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FACTORS REGULATING THE PRODUCTION OF
STX-2 IN *ESCHERICHIA COLI* O157:H7

BY

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B.S. Stonehill College, 2004

DISSERTATION

Submitted to the University of New Hampshire

in Partial Fulfillment

the Requirements for the Degree of

Doctor of Philosophy

in

Microbiology

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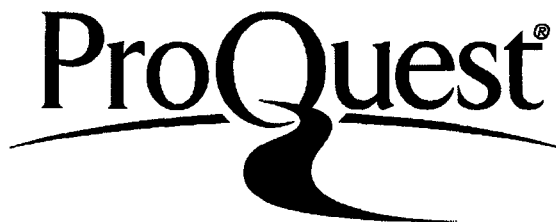
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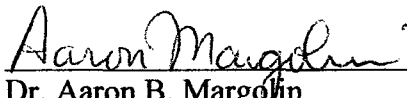
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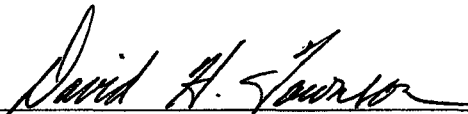
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DEDICATION

I would like to dedicate this dissertation to everyone who supported me throughout this endeavor, especially my best friend, Rich Shara; my mom, Patti Stefani; and my dad, Tom Stefani. Even when things were rocky, the three of you always told me that I could get through this. I appreciate everything you have always done for me.

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ABSTRACT

FACTORS REGULATING THE PRODUCTION OF STX-2 IN *ESCHERICHIA COLI* O157:H7

by

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University of New Hampshire, May, 2010

The severity of *Escherichia coli* O157:H7 disease is due in part to a major virulence factor produced by the microbe, the shiga-like toxin 2 (Stx-2). Antibiotic treatment to reduce pathogen numbers is controversial, as it is thought that antibiotics may increase the levels of Stx-2 released from the pathogen. Currently, recommended treatment for *E. coli* O157:H7 is palliative. The purpose of this study was to examine three critical factors potentially important to disease outcomes, and to determine their effect on expression of the *stx2* gene and on release of Stx-2 from the pathogen. Those factors selected for study were: i) various classes of antibiotics; ii) probiotic microorganisms; and iii) carbon source variation together with cAMP. Stx-2 was assessed using MTT cytotoxicity assays and ELISA analysis, while the expression of *stx2* was assessed using real time PCR. It was determined that antibiotics that affect microbial DNA increased *stx2* expression and Stx-2 production, and this was linked to an upregulation in the SOS DNA repair response. A link was also observed between the upregulation of *stx2* and those antibiotics that disrupt cell membrane integrity. However, these antibiotics did not increase the overall levels of Stx-2 released from *E. coli*

O157:H7. The probiotic microorganisms *Lactobacillus casei* and *L. plantarum* were found to decrease both *stx2* expression and Stx-2 release when grown in co-culture with *E. coli* O157:H7 at greater or equal numbers to the pathogen. This reduction in Stx-2 was at least in part attributable to organic acids produced by the probiotics, but other unknown factors produced by the lactobacilli cannot be excluded. Finally, it was determined that growth of the pathogen in glucose-supplemented media yielded significantly more *stx2* expression and Stx-2 production than growth in glycerol-supplemented media. This observation was confirmed by a decrease in *stx2* expression and Stx-2 production when exogenous cAMP was added to culture media. The examination of these three factors led to a clearer understanding of the intricacies involved in the regulation of *stx2*, and has demonstrated how such an apparently diverse group of external factors are interlinked through several complex mechanisms.

CHAPTER 1

INTRODUCTION TO *ESCHERICHIA COLI* O157:H7 AND THE SHIGA-LIKE TOXIN 2

1. **Historical perspective**

Escherichia coli O157:H7 was first detected as a potential human pathogen in the summer of 1982 after a number of people fell ill following the consumption of contaminated hamburger (127, 170). During the course of this outbreak, a total of 47 people in Oregon and Michigan had symptoms of hemorrhagic colitis; severe abdominal cramping and watery diarrhea that gave way to bloody diarrhea with little or no fever (127, 170). Laboratory testing at the time excluded enterotoxigenic *E. coli* (ETEC) or enteroinvasive *E. coli* (EIEC) as the causative pathogens (127, 170). Further testing revealed a serotype (*E. coli* O157:H7) not yet associated with human disease but present in affected patients in 24 of 34 stools (103, 170).

The cause of this outbreak was presumed to be undercooked ground beef. All patients reported to have eaten sandwiches at a fast food restaurant, with at least one of three items in common: ground beef patty, rehydrated onion, or pickles (127). Subsequent testing of ground beef samples held for examination revealed the presence of *E. coli* O157:H7, confirming this as the causative agent (127).

Two months later in November 1982, a second outbreak occurred in Ottawa, Canada in a nursing home (103). Of 353 residents, 31 became sick, 8 with diarrhea and

18 with hemorrhagic colitis (103). *E. coli* O157:H7 was isolated from the stools of 17 of 31 patients with isolation of this serotype more common from those with hemorrhagic colitis (103). Ground hamburger meat was again suspected as it had been served repeatedly during the time frame of the outbreak; however, no *E. coli* O157:H7 was isolated from the suspected meat (103).

The outbreak that brought *E. coli* O157:H7 to public attention as a major pathogen was the widely-publicized outbreak at the fast food restaurant chain Jack-in-the-Box in 1993 (8, 125). In the states of Washington, Idaho, California, and Nevada, there were more than 500 laboratory-reported cases and four deaths (125). Eventually these cases were directly linked to undercooked hamburger purchased at the Jack-in-the-Box restaurants. In the wake of this outbreak, several recommendations were put forth from state and federal government agencies regarding *E. coli* O157:H7 and other strains of enterohemorrhagic *E. coli* (EHEC) (26, 162). The following year *E. coli* O157:H7 infection became a nationally notifiable disease (125). That same year, the Council of State and Territorial Epidemiologists (CSTE) recommended that all bloody stools be screened for EHEC using growth on a sorbitol-MacConkey agar medium (26), as most EHEC strains are unable to ferment sorbitol, and are thus distinguished using this agar. Likewise, in the wake of this major outbreak, the United States Food and Drug Administration (FDA) released new guidelines for the cooking temperatures of ground beef (162). By 2000, 48 of 50 states required state-level notification of this pathogen (125).

2. **The Organism**

2.1. **Taxonomy and Nomenclature**

E. coli was first described by the German bacteriologist Theodore Escherich in 1885. *E. coli* O157:H7 belongs to the kingdom Bacteria, the phylum Proteobacteria, the class Gammaproteobacteria, the order Enterobacteriales, and the family *Enterobacteriaceae*. Both benign and human pathogenic serotypes are known to inhabit the normal intestinal tract of warm-blooded mammals (56). A variety of *E. coli* serotypes are found in the intestines of humans and are thought to contribute to intestinal health through the production of vitamin K, the digestion of food, and by offering passive immunity against other gastrointestinal pathogens (28, 45, 152). *E. coli* O157:H7 is not found in healthy individuals but is transiently cultured from the guts of cattle, and this results in the incidence of this pathogen in ground beef (20, 58, 87).

2.2. **Physicochemical Properties**

E. coli organisms grow optimally at gut temperature in the human body, 37°C. Although this is the preferred temperature for *E. coli*, it is able to survive a much larger range, from 19°C to 46°C (89). The O157:H7 serotype has a slightly narrower temperature range of approximately 19°C to 41°C (124). It is interesting to note that this serotype is unable to grow at 45°C, the temperature used to assess water samples for fecal coliform contamination (124), and therefore would not be detected by traditional fecal coliform assays. *E. coli* O157:H7 can survive freezing when inoculated into ground beef and remain viable when the meat is thawed at a later date. When stored initially at -80°C

and held at -20°C, organisms remain viable for 9 months or more (96).

Thermal inactivation of *E. coli* O157:H7 occurs optimally at 155°F (68°C) and all organisms are killed within 15 sec at this temperature; indeed it shows no more tolerance to cooking temperatures than do salmonellae (89). The recommended internal temperature for ground beef is 160°F (71.2°C), which will kill all *E. coli* O157:H7 (89). However, the 1997 Food and Drug Administration Food Code (96) requires a minimum internal temperature of 155°F (68.5°C) with a 15 second holding time (96).

E. coli O157:H7 has no growth factor requirements; metabolically it can utilize glucose as a sole carbon source to produce any macromolecular components necessary for growth of the cell (101). The organism is a facultative anaerobe; it is capable of growing aerobically, but also can grow anaerobically by using NO₃, NO₂ or fumarate as final electron acceptors (101). This ability allows *E. coli* to colonize the anaerobic lower colon.

Although *E. coli* can survive at low pH, (as low as pH 2 for several hours), growth of these organisms is limited to a pH range of 4 to 9 (145). It is believed that the acid tolerance displayed by these organisms contributes to their low infectious dose (thought to be 10-100 organisms total ingested) (9, 51). Three general mechanisms have been proposed as to how *E. coli* can withstand such extreme pHs: i) a buffering due to the *E. coli* cell's cytoplasm, ii) low proton permeability of the bacterial membranes, and iii) the exclusion and excretion of protons from the bacterial cytoplasm by membrane-bound proton pumps (9). These theories are in part supported by the limited growth and survival of *E. coli* in low pH due to weak acids (133). In a study by Benjamin, et al., microbial growth was severely reduced in benzoic acid (9). Unlike a strong acid, which

is dissociated outside of the cell, weak acids enter cells in an undissociated state and dissociate within the microbe, releasing the proton and subsequently lowering cytoplasmic pH (60, 131, 133). In the case of the three suggested mechanisms for how *E. coli* tolerates low pH due to a strong acid, both ii) low proton permeability of the membrane, and iii) the exclusion and excretion of protons, are null in the case of a weak acid. The buffering capability of the cytoplasm (i) above) may not be sufficient on its own to withstand an extreme pH by weak acids. Of interest is the observation that once these acid resistance systems are turned on, they remain on for prolonged periods, particularly when the organisms are stored at cold temperatures (4°C) (96). This acid tolerance allows *E. coli* O157:H7 organisms to grow in a variety of acidic foods, such as mayonnaise, apple cider and salami (96), all of which have been associated with outbreaks of disease due to this agent.

In contrast to its relative tolerance to acidic conditions, *E. coli* O157:H7 shows no outstanding tolerance to high sodium chloride concentrations. Unlike other food borne pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus*, this pathogen cannot survive in more than 6.5% NaCl (96).

2.3. Cultural and Morphological Characteristics

E. coli O157:H7 displays similar colony morphology to other strains of *E. coli*. Colonies readily appear after overnight growth at 37°C. On a standard medium such as Luria-Bertani (LB) agar, growth appears as slightly translucent, small to medium sized, off-white, convex, smooth colonies. On a slightly richer medium such as Mueller-Hinton agar or trypticase-soy agar, the morphological characteristics are identical but colonies

are usually somewhat larger.

The cellular morphology of *E. coli* O157:H7 is identical to non-pathogenic strains of *E. coli*. All are found as individual gram-negative cells in a classic coccobacillary shape and divide by binary fission at the polar ends. Table 1.1 summarizes the cellular morphology of *E. coli* O157:H7. The serotype O157:H7 refers to the somatic (surface antigen) serotype number 157 and the flagellar antigen serotype number 7 (11, 71, 122). In the United States, the O157:H7 serotype is the most prevalent and has attained the greatest notoriety, however, numerous other serotypes of EHEC are present, and these are often referred to as non-O157 isolates when citing incidence and outbreak details. These serotypes include (but are not limited to): O26:H11, O91:H21, O22:H8, O103:H2, O4:NM, O5:NM, O111:NM, and O145:NM (where NM is non-motile) (96).

E. coli O157:H7 produces lipopolysaccharide (LPS) as part of its surface structure. LPS is highly antigenic to patients suffering from these infections, and often illicit a strong inflammation response. LPS triggers platelet activation, one of the hallmark features of the deadly complication, hemolytic uremic anemia (HUS) (149), as discussed later.

2.4. Biochemical Properties

The biochemical profile of *E. coli* O157:H7 is similar to that of the commonly used laboratory strain *E. coli* K-12. Results are identical on eosin methylene blue (EMB) agar, MacConkey (MAC) agar, Hektoen enteric (HE) agar, triple sugar iron agar (TSI) slants, citrate (CIT) slants, motility-indole-ornithine (MIO) agar tubes, urea (URE) broth, as well as the oxidase (OX) test. The majority of *E. coli* O157:H7 strains are unable

Table 1.1. Morphological Characteristics of *E. coli* O157:H7 Cells

Cell shape	Coccobacillus, short bacillus
Cell arrangement	Singular
Cell size	1.1 to 1.5 μm wide by 2.0 to 6.0 μm long
Gram reaction	Gram negative
Motility	Yes, multiple polar flagella
Attachment features	Fimbriae, intimin
Oxygen needs	Facultative anaerobe

to ferment sorbitol, making sorbitol-MacConkey agar plates (sMAC) useful for the differentiation between O157:H7 and nonpathogenic serotypes (52). Table 1.2 provides a summary of the biochemical properties of *E. coli* O157:H7.

2.5. Genetics of *E. coli* O157:H7

E. coli O157:H7 has a single, circular chromosome with a genome size of 5.49 Mb. This allows for 5361 protein coding genes, 104 tRNA genes and 22 rRNA genes. The genome has a G+C content of 50.53% (64). There is also a 92 kb plasmid, pO157, which codes for several virulence factors in the organism including *espP* (an extracellular serine protease-coding gene), *hlyA* (a hemolysin-coding gene), *katP* (a periplasmic catalase-coding gene), and *toxB* (a toxin gene, similar to one of *Clostridium difficile*) (18). Due to the number of virulence genes located on it, it is thought that this plasmid is crucial to the full pathogenicity of *E. coli* O157:H7 (70). A particularly important bacterial feature on this plasmid is the gene for the production of fimbriae. These structures allow adherence of *E. coli* O157:H7 to host cells, and in the absence of this plasmid, *E. coli* O157:H7 has reduced virulence (70).

2.6. Environmental Aspects

E. coli O157:H7 is a disease of the human gastrointestinal tract; however, it is rarely transmitted person-to-person. Most individuals contracting the disease do so through a non-human reservoir, usually food or beverages that have been contaminated by cow manure or pasture runoff (50). The organism is near ubiquitous in cattle farms

Table 1.2. Biochemical Profile of *E. coli* O157:H7 and *E. coli* K-12

Medium/Test	<i>E. coli</i> O157:H7	<i>E. coli</i> K-12
EMB	Growth, Lac +	Growth, Lac +
MAC	Growth, Lac +	Growth, Lac +
sMAC	Growth, Sor -	Growth, Sor +
HE	Growth, Lac/Suc/Sal +	Growth, Lac/Suc/Sal +
TSI	A/A, Gas+, H ₂ S-	A/A, Gas+, H ₂ S-
CIT	Cit -	Cit -
MIO	Mot +, Ind +, Orn +/-	Mot +, Ind +, Orn +/-
URE	Ure -	Ure -
OX	Ox -	Ox -

across the United States, with a positive carriage rate of 1-2% of animals on a farm or feedlot on average (some seasonal variability occurs with carriage rates higher in warmer weather) (50, 56, 126). Despite this high carriage rate, the organism is a transient member of the normal flora of the cattle (56). Sampling populations of cattle reveals a steady rate of carriage, although carriage by the individual animals varies with time. Put another way, although a constant rate of 1-2% of animals carry the organism on a particular farm, the individuals that make up that percentage varies with each sampling. Therefore, the microbe is quite mobile between animals living in close contact and sharing water and food troughs (56, 126). These organisms are not pathogenic to cattle, but rather act like any other nonpathogenic strain of *E. coli* with colonization coming and going with an average duration of two months (87). It should be noted that juvenile animals are normally more prone to carriage of *E. coli* O157:H7, possibly due to a lower stability in their normal microbial gut flora (50).

Because of the close association of EHEC infections with cattle runoff and ground beef, numerous models have been proposed for the control of this pathogen within cattle populations. Eradication is likely an unachievable goal as this organism is carried within environmental niches (such as contaminated food and water troughs) as well as in wild animal populations (56, 87). In addition, *E. coli* O157:H7 has been found in sheep, dogs, horses and birds, and these may also serve as reservoirs for the pathogen (56, 87).

Pre-slaughter testing of cattle for EHEC has been suggested, but this is also likely to prove unsuccessful. Indeed, current regulations state that a group of cattle must be rejected if any animal tests positive for EHEC. As most slaughter groups are greater than 100 animals and with a 1-2% carriage rate, most groups would be rejected at slaughter

with huge economic losses. New guidelines are required before this type of pre-slaughter testing would become enforceable or even feasible (56).

