but enteric bacteria

such as *E. coli* have developed various physiological adaptations in response to bile to ensure their survival [229]. Bile salts have been shown to effect the expression of many STEC O157:H7 genes, including LEE, flagella, iron-acquisition and bile resistance [238]. Along the digestive tract, the concentration of bile salts may acts as a cue for O157 to prepare for the entry into a low-iron environment. Interestingly, several microcins, such as E492 (identified in *Klebsiella*) mimic catecholate siderophores and utilize the iron siderophore receptors to invade competing cells [239]. Considering that the molecule produced by *E. coli* O103F is thought to be a microcin [231] and turned on in the presence of bile salts, which has been shown to affect production of iron acquisition genes, suggests that O103F produces a siderophore microcin.

A4.3.3. Effect of Growth Time on Molecule Production

The results examining growth time on molecule production demonstrated that *E. coli* O103F produces the antimicrobial molecule during exponential phase. Contrary, almost all reported microcins are produced when bacterial cells enter stationary phase. However this is not true for microcin E492 which is produced in exponential phase [132]. This result along with the finding that the molecule produced by O103F is activated by bile salts suggests that O103F produces a microcin with a metabolic pathway similar to E492.

A4.3.4. *E. coli* O103F Molecule Specificity

Microcins and colicins generally have a restricted killing spectrum, targeting closely related species and/or the same species [189, 193]. They are being considered an alternative for antibiotics due to this unique trait. Previous results have shown that the diffusible from *E. coli* O103F inhibits growth of various STEC and non-STEC strains

[127]. The results from this study supplement previous data by illustrating the diffusible antimicrobial has an inhibitory effect on *Salmonella* (24 h) and *Klebsiella* (48 h). The data from this study is in accordance with previous studies [193] since both species tested are closely related to *E. coli* and belong all to the family of *Enterobacteriaceae* [240]. The narrow target range of microcins is one of the features that make microcins the weapon of choice to mitigate specific pathogens without affecting the remaining microbiome in the GIT [190].