In order to reduce both the percent of animals carrying *E. coli* O157:H7 and to reduce the number of *E. coli* O157:H7 carried by a particular animal, several measures have been put in place. One practice suggested was the withholding of food before slaughter in order to reduce the amount of waste in the gut of the cattle, thereby lessening the chance of contamination of due to gastrointestinal puncture during the slaughter process. However, fasting decreases the stability of the normal flora within the animal, which increases both the likelihood of colonization by transient *E. coli* O157:H7, and increases the amount of *E. coli* O157:H7 organisms within already-colonized animals, due to the reduction of normal flora organisms (126). Another possibility is the purposeful colonization of the cattle gut with competing organisms. This strategy has been successfully applied in the broiler chicken industry to reduce contamination by species of *Salmonella* (107). This method may be of interest especially in the case of juvenile cattle in which it has been proposed to stabilize their intestinal flora with benign microbes, so that EHEC colonization would prove difficult. This proposal is referred to as competitive exclusion (20, 56). Competitive exclusion cultures can be made up of a single species or of many types of bacteria and are designed to compete against undesirable organisms. Three mechanisms are proposed by which these probiotics inhibit the colonization of pathogens: i) competition for nutrients; ii) competition for binding sites on the epithelium; and iii) production of various compounds that prove toxic to the invading organisms (20, 28, 107, 150). Studies are on-going to determine the efficacy of this approach.

3. Shiga-like toxin 2

Although there are numerous virulence factors associated with *E. coli* O157:H7 that distinguish it from benign *E. coli* serotypes, the most notable and most deadly are the shiga-like toxins, Stx-1 and Stx-2. Stx-1 and Stx-2 are so named for their similarity to the closely related Shiga toxin (Stx) produced by *Shigella dysenteriae* serotype 1 (43). The most devastating of infections are usually associated with Stx-2-producing strains of EHEC, and it is these strains that are most often associated with the development of the life-threatening condition hemolytic uremic syndrome (HUS) (75,113). This research concentrates on the more deadly Stx-2.

Although Stx is produced by *S. dysenteriae*, and Stx-1 and Stx-2 are produced by EHEC, Stx and Stx-1 are a relatively homologous group compared to Stx-2. In fact, the A subunit of Stx and Stx-1 differ only by one amino acid residue, with position 45 in Stx being serine while in Stx-1 it is threonine (5, 43). The A subunits of Stx-1 and Stx-2 have only 55% similarity (5). Also of note is that while Stx-1 is homogenous, Stx-2 exists as variants. Within the Stx-2 group are Stx-2c (63, 83), Stx-2d (115, 116, 119), Stx-2d-activable (115, 116, 119), Stx-2e (54, 91), and Stx-2f (49, 139). These variants are distinguishable by biological and immunological activity and each one binds unique receptors (80). It should be noted, however, that all Stx-2 and its variants preferentially bind the Gb₃ receptor (Gal1–4Gal1–4glucosyl ceramide) (84, 163, 169).

3.1. Structure of Stx-2

The structure of Stx, Stx-1 and Stx-2 all conform to the classic AB₅ toxin structure. The A subunit is responsible for the catalytic activity of the toxin, and thus the

damage it wreaks on target cells (43, 84, 136, 169). The A subunit is further divided into the A1 and A2 fragments. The A1 fragment, at 27.5 kDa, causes the enzymatic activity of the toxin while the A2 fragment, at 4.5 kDa, connects the A subunit to the B subunit pentamer. The five identical B subunits are responsible for binding to target cells, allowing entry of the toxin (43) (Figure 1.1.).

Several key differences have been cited between the structures of Stx/Stx-1 and Stx-2. In Stx and Stx-1, the active site of the enzyme is blocked by the polypeptide A2 fragment and remains so until the A subunit is cleaved from the B subunit during entry into a host cell (42). In Stx-2, the active site of the enzyme is active in the holotoxin. The increased availability of the Stx-2 active site may be a factor contributing to the greater pathogenic potential of Stx-2 and its documented links to the development of HUS (43). In addition, the conformational arrangement of the Stx-2 B subunit pentamer appears to be different from that of the Stx and Stx-1 B pentamers. These different conformational perspectives could explain the different binding affinities of these pentamers to the host cell Gb₃ receptors (43). Likewise, the A1 peptide of the A subunit in Stx-2 appears to bind, and therefore block, one of the receptor sites in Stx-2, reducing by one the number of available binding sites on the pentamer. This too could contribute to the different affinities of Stx-2 for Gb₃ (43, 169).

3.2. Mode of Action of Stx-2

The AB₅ toxin structure is essential to the mode of action of the toxin. In essence, toxins enter human intestinal cells by attachment of the B subunit to the Gb₃ receptor, a globotriaosyl ceramide (Gal α (1-4)-Gal β (1-4)-Glc β 1-ceramide) receptor (84, 114, 163).

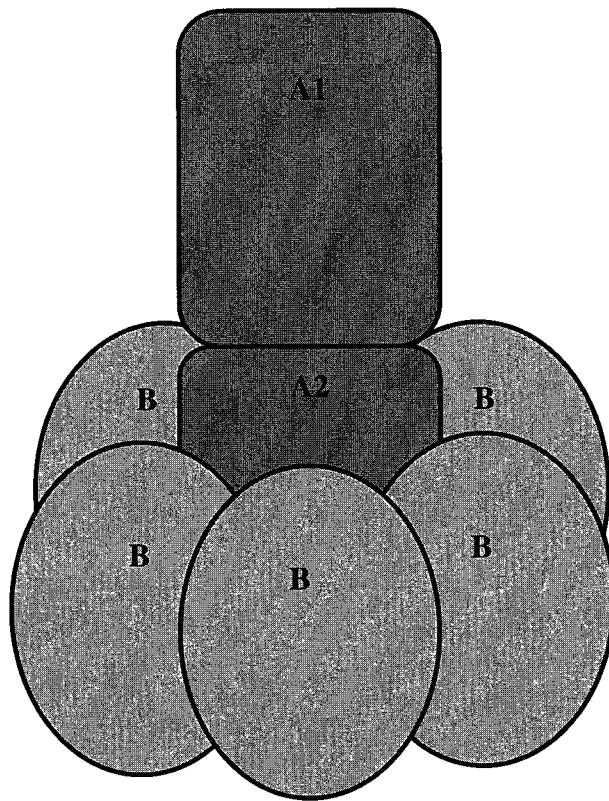


Figure 1.1. Schematic Representation of Stx-2. Stx-2 is an AB₅ toxin. The A subunit is comprised of two separate fragments, A1 and A2. The A1 fragment has the enzymatic activity of the toxin and the A2 fragment serves as a connector to the five identical B subunits. The B subunit pentamer binds the toxin to appropriate receptors on host cells.

The holotoxin is endocytosed and retrograde transported to the Golgi network and subsequently to the endoplasmic reticulum (95). At this post-endocytosis point, the A subunit is cleaved by furin, a membrane-bound protease similar to the enzyme trypsin, and this yields the A1 and A2 fragments. The A1 fragment then targets the ribosome (95). A1 has RNA N-glycosidase activity that cleaves a specific N-glycosidic bond in the 28S rRNA of the 60S ribosomal subunit, removing an adenine base. This base is an integral component of elongation factor binding to the ribosomal subunit, and therefore prevents the affected cell from carrying out protein synthesis (39, 114, 136, 144). Toxins may also enter the blood stream, travel to the kidneys, brain, and other organs, and cause extensive cell and tissue death leading to major organ failure and system collapse (71).

3.3. Genetics of *stx2*

The *stx2* gene is located on a lambda-like bacteriophage (BP-933W) inserted as a prophage into the chromosome of *E. coli* O157:H7. When the organism is metabolically stable, the prophage remains in a lysogenic state, leading to low or baseline level expression of *stx2*. However, when the *E. coli* O157:H7 host bacterium is subjected to stress, the prophage converts to a lytic state and is excised from the chromosome (166). During excision, the *stx2* gene is co-expressed with phage genes due to their proximity on the phage chromosome and this leads to an increase in toxin production (57, 78, 104, 164). Upregulation of *stx2* gene expression is believed to be the end result in a cascade pathway that begins with the activation of the RecA protein by damaged DNA (57, 164).

The genetic system of the *E. coli* BP-933W bacteriophage is complex, self-repressing, and self-regulating (Figure 1.2, 1.3). When the regulating proteins cII and

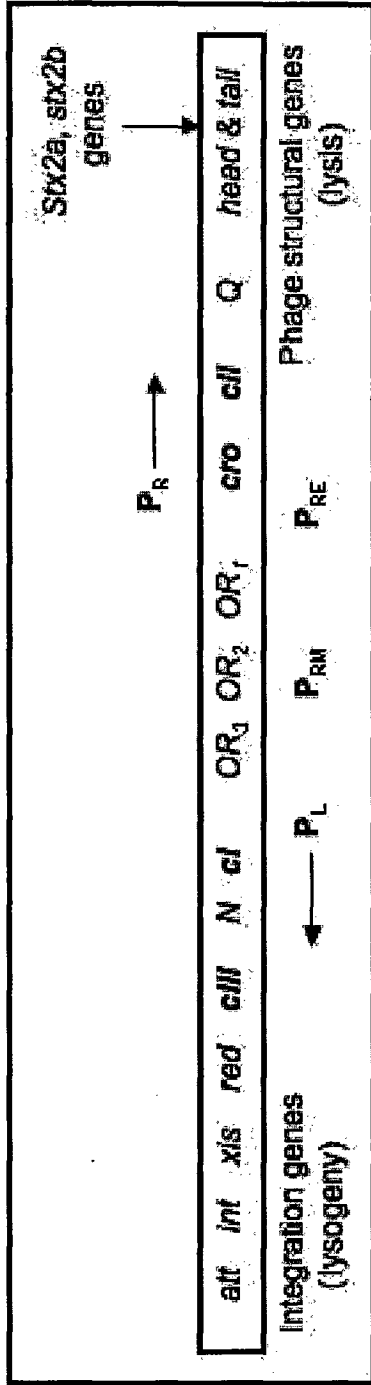


Figure 1.2. Simplified Genetic Map of the 933-W Operon. The P_R promoters is repressed during a healthy metabolic state of the *E. coli* O157:H7 host bacterium. When the host bacterium is stressed and the SOS response is activated, the *cII* regulatory protein is depleted leading to a high level of expression off the P_R promoter. This in turn leads to excision of the phage, and an increased level of *stx2* activity and *Stx-2* production



Figure 1.3. Complete Genetic Map of the 933-W Operon. The *stx2A* and *stx2B* genes are located within the genes required for lysis of the 933-W phage. This special proximity leads to similar rates of transcription due to a common promoter, P_R (120). Note the location of the *stx* genes within the genes for late regulation and packaging.

cIII are expressed at low levels they activate the repressor cI. CI in turn binds and represses the promoters, Pro_L and Pro_R, to stop transcription of a majority of the phage genome, including cII and cIII and the toxin gene, *stx2* (44, 86, 164). Thus, the system is self-repressing and self-regulating, i.e. it blocks transcription of the phage genome, maintaining the phage in a lysogenic state, unless a stress trigger interrupts the cycle. This interruption can occur following DNA damage, which leads to initiation of the SOS DNA repair system that in turn activates RecA. When the SOS response is initiated, activated RecA degrades the regulating protein, cII and hence the expression of cI is also arrested. Without expression of the cI repressor, the late phage genes are expressed, and these include *stx2* (44, 78, 104, 120, 164). Conversion to the lytic cycle is a bacteriophage survival mechanism effected when host DNA is compromised. The *stx2* gene is located between the late phage promoter and phage-excision genes and is co-transcribed when host cell damage is sensed. Therefore, under DNA damaging conditions, induction of phage genes and *stx2* occur simultaneously (44, 74, 86, 164).

4. Clinical and Epidemiological Findings

4.1. Clinical Presentation

E. coli O157:H7 causes a variety of disease states, depending on the age and general health states of the infected individual, as well as the dose of organism ingested (123, 101, 127, 114). In its mildest form, the disease presents with symptoms of diarrhea and stomach cramping lasting up to five days. More severe cases often show hemorrhagic colitis (HC) or bloody diarrhea (Table 1.3.). In the most severe cases

Table 1.3. Different Clinical Manifestations of EHEC Disease

Severity	Mild	Modest	Severe
Approximate percentage of cases	10%	70-85%	5-15%
Symptoms	Diarrhea (non-bloody) Abdominal cramps	Hemorrhagic colitis Abdominal cramps	Hemorrhagic colitis Abdominal cramps
Complications	None	None	HUS, TTP

(approximately 5% of patients) these gastrointestinal symptoms are followed by a complex presentation of symptoms culminating in hemolytic uremic syndrome (HUS) (114); an often fatal complication especially in children, the elderly, and the immunosuppressed. HUS is characterized by hemolytic anemia, thrombocytopenia, and renal failure. Hemolytic anemia results from the fragmentation of red blood cells as they pass through areas of thrombi in the small blood vessels. These sheared cells are characteristic of HUS during microscopic examination of patients suspected of having this condition. Low circulating platelet counts are a predictor, as platelets adhere to the damaged vascular endothelium and are removed from circulation. It has also been suggested, but not proven, that Stx-2 may interact directly with platelets, reducing their count (123). Renal damage occurs as these sheared red blood cells and platelets aggregate in the small blood vessels of the kidneys (123).

In some cases there may be neurological involvement and a fluctuating fever. Often these neurological symptoms are associated with a distinct but very similar condition, thrombotic thrombocytopenia purpura (TTP) (101, 123, 130). Both TTP and HUS are microangiopathic disorders that are characterized by abnormalities in the small blood vessels of the body. The key difference between these conditions is the location of the microvascular thromboses. In TTP they can be found in the brain leading to neurological symptoms, and also in the skin, intestines, skeletal muscle, pancreas, spleen, adrenal glands, and heart. In HUS, these microthrombi are confined to the kidneys (123) (Table 1.4).

Table 1.4. Comparison of HUS and TTP

	HUS	TTP
Age affected	Children	Adults
Microscopic examination of blood	Anemia	Anemia and thrombocytopenia
Peripheral smear	Microangiopathic hemolytic anemia	Microangiopathic hemolytic anemia
Organs affected	Kidneys	Predominantly CNS, but also intestines, pancreas, skin, heart, spleen and others
Treatment	Palliative, dialysis, plasmapheresis	Palliative, plasmapheresis, steroids
Prognosis	Good	Poor

It is important to note that, as mentioned previously, HUS occurs considerably more often in children than in adults, and in fact is the most common cause of kidney failure in children in the United States (105). Indeed, in the United States, the average annual incidence is 2.65 cases per 100,000 individuals under 5 years old. In individuals 5-18 years old the incidence is notably lower, 0.97 cases per 100,000 (123, 125).

2.1. Pathology

Symptoms of an *E. coli* O157:H7 infection usually occur within 3-8 days of ingestion of the bacterium. The symptomatic phase of disease can be divided into two phases: 1) before blood is present in the stool, and 2) after blood is present in the stool (101, 123, 130). However, in approximately 10% of reported cases there is no progression beyond phase 1 (123).

The first symptoms, preceding even the non-bloody diarrhea, include severe abdominal cramping and general irritability. These symptoms are often enough to induce a missed day of school or work, but do not often warrant a visit to the physician. After the onset of non-bloody diarrhea, an over-the-counter anti-diarrheal medication is often administered. Such treatments often include an antiperistaltic agent (101, 123, 130). In the case of EHEC infections, these medications are generally contra-indicated as they can slow the clearing of the organism from the body, allowing more time for colonization and toxin production (123, 130). Although most physicians are aware that antiperistaltic medications are detrimental in cases of EHEC infections, before the presence of blood in the stool there may be little or no reason to consider that an EHEC infection exists; non-bloody diarrhea is a common enough event. Bloody diarrhea often occurs within 1-3

days of the non-bloody diarrhea, and in most cases prompts a visit to a physician (123). At this point an EHEC infection is usually suspected and can be confirmed through laboratory tests (52). Lastly, in 5-15% of EHEC cases, infections progress to HUS. Often the first clinical symptoms of HUS are a diminished platelet count, followed by hemolytic anemia (123).

Hemolytic anemia is the cardinal feature of HUS; the patient may appear more pale than normal, and have abnormal swelling in the extremities. In blood smears, fragmented red blood cells are common. Thrombocytopenia occurs due to the increased clotting and destruction of platelets (123). Immunoglobulin G (IgG) concentrations in the blood serum decrease, while IgA and IgM elevate. Often IgM deposits are noted in the injured glomeruli of the kidneys (80, 123). In one-third of patients, blood pressure is elevated due to a combination of fluid replacement therapy and kidney dysfunction. This requires close monitoring to avoid harm to the patient. Urinalysis is abnormal with hematuria, proteinuria, and red blood cell cast-off cells (101).

Commonly, hepatomegaly is a finding in cases of HUS due to fluid overload and hypoalbuminemia. Despite these symptoms, liver failure and long-term hepatic sequelae have not been reported. In 10-20% of HUS cases, the pancreas is affected, though clinical pancreatitis is unusual. In 4-15% of patients, insulin-dependent diabetes mellitus occurs due to islet cell necrosis (130).

2.2. Pathogenesis and Virulence Factors

The pathogenesis of EHEC infections begins shortly after ingestion of the bacterium. It is believed that the infectious dose of *E. coli* O157:H7 may be as low as 10

to 100 organisms (81, 114). These organisms are able to survive the acidity of the human stomach and proceed to colonize the lower gastrointestinal tract (82, 93, 133). *E. coli* O157:H7 is not considered invasive. That is, it does not burrow into the epithelial cell layer, as organisms like *Shigella dysenteriae* do. It does associate closely with the intestinal epithelial layer, resulting in the formation of attaching and effacing lesions (69, 70, 108, 117, 118).

Attaching and effacing lesions are induced in part by the production of the 94 kDa to 97 kDa outer membrane protein intimin (69). Attaching and effacing lesions cause polymerization of actin filaments in the epithelial cells of the intestines, forming a characteristic pedestal that binds the *E. coli* O157:H7 cell. The microvilli are effaced from the intestinal lining (69, 117, 118). The intimin protein is encoded by a gene, *eae* (*E. coli* attaching and effacing), which is located chromosomally on the 35 kb locus of enterocyte effacement (LEE) pathogenicity island (37, 38). Upstream of the *eae* gene is the *tir* gene, which encodes the translocated intimin receptor, or Tir protein. Interestingly, Tir becomes the receptor on the eukaryotic cell for intimin, but it is produced by the *E. coli* O157:H7 cell. Tir is then shunted into the eukaryotic host cell through a type III secretion system. Once inside the host cell, Tir is phosphorylated and interacts with the host cytoskeleton and intimin (69). Intimin-independent adherence to host epithelial cells has been suggested through type 1 fimbriae, however, it should be noted that only a minority of *E. coli* O157:H7 strains produce fimbriae *in vitro*, and that the role of fimbriae in *E. coli* O157:H7 colonization is not established (159). For *E. coli* O157:H7, investigation of microbial adherence is problematic, as the toxins produced are often lethal to the host cells before adherence can be observed.

The LEE pathogenicity island (Figure 1.4.) is thought to have been horizontally transferred from another microorganism. The most striking proof of this is that the G+C content of the LEE pathogenicity island is 38.3%, compared to the 50.8% G+C content of the rest of the *E. coli* O157:H7 chromosome (37). Besides *eae* and *tir*, other virulence genes are located on the LEE pathogenicity island. Upstream of *eae* and *tir* are numerous genes, including *esc* and *sep*, that encode the type III secretion system necessary to translocate Tir into the host epithelial cell. Downstream of *eae* are a number of *esp* genes, which encode proteins responsible for epithelial cell signaling in the formation of the attaching and effacing lesion. Overall, the LEE pathogenicity island encodes all the necessary proteins for the production of an attaching and effacing lesion (37).

E. coli O157:H7 has a unique iron scavenging system efficient enough to utilize heme or hemoglobin as an iron source. This 69 kDa outer membrane protein is encoded by the *chuA* gene, which is turned on in iron-limiting conditions (111). The lysis of red blood cells (a known symptom of HUS) could aid in the pathogenesis of *E. coli* O157:H7 through the release of hemoglobin thus increasing the amount of iron available to the pathogen. Likewise, *E. coli* O157:H7 produces several hemolysins; the most characterized being the extracellular alpha-hemolysin (70, 140). Distinct from this is an enterohemolysin, named Ehy11, which is carried on the plasmid pO157. Although this phenotype is often lost quickly *in vitro*, anti-Ehy11 IgG has been detected in patients recovering from HUS, suggesting its presence during EHEC infections (70). Also located on the pO157 plasmid is the gene *katP*, which encodes a catalase-peroxidase enzyme, KatP. This enzyme contains an amino-terminal signal peptide that suggests it is transported through the cytoplasmic membrane, and is mostly found in the periplasm.

The locus of enterocyte effacement (LEE) of E2348/69

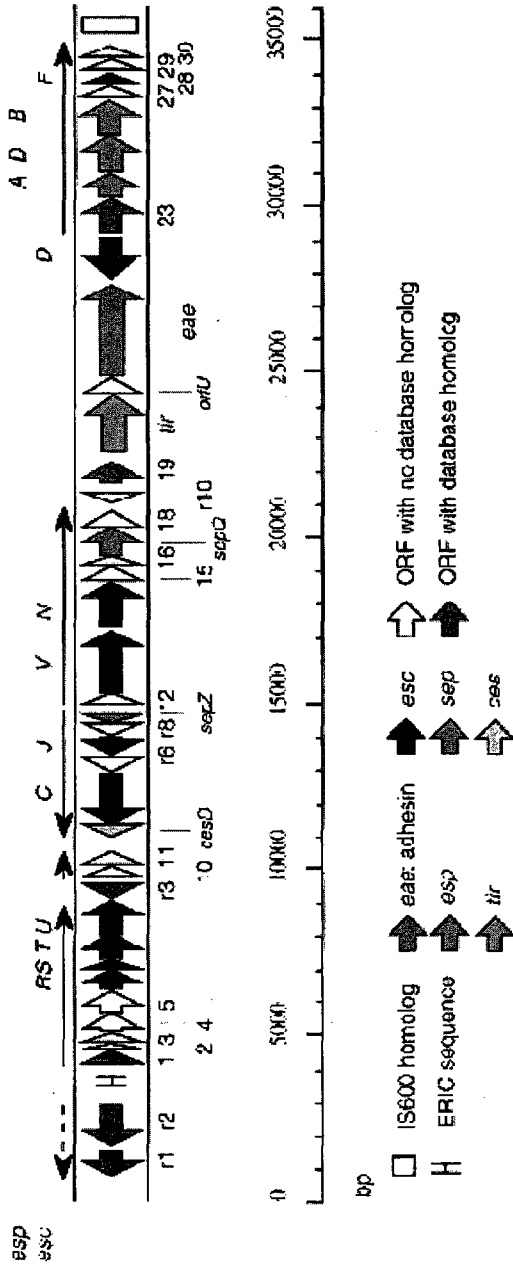


Figure 1.4. LEE Pathogenicity Island Gene Map. The LEE pathogenicity island contains the genes for production of intimin and its receptor, Tir. Also on this island are the genes for type III secretion of Tir into the eukaryotic cell, and genes for cell signaling of the eukaryotic cell (37).

KatP is deemed a bifunctional enzyme, possessing both catalase and peroxidase activities, making it a potent virulence factor when expressed inside the human host (70). The genes and the products they encode for the principal virulence factors of *E. coli* O157:H7 are outlined in Table 1.5.

Once colonization of the pathogen is established (through the aid of intimin, Tir, attaching and effacing lesions and the other virulence factors), Stx-2, arguably the most important of the diverse virulence factors, is released from the *E. coli* O157:H7. Stx-2 molecules translocate across the epithelial layer using an energy-dependent process, though the mechanisms of this movement are not elucidated (62). What is clear, however, is that the toxin crosses the epithelial cell barrier in a transcellular pathway, as tight junctions between cells remain intact. Stx-2 appears to bind less tightly to epithelial cells than does Stx-1 and may allow a greater proportion of toxin to enter the blood stream from the intestines (62). This could offer, at least in part, a rationale for the higher virulence and greater severity of disease reported for Stx-2 producing strains. Data suggest that Stx-2 could stimulate the secretion of interleukin-8 (IL-8) by intestinal epithelial cells, resulting in neutrophil migration to that tissue. Neutrophils are possible carriers of Stx-2 to the various organs of the patient, including the kidneys (165).

Once translocated across the epithelium, Stx-2 acts on endothelial cells and platelets in the microvascular network, leading to thromboses as seen in pathology studies. Endothelial cells are highly sensitive to Stx, and those of the microvascular endothelial cells are especially so (108). Such findings confirm the pathological data

Table 1.5. Virulence Factors of *E. coli* O157:H7

Gene	Location	Gene product/function
<i>stx2A</i>	Chromosome, 933-W phage	A subunit of Stx-2
<i>stx2B</i>	Chromosome, 933-W phage	B subunits of Stx-2
<i>eae</i>	Chromosome, LEE island	Intimin, A/E formation
<i>tir</i>	Chromosome, LEE island	Tir, A/E formation
<i>esp</i>	Chromosome, LEE island	Esp proteins, signal transduction for A/E formation
<i>esc</i>	Chromosome, LEE island	Type III secretion for the Esp proteins
<i>sep</i>	Chromosome, LEE island	Type III secretion for the Esp proteins
<i>chuA</i>	Chromosome	Heme utilization, iron transport system
<i>ehylI</i>	pO157 plasmid	hemolysin
<i>katP</i>	pO157 plasmid	Catalase-peroxidase
<i>etpC- etpO</i>	pO157 plasmid	Type II secretion system

that show that the colonic, neural, pancreatic, pneumonic microvasculature are much involved in HUS and TTP (123).

2.3. Epidemiology

E. coli O157:H7 causes 73,000 reported cases of disease in the US each year. Kidney damage and HUS occurs in 5-15% of cases. This condition usually occurs in children under 5 years old, and is the leading cause of acute kidney failure in children in the US (105). Annually approximately 60 of these illnesses result in death (125).

E. coli O157:H7 is usually foodborne or waterborne and a number of foods and beverages have served as the source of outbreaks. These include ground beef, lettuce, alfalfa sprouts, apple juice, and apple cider (50) (Figure 1.5). Many waterborne cases have also been reported from both drinking and recreational waters. Less commonly are outbreaks and sporadic cases caused by non-food and water sources, such as petting zoos and contact with farm animals. Person-to-person spread is rare (50, 125).

A distinct seasonality of *E. coli* O157:H7 is seen with warmer months having higher incidence, due possibly due to increased shedding by cattle coupled with increased consumption of ground beef and less careful food handling during barbeque season (40) (Figure 1.6).

A number of risk factors may increase the likelihood and severity of the disease. Children under 5 years old are at the most risk and may develop HUS. It is unknown why children are so exquisitely susceptible to develop this condition although the relatively small size of children's blood vessels may facilitate easier damage by Stx-2 (125).

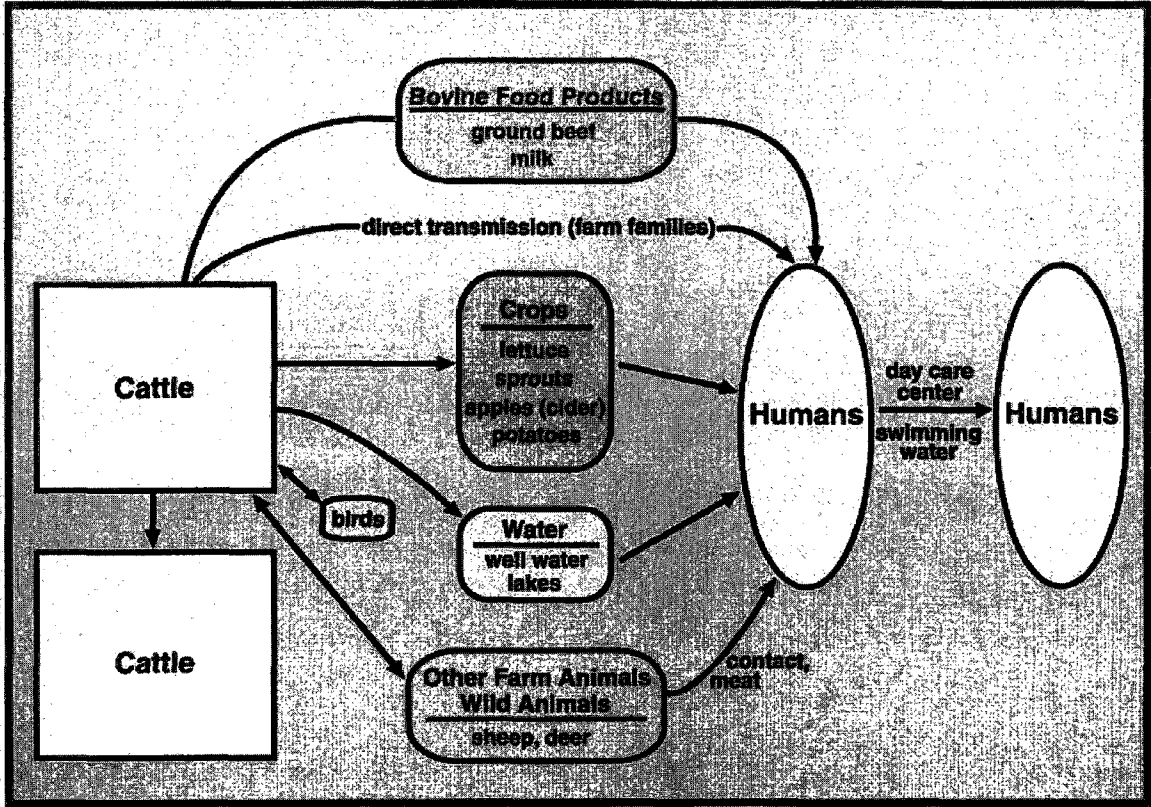


Figure 1.5. Transmission Routes for *E. coli* O157:H7. Note that the origin is invariably cattle (50).

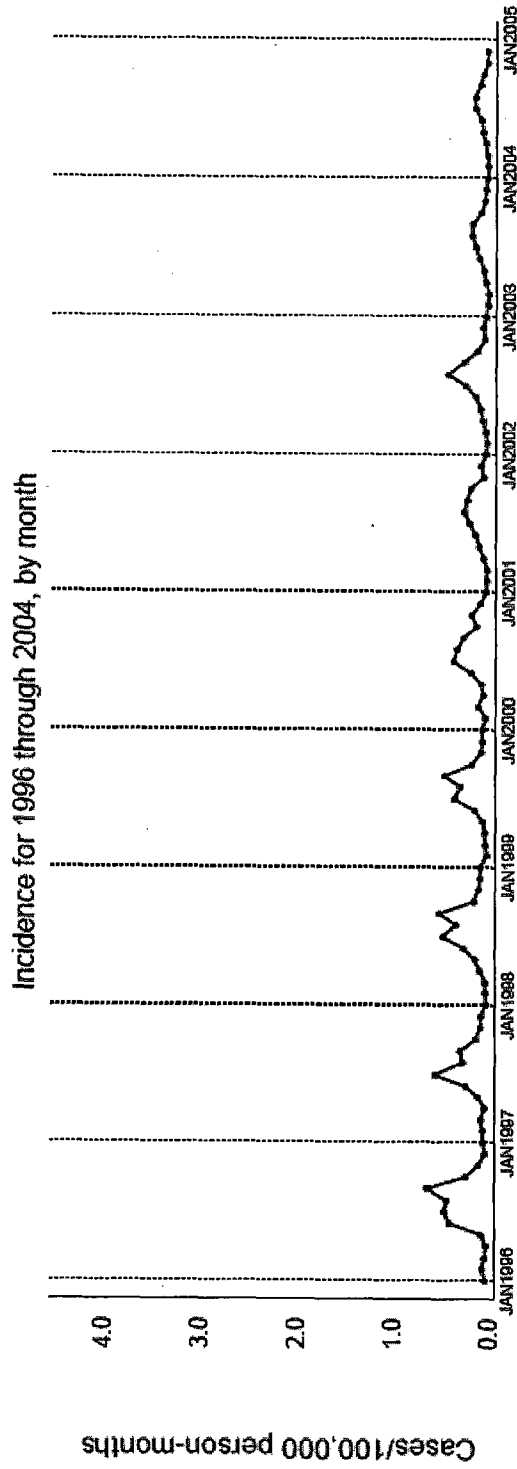


Figure 1.6. Seasonality of *E. coli* O157:H7 infections from 1996 until 2004. A clear pattern of seasonality is observed showing a higher summer incidence (40).

To offer confirmatory evidence, even the smaller blood vessels of adults are more susceptible to damage by the toxin than are the large blood vessels. Damage to the kidneys is due largely to microthrombi leading to blockages in the microvasculature of these organs (125). It is possible that a smaller dose of toxin would cause greater damage overall to children than to adults. The less mature immune system of children further complicates this scenario.

Other factors that increase the risk of *E. coli* O157:H7 infection include unsafe handling of raw meats, as well as consumption of raw and undercooked meats, unpasteurized beverages (such as apple cider and milk), and contaminated produce. Gender does not appear to be a factor in the likelihood of contracting disease (40).

As would be expected, the reported incidence of outbreaks increased after the first cases in 1982. However, such increase was likely due to a heightened awareness of the pathogen, and the mandatory reporting of cases of EHEC infections (Figure 1.7) (125). The trend in *E. coli* O157:H7 infections has declined between 1996 and 2007 (Figure 1.8) (40). In 2003 and 2004, the lowest rates of infection were reported but these were followed by two years of higher incidence followed by a decrease again in 2007 (40). Hand-in-hand with the incidence of *E. coli* O157:H7 disease is the incidence of HUS. Fortunately, the number of outbreak-associated HUS cases has declined since 1982, with a few spikes in frequency. The relative number of HUS cases also dropped in 2003 and 2004, coinciding with the lower incidence of *E. coli* O157:H7 cases (Figure 1.9) (40). Likewise, as can be seen in Figure 1.10, the number of HUS-associated deaths has declined since the 1982 outbreak (125). This is most likely due to greater awareness of the organism.

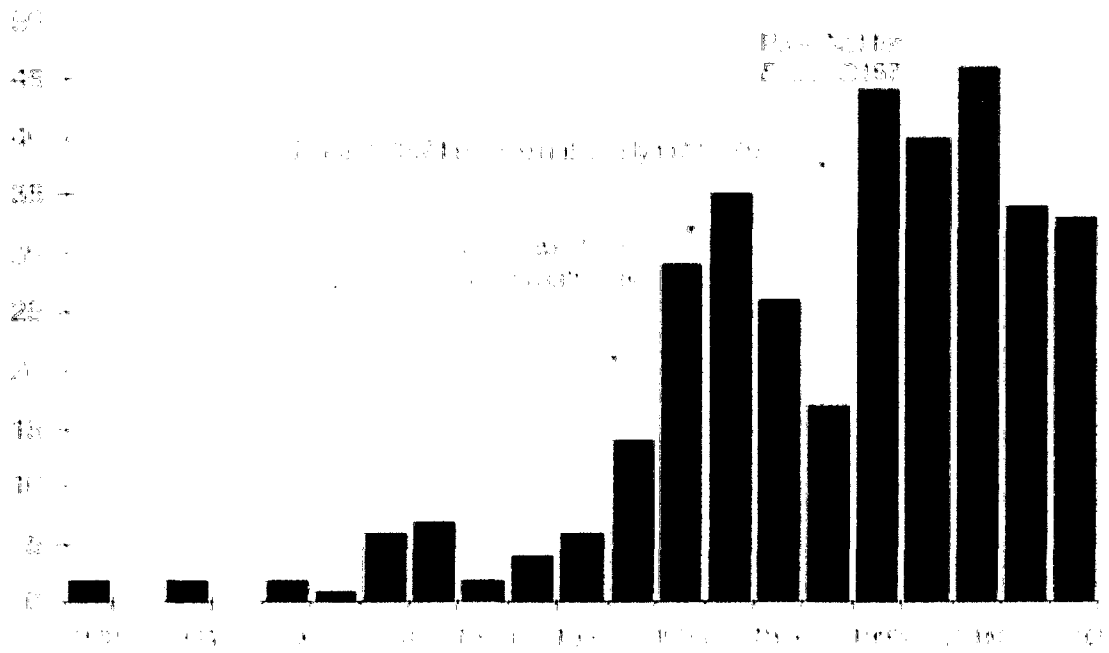


Figure 1.7. Reported Cases of *E. coli* O157:H7 from 1982 to 2002. Note the dip in incidence in 1997 when PulseNet for *E. coli* O157:H7 was implemented (125).

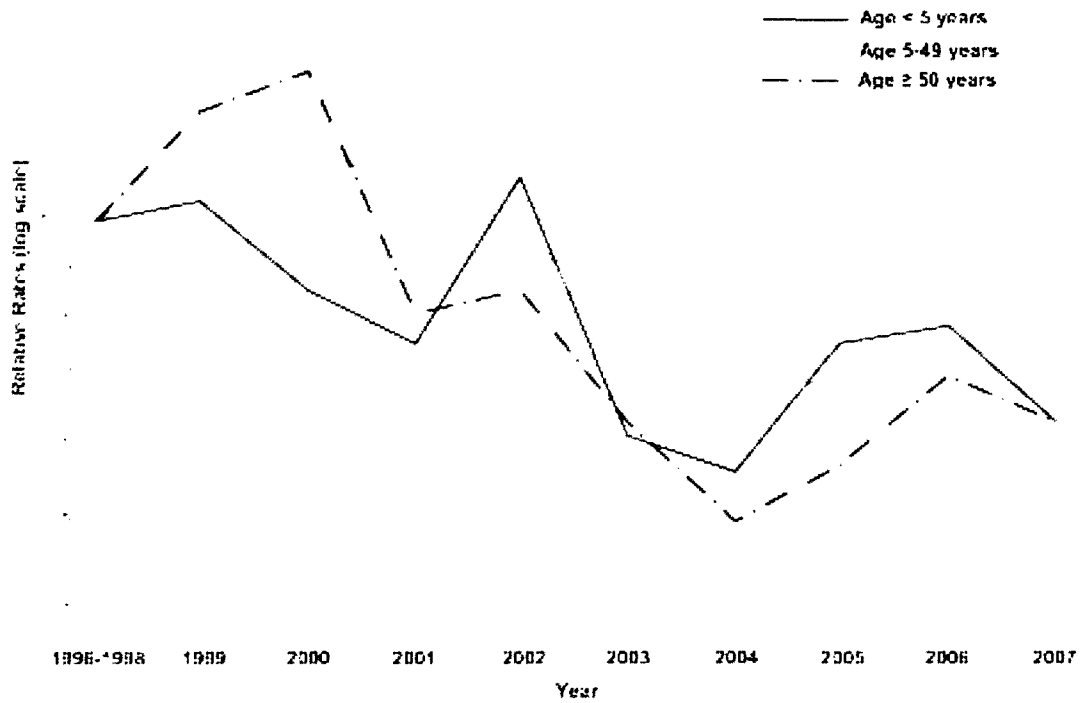


Figure 1.8. Relative Rates of *E. coli* O157:H7 Infection. The overall trend in disease is declining. The years of 2003-2004 had the lowest rates of infection, but were followed by two years of higher incidence before decreasing again in 2007 (40).

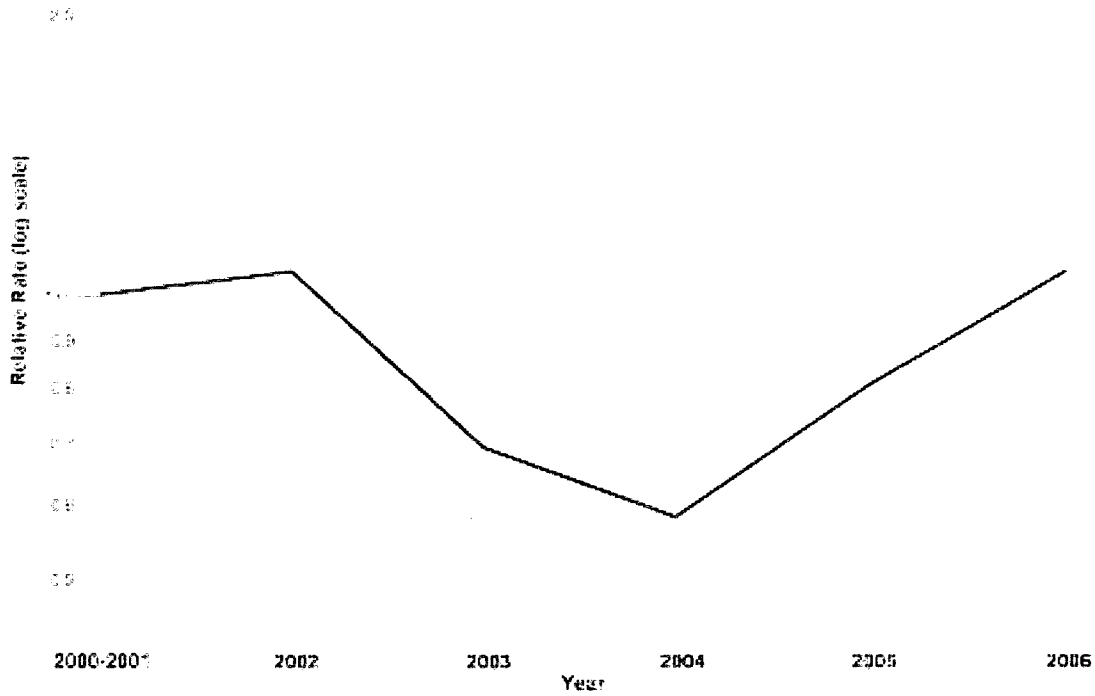


Figure 1.9. Relative Rates of *E. coli* O157:H7-associated HUS. The relative number of HUS cases dropped dramatically in 2003 and 2004, coinciding with the lower incidence of *E. coli* O157:H7 cases (40).

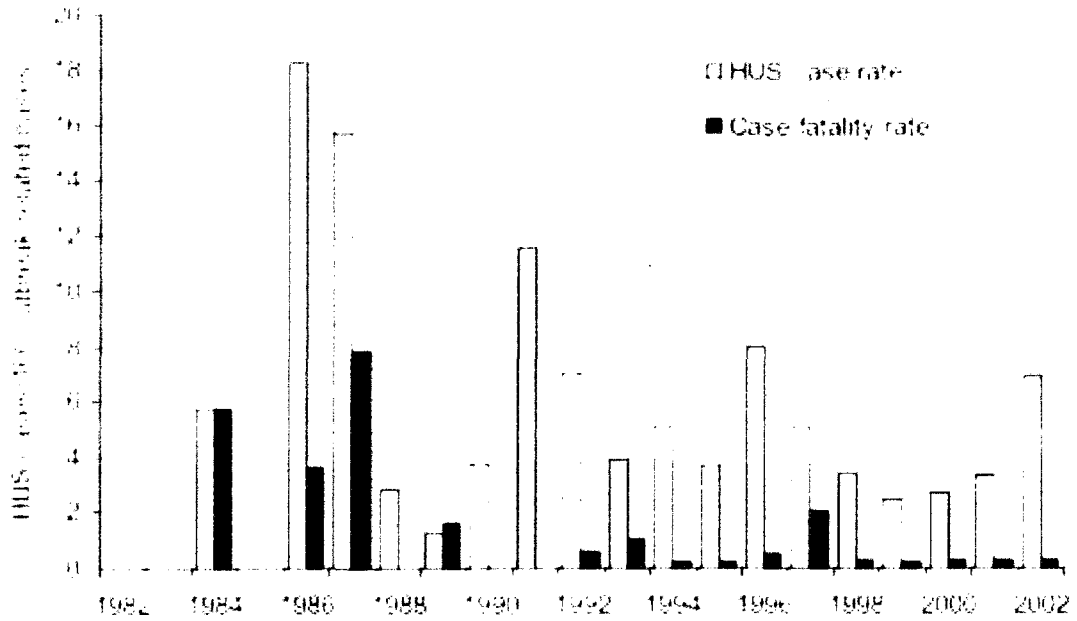


Figure 1.10. Number of Cases of HUS in Comparison to Case-fatality Rate. Overall, the number of cases of HUS has decreased, along with the proportion of fatal cases. This could be due to increased awareness of *E. coli* O157:H7 infections and their association with HUS (125).

Disease prevention is the same as for any gastrointestinal illness. Ground beef should be cooked to a minimum of 160°F to kill all *E. coli* O157:H7 (124).. It is also important to keep raw meats separate from ready-to-eat foods, or foods that will not be cooked. For example, knives and cutting boards should always be thoroughly washed if they have been in contact with raw meat before re-using for vegetables or other foods. Likewise, any fruits and vegetables should be washed thoroughly, particularly if they will not be cooked before consumption. Only pasteurized milk and juices should be consumed, to prevent the risk of ingesting *E. coli* O157:H7 from these beverages. When swimming, care should be taken to avoid swallowing lake or pool water. People with diarrhea should not be allowed to partake in recreational water activities until approved by a physician. As with the prevention of any infectious disease, careful and thorough hand washing with soap and warm water can greatly reduce the risk of acquiring or passing on an infectious agent (125).

2.1. Immune Response

In patients with HUS, increased concentrations of IL-6, IL-8 and TNF- α have been reported (80). These cytokines are produced by monocytes, which may be induced to release these inflammatory mediators by Stx-2. Likewise, a significant correlation exists between the neutrophil count in HUS patients and patients' outcomes. Children with poor outcomes (severe disease) had higher neutrophil counts on admission to the hospital. Similarly, patients who died of EHEC-associated HUS had significantly larger numbers of neutrophils in the renal glomeruli than in controls (80). These data add to the

suggestion that neutrophils are involved in Stx-2 carriage around the body during *E. coli* O157:H7 infections.

2.4. Treatment and Palliative Measures

Treatment of *E. coli* O157:H7 infections poses a true dilemma for most physicians. Most often, the patient is a child whose parents insist on treatment, yet traditional treatments (such as anti-diarrheal agents and antibiotics) are contra-indicated (97, 132, 171).

In the history of *E. coli* O157:H7 infections, the use of antibiotics has been both variable and controversial. In the first report on *E. coli* O157:H7 disease, two outbreaks were outlined. A total of 47 cases were identified and 23 patients had available treatment histories. Of those 23 patients, 11 were treated with antibiotics (8 with tetracycline and 3 with erythromycin). None of the 47 cases (including the 11 treated with antibiotics) developed any complications (such as HUS) (127). This information indicates that antibiotics are not detrimental to the outcome of *E. coli* O157:H7 infections. In a subsequent outbreak in a nursing home, 22% of the 55 affected patients developed HUS, and the mortality rate was 31%. Antibiotic therapy provided after onset was associated with increased fatalities (103). In this outbreak, the majority of patients were elderly, which may have contributed to the higher rate of complications and death. Nonetheless, this outbreak caused physicians and scientists to rethink the use of antibiotics for treating infections with this organism. A number of retrospective studies have been conducted on the efficacy of antibiotics in treatment protocols and this topic is a key component of this thesis. These details are discussed further in Chapter II. Currently, antibiotic treatment is

divisive (132). Although some physicians may continue to treat *E. coli* O157:H7 infections with antibiotics, a majority agrees that antibiotics are more harmful than helpful for combating this pathogen. Likewise, the use of antimotility agents (to reduce diarrhea symptoms) has been associated with a higher risk of developing HUS, and particularly HUS with central nervous system manifestations (97, 171).

Treatment is mainly palliative. Careful monitoring and rehydration therapy is often used to replace fluids lost to vomiting (in some cases) and severe diarrhea in non-HUS *E. coli* O157:H7 cases. In cases of HUS, the amount of fluids must be monitored carefully as the kidneys may be under some considerable stress, and extremely high blood pressure can result. Dialysis is often implemented to support kidney function and in severe cases plasmapheresis may be required (123). In essence, the objective is to remove circulating Stx from the blood in the hopes of reducing the damage to the microvascular structure of affected organs (123).

CHAPTER 2

THE EFFECTS OF SUB-INHIBITORY CONCENTRATIONS OF SELECTED ANTIBIOTICS ON THE PRODUCTION OF STX-2 BY *ESCHERICHIA COLI* O157:H7

1. **Abstract**

Shiga toxin 2 (Stx-2) is a major virulence factor contributing to the pathogenesis of HUS and to overall *E. coli* O157:H7 disease. The *stx2* gene is located within the genome of a lambda-like prophage, 933-W, and thus the genetic and physiological mechanisms involved in the production of Stx-2 are complex and closely involve induction or repression of the prophage. It was hypothesized that growth of the *E. coli* O157:H7 in DNA-damaging antibiotics would increase *stx2* expression and Stx-2 release by initiating the bacterial SOS DNA repair response. Likewise, it was hypothesized that growth of the pathogen in antibiotics that do not affect bacterial DNA (β -lactams, protein synthesis inhibitors, cell membrane disruptors, and transcription inhibitors) would not initiate the SOS DNA repair response, and therefore would not increase Stx-2 production. *E. coli* O157:H7 was grown in 90% MIC-level antibiotics, and at 1 and 6 h post-induction, the *stx2* gene was assessed using quantitative Real-Time PCR. At 24 h post-induction, the production of Stx-2 toxin was measured using a capture ELISA, as well as by MTT cytotoxicity assay. It was determined that *stx2* expression and Stx-2 production increased following microbial exposure to the DNA-damaging antibiotics ciprofloxacin, norfloxacin, and trimethoprim, as well as with the positive control, mitomycin C. These

results were concurrent with an upregulation in the DNA damage inducible gene, *dinD*. No significant change was detected when the organism was grown in cefotaxime or chloramphenicol. Rifampin significantly decreased Stx-2 production and *stx2* expression. Gentamicin and polymyxin B increased *stx2* expression but did not increase Stx-2 release, and this may be associated with the cell membrane-altering properties of both these antibiotics. These studies suggest that *stx2* expression and subsequent release of Stx-2 are linked to the SOS DNA repair system, and that antibiotics that damage bacterial DNA should be avoided in clinical use for *E. coli* O157:H7 infections. Rifampin may serve as a viable alternative for treating these infections given the decrease noted in the *stx2* expression and Stx-2 release; however, the rapid development of resistance to this antibiotic may relegate it to a secondary role in combined therapy.

2. **Introduction**

Escherichia coli O157:H7 is the causative agent of “hamburger disease”, a severe type of food poisoning with symptoms ranging from profuse, watery diarrhea to the bloody diarrhea of hemorrhagic colitis (HC) (8, 103, 125, 127, 170). In 5-15% of cases, this disease escalates to HUS (75, 113), and unfortunately there is no curative treatment for these infections. In the past, antibiotics were prescribed to combat the bacterium but research has contra-indicated this approach (10, 97, 99, 109, 132, 156, 157, 171). Indeed, one study showed a 17x higher rate of HUS development in children treated with antibiotics compared to those not treated with antibiotics (171). Despite a wide range of studies on the pathogenesis of *E. coli* O157:H7, specific treatments aimed at the source of the disease have not been forthcoming. Rather, most treatments are palliative, that is,

they are associated with treating symptoms rather than eradicating the etiological agent causing the disease. As such, research on the efficacy of antibiotic treatment of *E. coli* O157:H7 infections is of value.

The principal virulence factors produced by *E. coli* O157:H7 that initiate this disease are the Shiga-like toxins (Stx), including Stx-2 (12, 14). The *stx2* gene is located on a lambda-like bacteriophage, BP-933-W, inserted as a prophage into the chromosome of *E. coli* O157:H7. When the microorganism is metabolically stable, the prophage remains in a lysogenic state. However, if the *E. coli* host is subjected to stress, the prophage converts to a lytic state and is excised from the chromosome (104, 164). During excision, the *stx2* gene is co-expressed with phage genes, leading to an increase in toxin production. Upregulation of *stx2* gene expression is believed to be the end result in a cascade pathway that begins with the activation of the RecA protein by damaged DNA (74). When the regulating proteins cII and cIII are expressed at low levels they activate the repressor cI. cI in turn binds and represses the promoters, Pro_L and Pro_R, to stop transcription of the majority of the phage genome, including the toxin gene, *stx2*. In this manner, the system is self-repressing and self-regulating; it blocks transcription of the phage genome unless something interrupts the cycle. This interruption occurs when RecA becomes activated by DNA damage. RecA is a protein normally involved in recombination events, but when activated it becomes a cellular protease. RecA causes cII to be degraded and hence the expression of cI is arrested. Without expression of the cI repressor, the late phage genes are expressed, including *stx2* (78, 120, 164). This conversion to the lytic cycle is a bacteriophage survival mechanism effected when host (*E. coli* O157:H7) DNA is compromised. The *stx2* gene is located between the late phage

promoter and phage-excision genes and is co-transcribed when host cell damage is sensed. Therefore, under DNA damaging conditions induction of phage genes and *stx2* occurs simultaneously (74, 86, 166).

It was previously unknown if increased Stx-2 levels were due to an increase in *stx2* expression, or if it were simply lysis of bacterial cells which released pre-formed, stored toxin. Kimmit et al. provided convincing evidence that genuine toxin gene expression occurred in response to environmental stress (74). Specifically, Kimmit's research showed that DNA-damaging antibiotics such as the fluoroquinolones and folate synthesis inhibitors are the most potent inducers of the *stx2* promoter (74). In a genome-wide assay, Herold et al. demonstrated upregulation of a number of genes after treatment with sub-inhibitory concentrations of the DNA-damaging antibiotic norfloxacin (57). Of significance, their study yielded a 150-fold increase in *stx2* expression in the presence of high levels of norfloxacin. They surmised that with very low concentrations of the antibiotic, *E. coli* was using an alternate RecA-independent DNA repair pathway. However, when high, near-inhibitory concentrations were employed, the "last-ditch" SOS response of the bacterium was activated which increased *stx2* induction (57).

In 2007, Ochoa et. al showed that the transcription-inhibitor rifaximin did not induce Stx-2 or 933-W phage release, as opposed to the DNA-damaging antibiotic ciprofloxacin, which increased Stx-2 production in 96% of strains evaluated (109). This confirmed previous hypotheses that DNA-damaging antibiotics lead to an increased production of Stx-2, and thus should be avoided in clinical situations involving *E. coli* O157:H7. However, whether all antibiotics or just those that affect bacterial DNA should be avoided as clinical treatment for these infections has yet to be elucidated.

In the current study, the production of Stx-2 and the activity of *stx2* were measured when the organism *E. coli* O157:H7 was grown in sub-inhibitory concentrations of various antibiotics. These were selected from a wide variety of antibiotic classes, and included a broad range of bacterial targets. Those antibiotics studied were: i) cefotaxime, a cell wall inhibitor; ii) ciprofloxacin, a DNA gyrase inhibitor; iii) chloramphenicol, a protein synthesis inhibitor; iv) gentamicin, a protein synthesis inhibitor; v) polymyxin B, a cell membrane inhibitor; vi) rifampicin, a transcription inhibitor; vii) norfloxacin, a DNA gyrase inhibitor; and viii) trimethoprim, a folate metabolism inhibitor. Mitomycin C was used as a positive control, as it has been shown to be a potent mutagen that upregulates Stx-2 production (138). These antibiotics were selected to comprise both DNA-affecting and non-DNA affecting drugs. Likewise, polymyxin B was included because of its cell-membrane affecting abilities; it has been suggested that Stx-2 is increased by some antibiotics simply due to bacteriolysis. Polymyxin B is known to cause bacteriolysis (137) and in that respect serves as a comparison control for other antibiotics.

Stx-2 production was measured by both cytotoxicity assays and by enzyme-linked immunosorbent assay (ELISA). The expression of the *stx2* gene was measured by quantitative Real Time PCR (q-PCR). In q-PCR studies, two additional genes were investigated, *serC* (serine synthase) and *dinD* (DNA-damage inducible). The *serC* gene was included because it is considered a “housekeeping gene”; it is constitutively transcribed despite growth conditions (57). All results were expressed as a function of *serC* activity to allow for differences in experimental techniques (efficacy of RNA extraction, cDNA synthesis, etc.). The *dinD* gene is expressed when DNA damage is

sensed within the *E. coli* O157:H7 cell. If *stx2* is upregulated because of damage to DNA then the *dinD* gene should also be upregulated.

3. Hypothesis

It was hypothesized that an upregulation in *stx2* expression and an increase in Stx-2 production would occur when the *E. coli* O157:H7 was grown in DNA-damaging antibiotics. Conversely, growth of the pathogen in antibiotics that target other parts of the bacterial cell (excluding the DNA) were hypothesized to have no effect on *stx2* expression or Stx-2 production.

4. Materials and Methods

Media and reagent preparations are provided in detail in Appendix A.

4.1. Bacterial Cultivation

The *E. coli* O157:H7 strains 90-2380 and 43888 stock cultures were acquired and maintained as described in Appendix B. Overnight and experimental cultures used in antibiotic experiments were grown on Mueller-Hinton (MH) agar plates, or in Mueller-Hinton broths (MHB). All cultures were grown statically at 37°C.

4.2. Vero Cell Growth and Maintenance

African green monkey kidney (vero) cells were maintained as described in Appendix B. In preparation for cytotoxicity assays, vero cells were passaged into a 96-well microtiter plate (Corning Life Science, Lowell, MA) as follows: Log-phase (60-

70% confluent) vero cells in a T₂₅ flask were rinsed twice with Hank's balanced salt solution-modified (HBSS-mod). Following thorough rinsing, trypsin-EDTA solution was added to the flask and allowed to incubate at room temperature for approximately three min. The cells were observed through an Olympus CK2 inverted phase-contrast microscope (Olympus, Center Valley, PA) for removal from the bottom of the flask. This was noted by the rounding up of cells as they released from the flask surface and became free-floating in the liquid. The action of the trypsin was ceased by the addition of five times the volume of Dulbecco's modified Eagle's minimal essential media (DMEM) with three percent bovine calf serum (BCS). This suspension of free-floating vero cells was centrifuged (IEC Centra, Waltham, MA) for five min at room temperature at 1000 rpm to pellet the cells. The supernatant fluid was carefully removed and discarded, and the resulting pellet was resuspended in DMEM with 3% (v/v) BCS. A cell count was performed in a hemocytometer using a dilution in the viability stain, trypan blue. Vero cells were then seeded into the 96-well plates at a concentration of 10,000 cells/well.

4.3. Minimum Inhibitory Concentration (MIC) Assays for Antibiotics

A minimum inhibitory concentration (MIC) assay was performed for each antibiotic under investigation to determine the lowest concentration that could still inhibit the growth of *E. coli* O157:H7 strain 90-2380. A microtiter plate method was utilized (see Table 2.1), in which each row of the 96-well plate contained a 1:2 dilution of a specific antibiotic; the last two columns did not contain antibiotic. Column 11 was used as a no-antibiotic control and column 12 was a media-only control. The eight antibiotics were prepared as listed in Appendix A. A standardized suspension of *E. coli* O157:H7

Table 2.1. MIC Plate Set Up.

	Columns 1-10	Column 11	Column 12
A	Cefotaxime, 1:2 dilution, 10 mg/mL to 19.5 µg/mL	<i>E. coli</i> only (no antibiotic)	Media only
B	Ciprofloxacin, 1:2 dilution, 1 µg/mL to 1.95 ng/mL	<i>E. coli</i> only (no antibiotic)	Media only
C	Chloramphenicol, 1:2 dilution, 100 µg/mL to 195 ng/mL	<i>E. coli</i> only (no antibiotic)	Media only
D	Gentamicin, 1:2 dilution, 100 µg/mL to 195 ng/mL	<i>E. coli</i> only (no antibiotic)	Media only
E	Polymyxin B 1:2 dilution, 10µg/mL to 19.5 ng/mL	<i>E. coli</i> only (no antibiotic)	Media only
F	Rifampin, 1:2 dilution, 100 µg/mL to 195 ng/mL	<i>E. coli</i> only (no antibiotic)	Media only
G	Norfloxacin, 1:2 dilution, 1 µg/mL to 1.95 ng/mL	<i>E. coli</i> only (no antibiotic)	Media only
H	Trimethoprim, 1:2 dilution, 10µg/mL to 19.5 ng/mL	<i>E. coli</i> only (no antibiotic)	Media only

strain 90-2380 was added to each well of the plate, except column 12, the media-only control column. The plate was incubated overnight at 37°C statically. Following incubation, the plate was visually observed for turbidity in each well. The plate was also assessed spectrophotometrically for absorbance values at 595 nm. The absorbance values were graphically represented to depict the lowest concentration of antibiotic that inhibited growth of the organism, as compared to the no-antibiotic control.

4.4. Growth Curves of *E. coli* O157:H7 in 90% MIC Antibiotics

E. coli O157:H7 strain 90-2380 was grown in stationary culture at 37°C overnight in MHB. The following day the culture was adjusted with pre-warmed MHB to give an optical density of 0.2 at 595 nm. This culture was grown for an additional 60 min at 37°C to achieve log phase of growth. The culture was then centrifuged at 4,000 rpm for 5 min (IEC Centra) and the pellet was resuspended in MHB media to the same volume as before centrifugation. The culture was then divided into 10 equal 5-mL aliquots. The eight antibiotics were added to each of the 5-mL aliquots to yield a final concentration equivalent to 90% of that antibiotic's MIC. A positive control containing 2.5 µg/mL of mitomycin C-induced culture was included as the ninth sample, and the tenth sample was left uninduced.

Three 200µL aliquots from each of these ten tubes were immediately removed and pipetted in triplicate into a sterile 96-well plate (Corning). The 96-well plate was inserted into a microplate reader (Tecan, Durham, NC) and maintained in static conditions at 37°C. Absorbance readings at 595 nm were taken every 5 to 15 min for 6 h

and recorded into an Excel spreadsheet. The data were plotted to determine a growth curve for *E. coli* O157:H7 in the presence of antibiotics at 90% of their MIC level.

4.5. MTT Cytotoxicity Assay Standards

A standard curve for the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) was prepared by seeding vero cells in quadruplicate into each well of a 96-well plate to give a dilution series. This was achieved in a similar fashion to the passaging procedure described, except instead of seeding 10,000 cells/well, wells were seeded at the concentrations outlined in Table 2.2. Vero cells in each well were stabilized for 1 h at 37°C in 7% CO₂ (Airgas, Salem, NH) to allow cells to settle and attach to the microtiter plate. Following the 1 h incubation, 10µL of MTT salt solution was added to each well and incubated for 3 h at 37°C in 7% CO₂. During this incubation, viable vero cells converted the yellow MTT salt to insoluble purple formazan crystals. After incubation, 100 µL MTT lysing solution was added to each well. This solution lysed the vero cells and dissolved the fomazan crystals to give a uniform shade of purple, which was evaluated in a Biorad 3550 spectrophotometer (BioRad, Hercules, CA). The absorbance data generated was graphically plotted in relation to the seeded number of vero cells, resulting in a linear equation over a particular concentration of vero cells. This standardization process allowed for unknown concentrations of vero cells to be enumerated based on a correlation to this standard.

Table 2.2. Vero Cell Seeding for MTT Standards.

Col 1	Col 2	Col 3	Col 4	Col 5	Col 6	Col 7	Col 8	Col 9	Col 10
0 cells	5×10^2 cells	1×10^3 cells	2.5×10^3 cells	5×10^3 cells	1×10^4 cells	2.5×10^4 cells	5×10^4 cells	7.5×10^4 cells	1×10^5 cells

4.6. Induction of Stx-2 by Antibiotics for MTT Assay

E. coli O157:H7 strain 90-2380 was grown in static culture overnight in MHB at 37°C. The following day the culture was adjusted with pre-warmed MHB to give an optical density of 0.2 at 595 nm. This culture was grown for an additional 60 min to stimulate the cells into log phase growth. This culture was centrifuged at 4,000 rpm for 5 min (IEC Centra), the supernate was discarded, and the pellet was resuspended in the identical type and volume of media as before centrifugation. This served to remove residual toxin from the overnight growth so that only newly-formed toxin would be assayed. The culture was divided into 10 equal 5-mL aliquots. Each of the eight antibiotics was added to each 5-mL aliquot to yield an overall concentration equal to 90% of that antibiotic's MIC. This value was recorded as the sub-inhibitory concentration (SIC). A positive control of culture containing 2.5 µg/mL mitomycin C to induce DNA damage to organisms was included, along with a negative control of uninduced bacteria. The control cultures and the test cultures (grown in the SIC of each antibiotic) were incubated under static conditions for 24 h at 37°C. Immediately following incubation, the cultures were centrifuged at 4°C at 4,000 rpm for 10 min to pellet. The supernates were removed and filtered through a low-protein binding 0.2 µm filter (Pall, East Hills, NY) to give cell-free preparations. These supernates were stored at -20°C for use in MTT assays.

4.7. Antibiotic-Induced MTT Assay

Supernates from antibiotic-induced cultures were added in triplicate (10µL) to a 100% confluent 96-well plate of vero cells. Controls (mitomycin C-induced and uninduced) were also added to the vero cell plate. The plate was incubated at 37°C in 7%

CO₂ (Airgas) for 72 h. Following the 3-day incubation, 10 µL of MTT salt was added to each supernate-containing well. The plate was incubated for 3 h at 37°C in 7% CO₂. After incubation, 100 µL of MTT lysing solution was added to each well. This solution lysed the vero cells and dissolved the fomazan crystals to a uniform shade of purple, which was then read in a Biorad 3550 spectrophotometer (BioRad) and recorded.

4.8. Induction of Stx-2 by Antibiotics for Real Time PCR and ELISA

E. coli O157:H7 was grown as static cultures overnight in MHB at 37°C. The following day the culture was adjusted with pre-warmed MHB to give an optical density of 0.2 at 595 nm. This culture was grown for an additional 60 min to stimulate the cells into log phase growth. This culture was centrifuged at 4,000 rpm for 5 min (IEC Centra) the supernate was discarded, and the pellet was resuspended in the identical type and volume of media as before centrifugation. This served to remove residual toxin from the overnight growth so that only newly-formed toxin would be assayed. The culture was divided into 10 equal 2-mL aliquots. Each of the eight antibiotics was added to each 2-mL aliquot to yield an overall concentration equal to the SIC for each antibiotic. A positive control culture containing 2.5 µg/mL mitomycin C to induce DNA damage to organisms was included, along with a negative control of uninduced bacteria. The control cultures and the test cultures (grown in the SIC of each antibiotic) were incubated under static conditions for 1 h or 6 h at 37°C. Immediately following incubation, 100 µL of each culture was removed to a sterile microcentrifuge tube and retained for RNA extraction, which was performed immediately. For the collection of supernates for ELISA analysis of Stx-2 concentration, the cultures were further incubated for a total of

24 h post-induction. The 2-mL cultures were then centrifuged at 4°C at 4,000 rpm for 10 min to pellet the bacterial cells. The supernates were removed and filtered through a low-protein binding 0.2 µm filter (Pall) to give cell-free preparations. These supernates were stored at -20°C for use in ELISA assays.

4.9. RNA Extraction and cDNA Synthesis

Directly following antibiotic induction of the cultures, 500 µL of Bacterial RNA Protect™ (Qiagen, Valencia, CA) was added to each of the 100 µL aliquots and incubated at room temperature for 15 min. The tubes were then centrifuged at 5400 rpm (Beckman, Center Valley, PA) for 5 min. RNA was collected using the Promega SV Total RNA Isolation System™ kit (Promega, Madison, WI), as follows. The supernates were discarded and the pellets resuspended in 100 µL of tris-ethylene diamine tetra acetic acid (TE) buffer along with 2 µL of lysozyme stock (20mg/mL,) and incubated at room temperature for 5 min. The cells were further lysed by the addition of 75 µL of RNA Lysis Buffer (Promega), and 350 µL of RNA dilution buffer (Promega). Nucleic acids were precipitated by the addition of 200 µL 95% ethanol (Acros, Morris Plains, NJ). Each solution was transferred to a separate filter spin column provided in Promega kit and centrifuged at 10,000rpm for 1 min. The filtrates were discarded and 600 µL of RNA wash solution were added to rinse remaining cellular debris from the columns. The tubes were re-centrifuged at 10,000 rpm for 1 min. DNA in each column was degraded by the addition of 40 µL yellow core buffer (Promega), 5 µL of 0.09M MnCl₂ (Promega), and 5 µL DNase I enzyme (Promega). The DNase mixture was incubated at room temperature directly on the spin column membranes for 30 min. After incubation, 200µL

of DNase stop solution (Promega) was added to the spin columns and centrifuged at 10,000rpm for 1 min. The RNA was washed twice, first with 600 μ L, then with 250 μ L, of RNA wash solution (Promega) to remove degraded DNA and residual buffer. The spin columns were transferred to elution tubes and 100 μ L of nuclease-free water was added to the membranes. The columns were centrifuged at 10,000 rpm for 1 min to elute the RNA. The spin columns were removed and discarded, and the eluted RNA was stored at -20°C for use in cDNA synthesis assays.

The RNA was checked for DNA contamination by PCR. As PCR amplifies DNA only, this step was included to verify that there was no DNA contaminating the RNA samples. PCR was run in a thermocycler (Perkin-Elmer GeneAmp, Waltham, MA) with a 5 min, 94°C denaturation step, followed by 30 cycles of amplification. The amplification cycles were 94°C for 30 sec, 54°C for 30 sec, 72°C for 30 sec. The PCR run was terminated with a 7 min elongation step at 72°C. Purified *E. coli* O157:H7 strain 90-2380 DNA was used as the positive control. All PCR products were stored at -20°C for agarose gel electrophoresis purity analysis.

A 2.5% agarose gel was made by melting 1.25 g of agarose (Omnipur, Lawrence, KS) in 50 mL of 1x Tris-acetate-ethylene diamine tetraacetic acid (TAE). The gel solution was poured into the casting stand and allowed to solidify, during which time the samples were prepared by mixing 6 μ L of the template (in this case, the PCR product of extracted RNA) with 2 μ L 6x loading buffer (Promega) and 4 μ L d.H₂O. The solidified gel was placed into the electrophoresis apparatus (Biorad) and covered with 1x TAE. Samples were loaded into the gel and run at approximately 100 volts (+/- 5 volts) until two-thirds of the way down the gel. A 1 kb ladder with 100 bp bands (New England

Biolabs, Ipswich, MA) was run simultaneously for band size comparisons. The gel was stained in ethidium bromide and visualized in an imaging system (Syngene Bio-Imaging, Frederick, MD). Samples that were DNA-free were deemed acceptable for cDNA synthesis. Any samples found to contain DNA were discarded.

cDNA synthesis was performed using the SuperScript III cDNA synthesis kit™ (Invitrogen, Carlsbad, CA). In thin-walled PCR tubes, 6 µL of RNA, 1 µL of 50 ng/µL random hexamers (Invitrogen), and 1 µL annealing buffer (Invitrogen) were combined and collected by a brief, 3-second mini-centrifugation. The tubes were heated for 5 min at 65°C in a heating block (VWR, Westchester, PA) and immediately placed on ice for 1 min. Following the ice-incubation, 10 µL of 2x First-strand reaction mix (Invitrogen) was added to the tube with 2 µL of Superscript III/RNase Out enzyme mix (Invitrogen). The samples were thoroughly mixed and placed in the thermocycler with the following settings: 10 min at 25°C, 50 min at 50°C, 85°C for 5 min, followed by a 4°C hold period. cDNA samples were amplified through a second PCR and agarose gel to ensure proper reverse-transcription of RNA into cDNA was achieved. cDNA samples which amplified in a PCR reaction were deemed acceptable and were stored at -20°C until for use in Real Time PCR assays.

4.10. Quantification of cDNA

cDNA samples were quantified using the Qubit High Sensitivity (HS) DNA Quantification kit™ (Invitrogen) according to the manufacturer's instructions. In brief, the 200x concentrated analyzing reagent was diluted 1:200 in the supplied diluent to give a single strength analyzing reagent. cDNA samples were diluted 1:100 in the analyzing

reagent and were incubated at room temperature for three min, after which each sample of cDNA was assayed for fluorescence activity in the Qubit fluorometer. The fluorometer was calibrated prior to each use using the kit-supplied standards.

4.11. cDNA standardization

Antibiotic-induced cDNA samples were standardized to a 100 ng/mL concentration using nuclease-free water prior to use in Real Time PCR experiments. Standardized cDNA samples were stored at -20°C.

4.12. Real Time Polymerase Chain Reaction

Real Time PCR was performed using an AP 7300 Thermocycler and software (Applied Biosystems, Carlsbad, CA). PCR was performed using 200 nM of dual-labeled (FAM/TAMRA) fluorogenic probes (Appendix C), 500 nM of both forward and reverse primers (Appendix C), and a 1x concentration of PCR enhancer (5Prime, Gaithersburg, MD) and a 1x concentration of RealMasterMix with Rox (5Prime). The RealMasterMix with Rox contains 0.1 U/ μ L Taq DNA Polymerase, 12.5 mM magnesium acetate, and 1.0 mM dNTPs. A standard curve was performed with each Real Time PCR experiment, and each standard curve was constructed using highly-purified PCR product (see Appendix C) at a final concentration of 100 ng prepared in a 10-fold dilution to give a terminal concentration of 10 pg.

Real Time PCR data were analyzed using the REST (Relative Expression Software Tool) program. The PCR efficiency for each Real Time PCR run was entered into the software program along with the cycle threshold values for each sample. The

program normalized each value to the supplied data for the *serC* housekeeping reference gene and to its PCR efficiency. Once normalized, the treated samples, consisting of the eight antibiotics and mitomycin C were compared against the untreated sample (uninduced) for up or down regulation in the genes of interest (*stx2*, *dinD*).

4.13. Enzyme-linked Immunosorbent Assay

ELISA was designed based after a protocol by Moody, 2003 (102). Murine anti-Stx-2 subunit A (monoclonal IgG 11E10, Toxin Technology, Sarasota, FL) was employed as the primary antibody. This was diluted 1:250 to give a working stock IgG preparation in sterile 1x PBS. This primary antibody was applied to the ELISA plate and incubated overnight on a plate rocker to allow the antibody to adhere to the wells of the microtiter plate. The following day, plates were drained and each well filled with blocking buffer containing 5% skim milk and allowed to rock at room temperature for 1 h to prevent subsequent non-specific binding. The plate was then emptied and rinsed three times with PBS. A ten-fold dilution series of purified Stx-2 (Toxin Technology) was applied in triplicate in order to derive a standard curve for the assay. The supernates to be tested were also added in separate wells in the plate in triplicate. Any unused wells were filled with blocking buffer and the entire plate was incubated at room temperature for 2 h while rocking. The plate was then emptied and rinsed three times with PBS. The secondary antibody, monoclonal murine antibody specific to Stx-2 subunit A and conjugated to horseradish peroxidase (Toxin Technology), was added to each well and incubated at room temperature for 2 h while rocking. After incubation, the wells were thoroughly washed (five times) with PBS to remove all traces of unbound secondary

antibody. The horseradish peroxidase chromogenic substrate (2,2'-Azinobis [3-ethyl-benzothiazoline-6-sulfonic acid]-diammonium salt, ABTS) solution (Pierce, Rockford, IL) was added to the wells and incubated for 1 h. The plate was spectrophotometrically analyzed at 405 nm and the data were recorded.

5. **Results**

Statistical significance was determined as described in Appendix B, Section 5.

5.1. **Minimum Inhibitory Concentration (MIC) Assay**

The MIC value for each antibiotic was determined to be the lowest concentration of antibiotic that still inhibited the growth of the *E. coli* O157:H7. In all cases, the visual (Figure 2.1) and spectrophotometric (Figure 2.2) results for the MIC were in agreement. The sub-inhibitory concentration (SIC) was calculated as 90% of these derived MIC values (Table 2.3).

MIC values were lowest for the DNA-damaging antibiotics ciprofloxacin and norfloxacin; a concentration of 31.25 ng/mL was sufficient to inhibit growth of the *E. coli*. Trimethoprim, an inhibitor of thymidine and uridine synthesis, and therefore involved in blocking the synthesis of DNA, was found to have an MIC value of 156.25 ng/mL. The same MIC value was calculated for gentamicin, an aminoglycoside. Polymyxin B, a cell membrane disruptor, required 1.25 µg/mL to inhibit growth of the microorganism. Cefotaxime, a beta lactam, and rifampin, an inhibitor of transcription

A)

	Columns 1-10	Column 11	Column 12
1	Cefotaxime, 1:2 dilution, 10 mg/mL to 19.5 μ g/mL	<i>E. coli</i> only (no antibiotic)	Media only
2	Ciprofloxacin, 1:2 dilution, 1 μ g/mL to 1.95 ng/mL	<i>E. coli</i> only (no antibiotic)	Media only
3	Chloramphenicol, 1:2 dilution, 100 μ g/mL to 195 ng/mL	<i>E. coli</i> only (no antibiotic)	Media only
4	Gentamicin, 1:2 dilution, 100 μ g/mL to 195 ng/mL	<i>E. coli</i> only (no antibiotic)	Media only
5	Polymyxin B 1:2 dilution, 10 μ g/mL to 19.5 ng/mL	<i>E. coli</i> only (no antibiotic)	Media only
6	Rifampin, 1:2 dilution, 100 μ g/mL to 195 ng/mL	<i>E. coli</i> only (no antibiotic)	Media only
7	Norfloxacin, 1:2 dilution, 1 μ g/mL to 1.95 ng/mL	<i>E. coli</i> only (no antibiotic)	Media only
8	Trimethoprim, 1:2 dilution, 10 μ g/mL to 19.5 ng/mL	<i>E. coli</i> only (no antibiotic)	Media only

B)

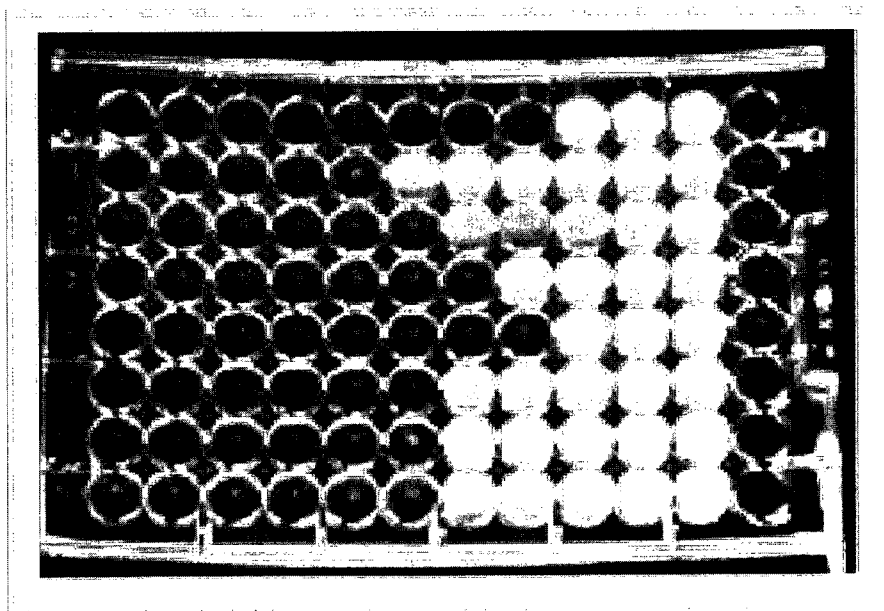


Figure 2.1. MIC Microtiter Plate. A) Schematic set up of the MIC microtiter plate. Each row of the microtiter plate represents an antibiotic to be tested. The first, left-most column of the microtiter plate is the stock concentration of the antibiotic, and each subsequent column represents a 2-fold dilution of that antibiotic. Column 11 was the no-antibiotic control (PBS only), and Column 12 was the no-*E. coli* control (PBS only). B) MIC plates were inspected visually for turbidity (opalescent) in a well, indicating growth. The lowest concentration of antibiotic that still inhibited growth was defined as the MIC.

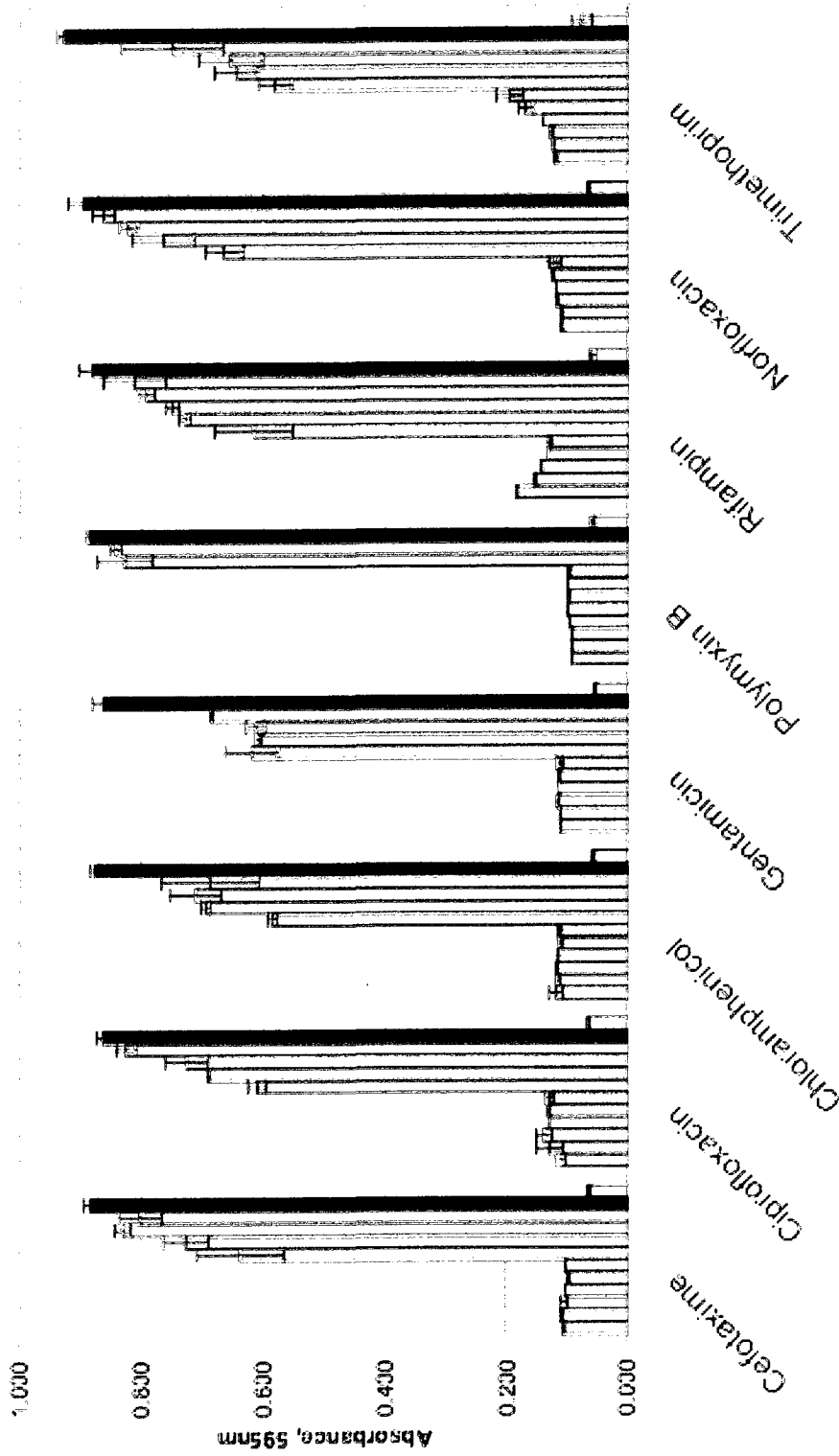


Figure 2.2. MIC Determination By Spectrophotometer. The MIC plate was read at 595nm to determine the optical density of each well. Each collection of gray bars (solid and hatched) represents the density of growth of the *E. coli* in each dilution of antibiotic. The black bars represent growth of the *E. coli* without antibiotic. The white bars represent media-only controls. The hatched gray bar represents the MIC value; this is the lowest concentration of antibiotic that still inhibited growth of the organism. See Table 2.3 for tabulated data.

Table 2.3. Determined MIC and Calculated SIC for *E. coli* O157:H7

Antibiotic	Target	MIC	SIC (90% MIC)
Cefotaxime	Inhibits peptidoglycan synthesis	5.0 µg/mL	4.5 µg/mL
Ciprofloxacin	Inhibits DNA gyrase	31.25 ng/mL	28.13 ng/mL
Chloramphenicol	Inhibits peptidyl transferase, 50S subunit	10 µg/mL	9 µg/mL
Gentamicin	Prevents formation of initiation complex with mRNA (30S subunit), Causes misreading of mRNA, possible other mechanisms.	156.25 ng/mL	140.63 ng/mL
PolymyxinB	Detergent, interacts with phospholipids.	1.25 µg/mL	1.13 µg/mL
Rifampin	Binds and inhibits RNA polymerase	5 µg/mL	4.5 µg/mL
Norfloxacin	Inhibits DNA gyrase	31.25 ng/mL	28.13 ng/mL
Trimethoprim	Inhibits <i>de novo</i> thymidine and uridine synthesis.	156.25 ng/mL	140.63 ng/mL

processes, both had calculated MIC values of 5µg/mL. Chloramphenicol was found to have an MIC value of 10µg/mL.

5.2. Growth Curves of *E. coli* O157:H7 in Antibiotics

E. coli O157:H7 strain 90-2380 was grown and induced with antibiotics in a fashion identical to that described for ELISA supernates and mRNA collection for Real Time PCR. Following induction, the cultures were measured for absorbance at 595nm every 5 to 15 min. The data were collected and used to generate a growth curve of the organism in the presence of the SIC of each antibiotic. As can be seen from Figure 2.3, no significant differences were detected between the growth of the organism in the SIC of each antibiotic, and in the uninduced control culture.

5.3. MTT Cytotoxicity Assay

An MTT cytotoxicity assay was developed to determine the effects on vero cells of the Stx-2-containing supernates. A standard control curve was developed for the MTT assay by seeding a 96-well plate with a known dilution of vero cells (without Stx-2) and comparing it to the absorbance created by those cells at the completion of the MTT test. The standard curve (Figure 2.4) showed excellent linearity between the number of vero cells and the absorbance value recorded by spectrophotometer. The correlation coefficient for the line was 0.9992.

Once the standard curve had been established for the assay, vero cells were exposed to the various Stx-2-containing supernates collected from *E. coli* O157:H7 strain 90-2380 grown in each of the eight antibiotics. Data were normalized against the uninduced control. Supernates from the organism grown in mitomycin C were used as

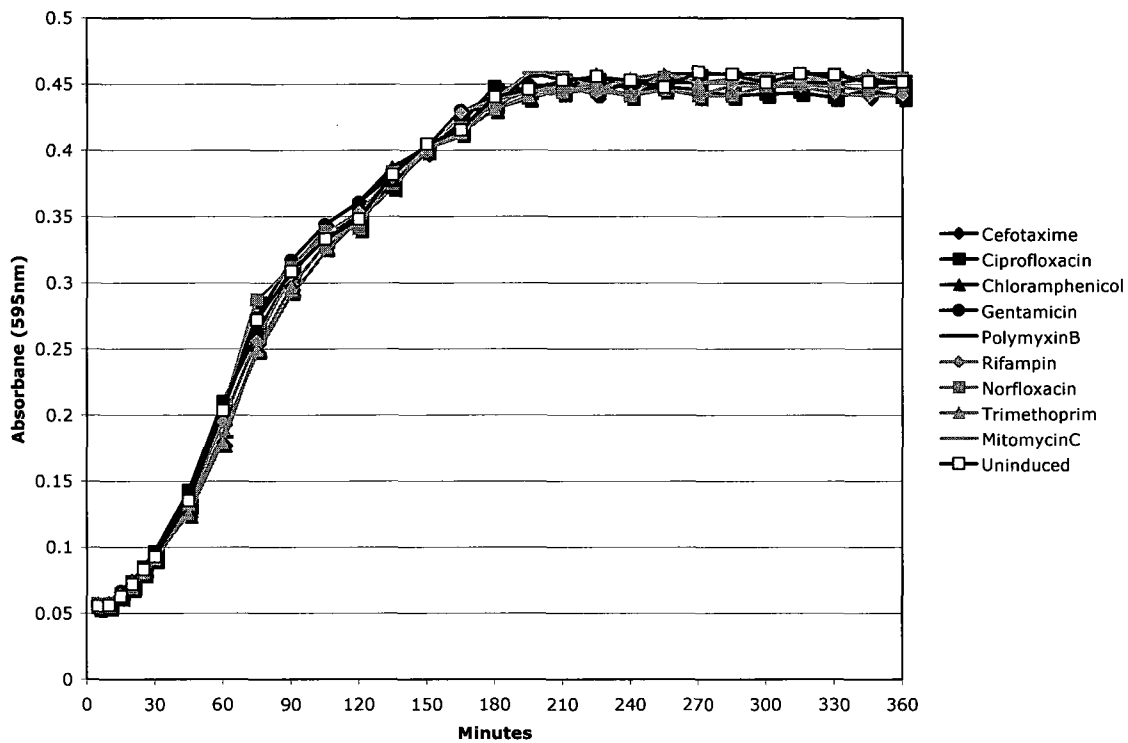


Figure 2.3. Growth Curve of *E. coli* O157:H7 strain 90-2380 in SIC-level Antibiotics Investigated. Note that growth curves of the organism in the antibiotics were similar to the uninduced controls.

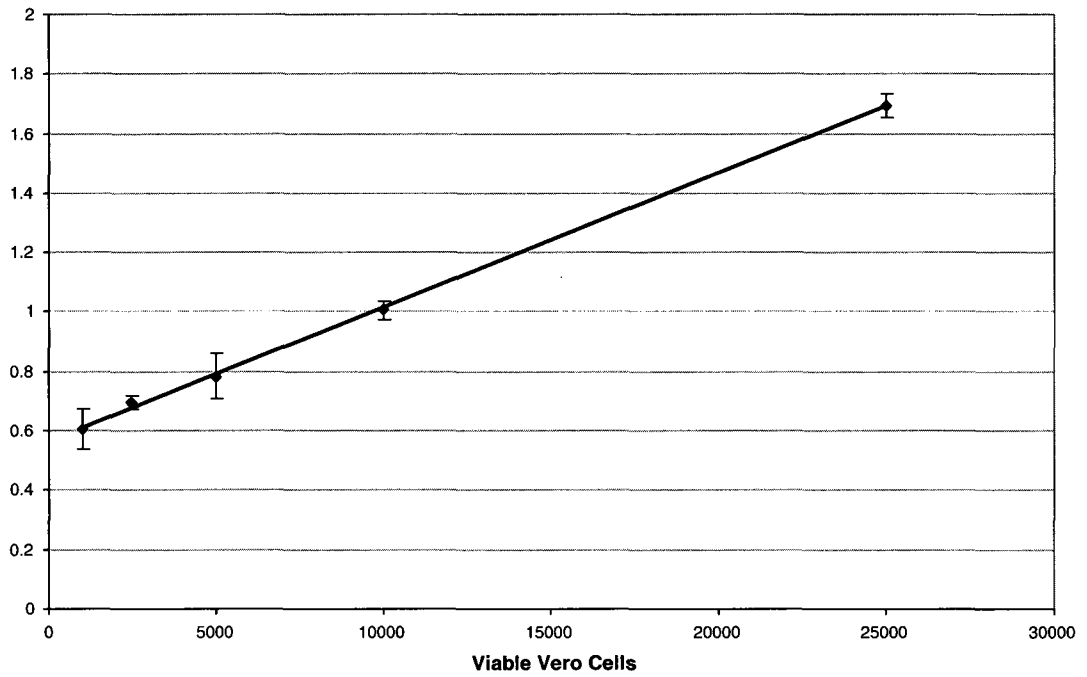


Figure 2.4. MTT Cytotoxicity Assay Standard Curve. Vero cells were seeded at known number densities and then measured by MTT cytotoxicity assay to develop a standard curve. Correlation coefficient = 0.9992.

positive controls while supernates from the organism grown in uninduced MHB were used as negative controls (Fig 2.5). Supernates collected from *E. coli* grown with either DNA-damaging antibiotic, ciprofloxacin or norfloxacin reduced vero cell viability to 70% and 85%, respectively. As predicted, the positive control supernates from growth in mitomycin C also decreased vero cell viability (75%). The average viability for the vero cells also declined when grown with supernates from trimethoprim-induced cultures, though this was not statistically significant. Vero cell viability increased to 125% of the uninduced control when the supernate from *E. coli* grown with polymyxin B was used. Treatment of *E. coli* with cefotaxime, chloramphenicol, gentamicin, or rifampin did not significantly alter the vero cell viability compared to the uninduced control.

5.4. ELISA Analysis of Antibiotic Induced *E. coli* O157:H7

Following antibiotic-induction of the *E. coli* O157:H7 strain 90-2380 cultures, supernates were assayed for Stx-2 concentration by ELISA. Dilutions of commercially obtained Stx-2 (Toxin Technology) were used to construct a standard curve for comparison (Figure 2.6). The standard curve showed exceptional linearity with a correlation coefficient of 0.9932.

Samples of supernates from antibiotic-induced cultures were collected. All antibiotic-induced samples were normalized against the uninduced control, and data is shown as fold-increase in Figure 2.7. As expected, the positive control mitomycin C increased Stx-2 production by 2-fold compared to the uninduced control. Likewise, the three antibiotics that act on the DNA of bacterial cells, ciprofloxacin, norfloxacin, and trimethoprim, all increased Stx-2 by some degree. Ciprofloxacin surpassed the positive

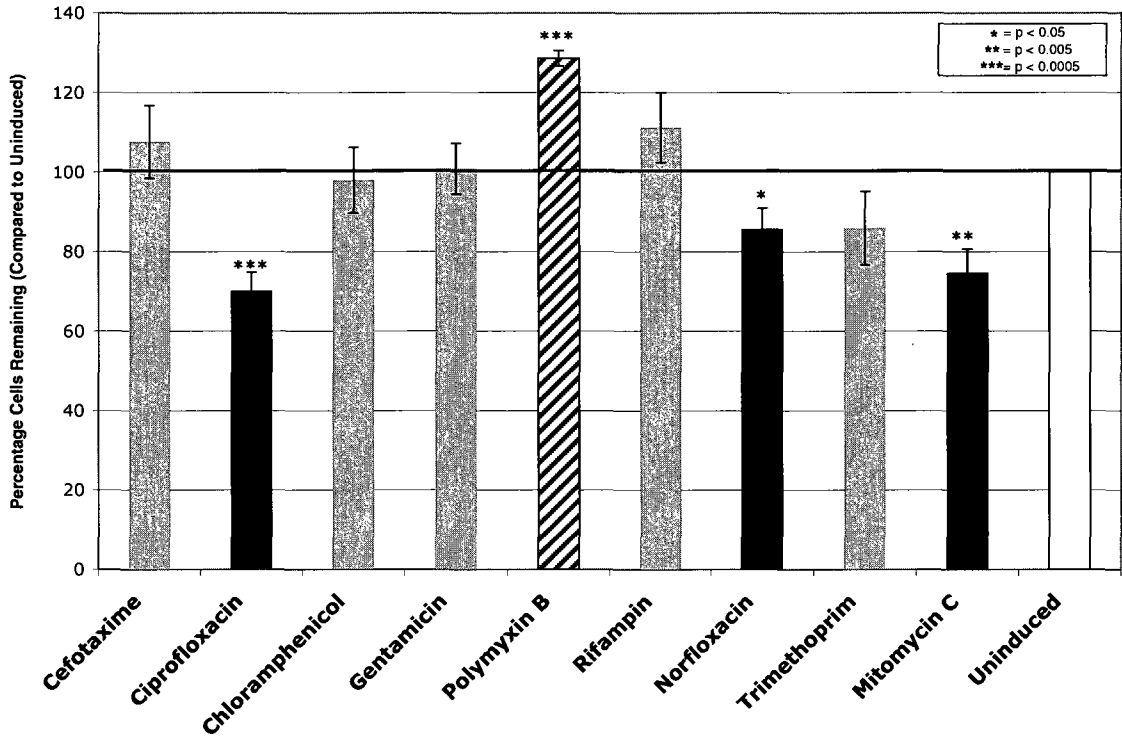


Figure 2.5. MTT: Cytotoxicity of *E. coli* O157:H7 Supernates from Antibiotic-induced Cultures. All data were normalized against the uninduced control (white bar). Data for cefotaxime, chloramphenicol, gentamicin, rifampin, and trimethoprim (gray bars) show no significant change from the uninduced sample. Data for ciprofloxacin, norfloxacin and the positive control mitomycin C (black bars) show significant decreases in vero cell viability. Polymyxin B (hatched bar) showed a significant increase in viability compared to the uninduced sample.

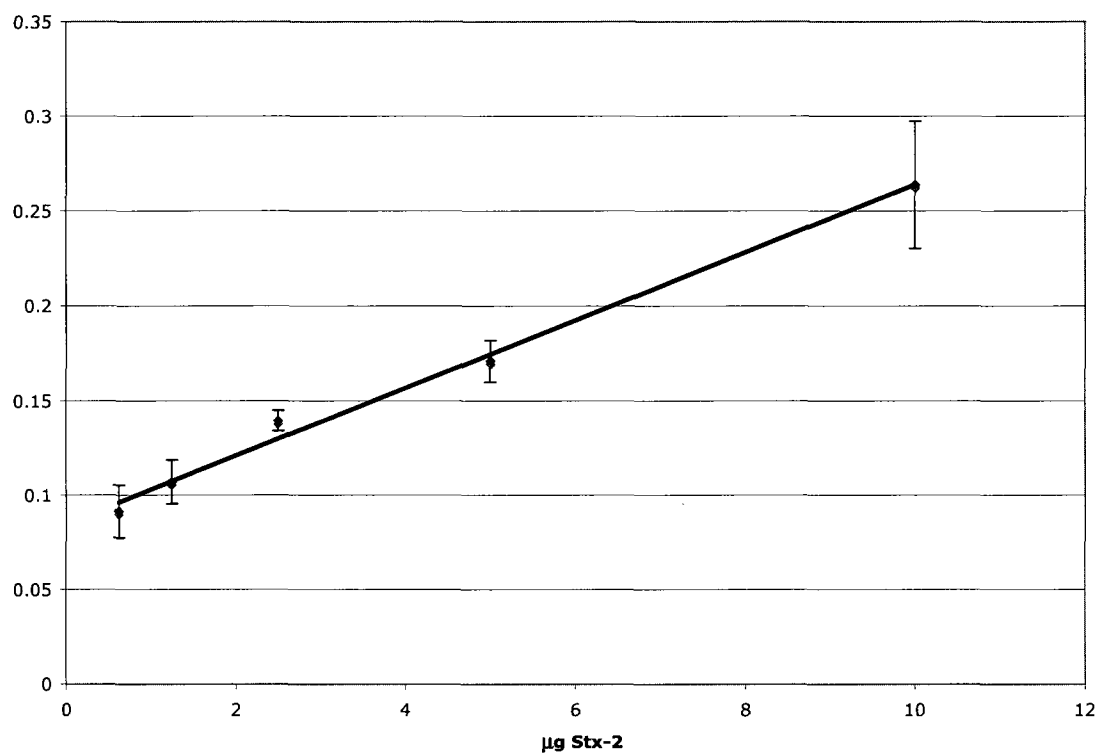


Figure 2.6. Stx-2 Standard Curve: ELISA. Standard concentrations of commercially purchased Stx-2 were evaluated in an ELISA to obtain a standard curve. Correlation coefficient = 0.9932. A separate standard curve was prepared for each ELISA determination, each of which yielded results similar to this example.

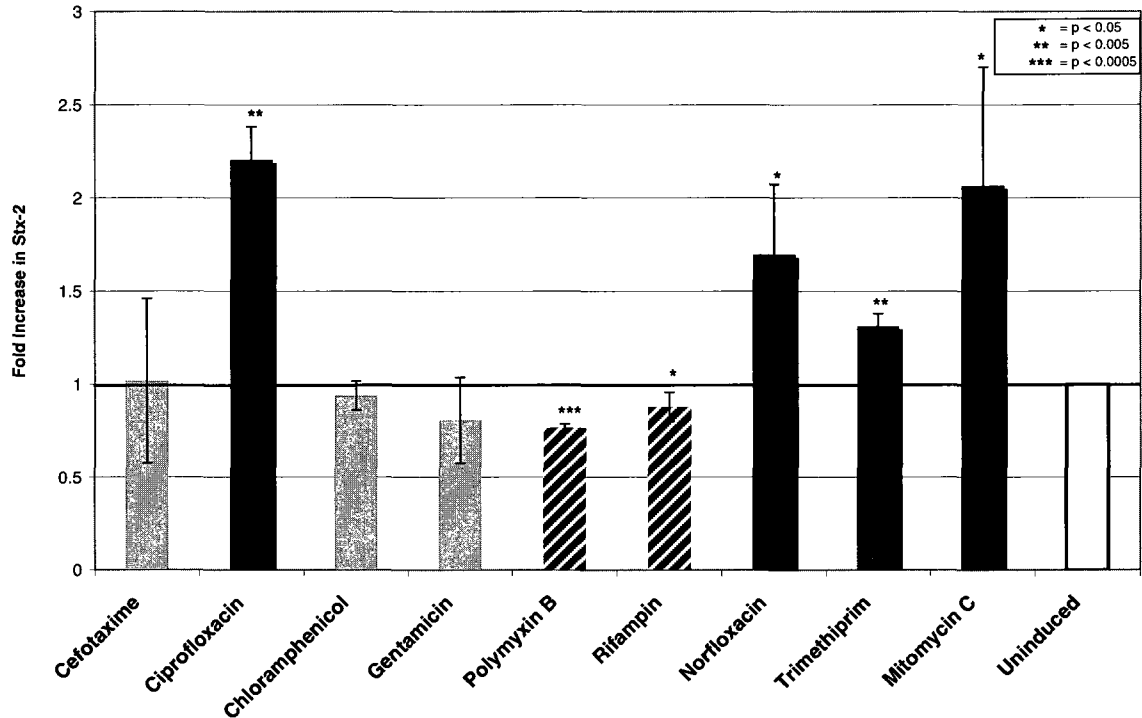


Figure 2.7. ELISA: Increase in Stx-2 Concentration After 24 h Induction with Antibiotics. All antibiotic-induced samples were normalized against the uninduced control (white bar). Data are shown as fold-increase compared to the uninduced sample. For the antibiotics cefotaxime, chloramphenicol, gentamicin, no significant increase beyond the uninduced control was noted (gray bars). Ciprofloxacin, norfloxacin, trimethoprim, and the positive control mitomycin C (black bars) gave significantly higher values than the uninduced control. Polymyxin B and rifampin (hatched bars) showed significant decreases as compared to the controls.

control and increased Stx-2 by 2.25-fold. Stx-2 production by *E. coli* O157:H7 increased to 1.65 times the uninduced control when treated with norfloxacin. Trimethoprim increased Stx-2 by 1.3-fold. Both polymyxin B (a cell membrane disruptor) and rifampin (a transcription inhibitor) decreased Stx-2 production to approximately 0.75% and 0.85% of the uninduced control. No significant change in Stx-2 production was seen when the pathogen was grown in the presence of cefotaxime, chloramphenicol, or gentamicin.

5.5. Real Time PCR Analysis of *E. coli* O157:H7 Transcripts

At 1 hour after induction with the various antibiotics, a marked difference in *stx2* and *dinD* expression compared to the uninduced control was noted (Figure 2.8). *dinD* expression was increased by 5.7-fold when *E. coli* was grown with the positive control, mitomycin C. Norfloxacin and ciprofloxacin also increased *dinD* expression by 4.0-fold and 4.8-fold, respectively. These results were expected, as all three agents act on the DNA of bacterial cells, and *dinD* is highly expressed in the presence of DNA damage. Chloramphenicol also increased *dinD* expression, though to a lesser degree (2.2-fold). *stx2* expression was increased by growth in mitomycin C (1.2-fold), ciprofloxacin (1.6-fold), and polymyxin B (3.7-fold). Gentamicin reduced *stx2* expression to 0.67-fold of the uninduced control. At 6 h of antibiotic induction, the profile of *dinD* and *stx2* expression changed slightly, as seen in Figure 2.9. *dinD* and *stx2* expression were both still upregulated by ciprofloxacin (4.1-fold, 8.5-fold), norfloxacin (2.2-fold, 4.5-fold), and the positive control mitomycin C (2.6-fold, 5.4-fold). Interestingly, whereas no significant change in either gene was seen with a 1 h induction with trimethoprim, at 6 h post-induction, the expression of *dinD* and *stx2* were both increased (1.9-fold, 2.4-fold).

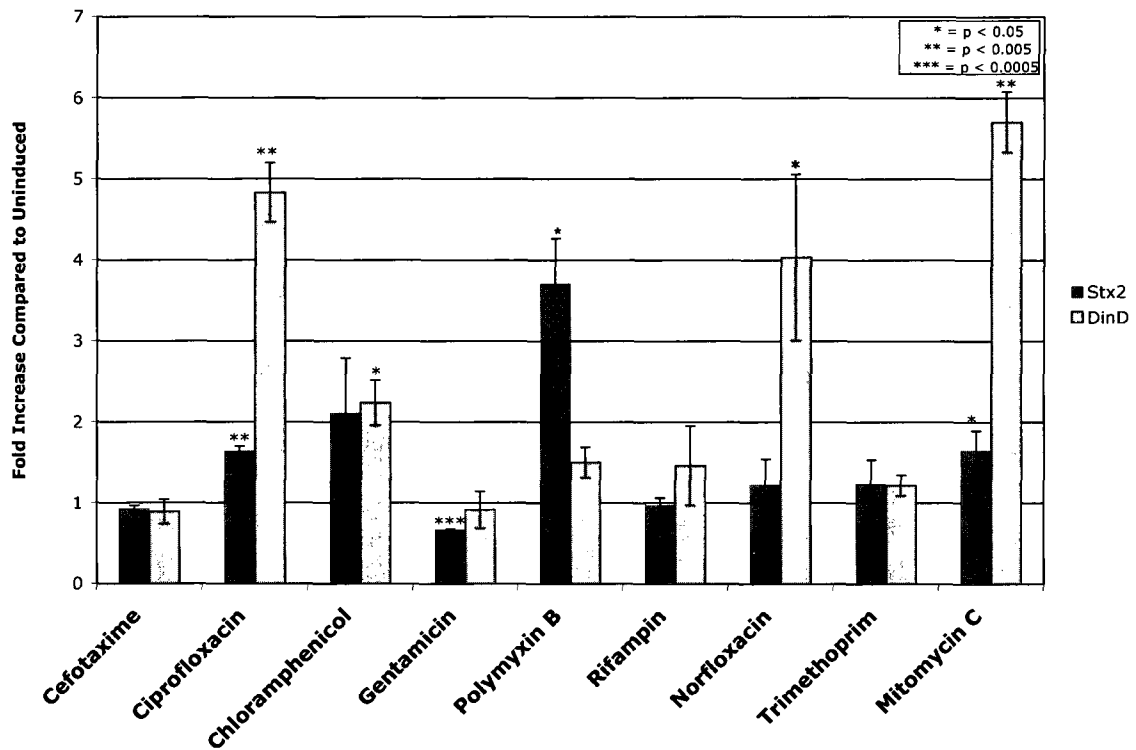


Figure 2.8. *stx2* and *dinD* Expression by Real Time PCR after 1 h Induction with Antibiotics. At one h post-induction, *stx2* expression was upregulated by growth in ciprofloxacin, polymyxin B, and mitomycin C, while *stx2* expression was downregulated in gentamicin. *dinD* expression was upregulated by growth in ciprofloxacin, chloramphenicol, norfloxacin, and the positive control, mitomycin C.

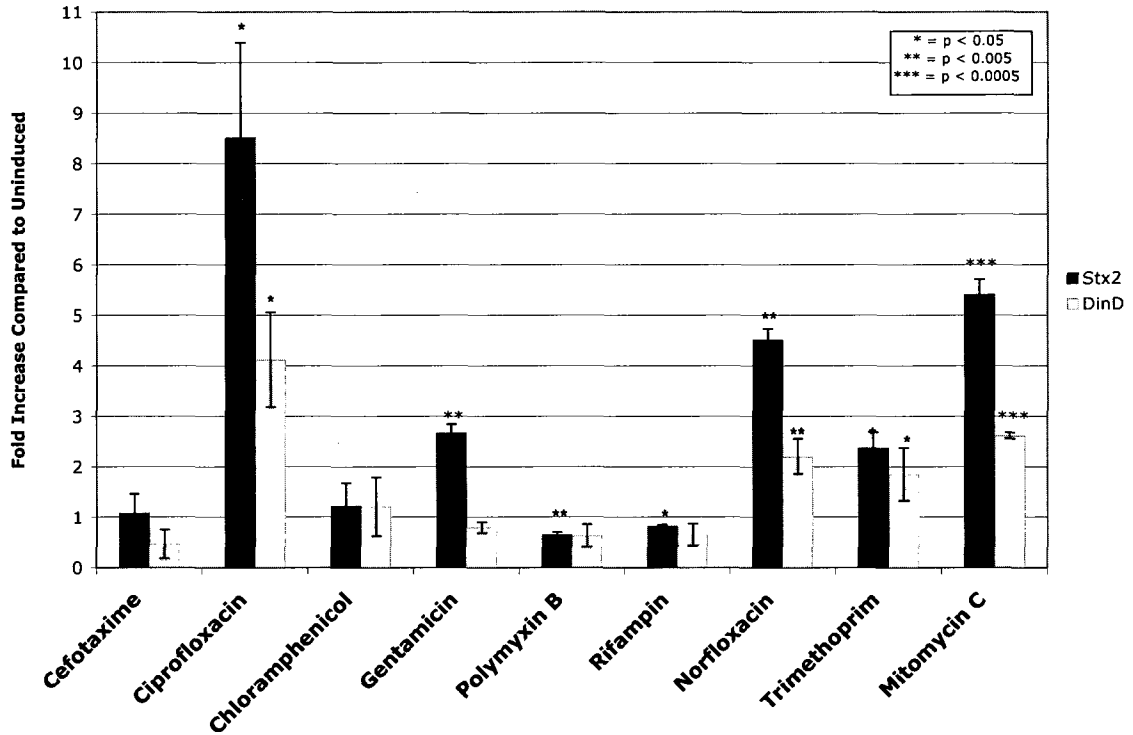


Figure 2.9. *stx2* and *dinD* Expression by Real Time PCR after 6 h Induction with Antibiotics. At 6 h post-induction, *stx2* expression was upregulated by growth in ciprofloxacin, gentamicin, norfloxacin, trimethoprim and mitomycin C. *stx2* expression was downregulated in polymyxin B, rifampin. *dinD* expression was upregulated by growth in ciprofloxacin, norfloxacin, trimethoprim, and mitomycin C.

Gentamicin, which was found to reduce *stx2* expression at 1 h actually increased *stx2* expression to 2.6-fold greater than the uninduced control after 6 h of induction. *stx2* expression was downregulated by growth in polymyxin B and in rifampin, by 0.65 and 0.83-fold, respectively. Table 2.4 summarizes the results of the MTT cytotoxicity assay, the ELISA studies, and q-PCR studies at both 1 and 6 h antibiotic induction.

6. Discussion

Despite the major clinical problems associated with the disease, there is currently no accepted treatment for patients infected *E. coli* O157:H7 (132). Retrospective studies have shown that treatment with antibiotics often worsened the course of disease, potentially leading to the deadly complication HUS (86, 171). Studies have reported that the principal virulence factor for this pathogen, the toxin Stx-2, is produced in greater concentrations when treated with DNA-damaging antibiotics (57, 74). It was originally hypothesized for the present studies that Stx-2 production and *stx2* expression would be increased when *E. coli* O157:H7 strain 90-2380 was grown in the SIC of each DNA-damaging antibiotic. Although some variability was noted, the data from these assays confirmed the validity of this hypothesis. Because the location of the *stx2* gene is within the phage structural genes, *stx2* is co-expressed when these phage genes switch from lysogeny to lysis. It was thought that DNA-damaging antibiotics would “turn on” the SOS DNA repair system, which would in turn activate the RecA protein. Activated RecA normally recognizes and cleaves the protein LexA, but because of the structural similarity of LexA to the 933-W repressor cI, this repressor is also degraded by RecA (104, 164). These studies have shown that *stx2* was indeed upregulated when this

Table 2.4. Antibiotic Induction – Summary of Results

Antibiotic	Target	MTT Assay % survival vero cell	ELISA (fold Stx-2) Stx-2 (24 h)	Real Time PCR (fold <i>stx2</i> or <i>dinD</i> expression)			
				<i>stx2</i> (6 h)	<i>stx2</i> (1 h)	<i>dinD</i> (6 h)	<i>dinD</i> (1 h)
Cefotaxime	Cell wall	107.7%	1.02	1.08	0.93	0.47	0.89
Ciprofloxacin	DNA (direct)	70.2% ***	2.20 ***	8.51 *	1.64 **	4.12 *	4.83 **
Chloramphenicol	Protein synthesis	97.9%	0.94	1.22	2.11	1.21	2.24 *
Gentamicin	Protein synthesis	100.8%	0.81	2.67 **	0.67 ***	0.79	0.91
Polymyxin B	Cell membrane	128.6% ***	0.77 ***	0.65 **	3.71 *	0.64	1.50
Rifampin	Transcription	111.23%	0.88 *	0.83 *	0.98	0.65	1.46
Norfloxacin	DNA (direct)	85.7%	1.69 *	4.53 **	1.23	2.21 **	4.03 *
Trimethoprim	DNA (indirect)	85.9%	1.32 ***	2.37 *	1.24	1.85*	1.22
Mitomycin C	Positive control	74.7%	2.80 *	5.41 ***	1.65 *	2.62 ***	5.71 ***
Uninduced	Negative control	100%	1.00	1.00	1.00	1.00	1.00

Significant results are shaded in gray. * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.0005$

toxigenic organism was grown in the DNA-damaging antibiotics, ciprofloxacin, norfloxacin, and trimethoprim, and that this upregulation coincided with an upregulation in the DNA damage inducible gene, *dinD*. Likewise, an increase in Stx-2 production was seen at 24 h post-induction with the same three antibiotics (ciprofloxacin, norfloxacin and trimethoprim), as assayed by ELISA. These data dovetailed closely with MTT cytotoxicity results, which showed an increase in vero cell death, presumably due to an increase in Stx-2, when vero cells were subjected to supernates from *E. coli* O157:H7 grown in either ciprofloxacin or norfloxacin. Supernates collected from *E. coli* O157:H7 trimethoprim induction also lowered vero cell viability, though not to a significant degree more than the uninduced control. Ciprofloxacin and norfloxacin are fluoroquinolones, and their mode of action is to bind to DNA gyrase and topoisomerase IV (59, 112). Through this binding, this class of antibiotics induces DNA breakages, which in turn induce the SOS DNA repair system in the microorganism (74). Trimethoprim affects DNA in a more indirect manner by binding to dihydrofolate reductase and inhibiting the formation of folic acid, a necessary precursor to nucleic acid bases (17). Although trimethoprim does not directly induce DNA damage, it has been shown to be a potent inducer of the SOS response (79). Growth of *E. coli* O157:H7 strain 90-2380 in any of the three DNA-damaging, SOS response-inducing antibiotics tested led to an increase in *stx2* gene expression and Stx-2 production. These studies confirmed the hypothesis that *stx2* expression and Stx-2 production by *E. coli* O157:H7 were increased by DNA damage and the SOS response.

Unexpectedly, the 6 h treatment with gentamicin caused an upregulation in *stx2* expression by 2.67-fold; however this did not correlate with an increase in *dinD*

expression and these results were not confirmed in the ELISA or MTT cytotoxicity assays. In fact, gentamicin was seen to significantly decrease *stx2* expression at 1 h by 0.67-fold. It was not clear why gentamicin, an antibiotic that acts on the 30S ribosome to block the binding of formylmethyl transfer RNA to prevent the formation of initiation complexes at the early stage in bacterial protein synthesis (55), thus leading to nonsense peptides, caused an upregulation in *stx2* at 6 h. However, the mode of action of the aminoglycosides such as gentamicin is complex, and it is possible that these observed results are due to other ill-defined action of the antibiotic on the pathogen. Indeed, gentamicin is known to be both bacteriostatic via the protein synthesis processes it induces, yet it has also been shown to be bactericidal by mechanisms not well defined. It is thought that gentamicin can increase bacterial membrane leakage, leading to cell death (66, 67). This additional mode of action may offer an explanation for the upregulation of *stx2* seen at 6 h post-induction. As with gentamicin at 6 h, polymyxin B increased *stx2* expression at 1 h post induction but this was not seen at 6-h post induction. Like gentamicin, polymyxin B disrupts cell membranes through ionic interactions (137). It is possible that the disruption of the bacterial cell membranes caused an upregulation in *stx2*. For polymyxin B, this upregulation was seen at 1 h post induction, yet for gentamicin, this upregulation was only seen in the 6 h post induction experiments. Gentamicin may require more time (6 h as opposed to 1 h) for this upregulation in *stx2* to be detectable. With increased cell membrane permeability would come an increase in diffusible bacterial signal molecules released by quorum sensing, which would then be better able to pass into the *E. coli* cells. Quorum sensing has been shown to be an upregulator of the several virulence genes in *E. coli* O157:H7, including *stx2* (3, 72, 147,

148). Further research into the effect of cell membrane disruptors on *stx2* and on the uptake of quorum sensing molecules is needed to fully elucidate this upregulation in *stx2*. Although gentamicin at 6 h post-induction and polymyxin B at 1 h post-induction increased expression of *stx2*, these antibiotics did not ultimately result in an increase in Stx-2, as shown by ELISA and MTT cytotoxicity assay. Studies on pertussis toxin, another AB₅ toxin with some similarities to Stx-2, have shown that polymyxin B inhibited the release of the pertussis toxin from the *Bordetella pertussis* cells (27). Pertussis toxin is assembled in the periplasm of the cell, and when polymyxin B is present, assembly and release of the toxin is inhibited (27). It is possible that a similar mechanism is engaged in *E. coli* O157:H7, however, the means by which Stx-2 molecules are secreted from the microbial cell are not fully elucidated (121). Despite the upregulation of *stx2*, these agents (gentamicin and polymyxin B) did not increase Stx-2 production, and in the case of polymyxin B, actually decreased Stx-2 production overall.

Of the eight antibiotics representing various classes of antibiotic groupings evaluated for their effects on *stx2* expression and Stx-2 production, only rifampin consistently caused less *stx2* expression and less Stx-2 production by *E. coli* O157:H7. Rifampin binds to DNA-dependent RNA polymerase and inhibits transcription by blocking the synthesis of mRNA. Rifampin was able to reduce *stx2* expression by 0.83-fold at 6 h post-induction, although reduction at 1 h post-induction was not significant. Stx-2 release from the *E. coli* O157:H7 cells was also reduced to 0.88-fold that of the uninduced control. Though not significant due to variations between trials, rifampin also increased vero cell survival to 110%, of the uninduced culture. In a study by Ochoa, et. Al (109), a related antibiotic, rifaximin was shown to not induce Stx-2 production or

phage release by *E. coli* O157:H7. As the authors point out, in *E. coli* O157:H7, sigma factor 70 (σ^{70}) and the antiterminator Q are involved in the expression of the lytic cycle of the phage; they allow the RNA polymerase to read-through a terminator, thereby allowing transcription of downstream genes, including *stx*. Rifampin and rifaximin inhibit σ^{70} action (109), halting the transcription of *stx*, and this is likely the reason why *stx2* expression and Stx-2 production were diminished when *E. coli* was grown in rifampin. Currently, antibiotic therapy for treating *E. coli* O157:H7 infections is controversial due to possible exacerbation of disease resulting from increased output of Stx-2 (74, 132). However, these studies have shown that *stx2* expression and Stx-2 production are reduced by growth in rifampin compared to no antibiotic treatment, and therefore this antibiotic should not exacerbate disease by an increase in Stx-2. In a patient with an *E. coli* O157:H7 infection, rifampin may reduce pathogen numbers without an increase in Stx-2 release, which is ultimately the goal of antibiotic treatment. Due to the relative ease with which microorganisms develop resistance to rifampin, this could prove problematic. However, these studies also showed no significant change in *stx2* expression or Stx-2 release when *E. coli* O157:H7 was grown in SICs of cefotaxime (a β -lactam). Synergy has been shown between rifampin and β -lactam antibiotics for treating difficult, resistant infections, such as those caused by *Pseudomonas aeruginosa* (41), *Stenotrophomonas maltophilia* (24), and methicillin-resistant *Staphylococcus aureus* (15, 41). Perhaps combined therapy of rifampin and a newer β -lactam may prove effective for treatment in *E. coli* O157:H7 infections. It should be noted that not all antibiotics should be discounted as treatments for these infections, as not all antibiotic classes have been demonstrated to cause an increase in Stx-2.

CHAPTER 3

LACTOBACILLI AND THEIR EFFECT ON THE PRODUCTION OF STX-2 BY *ESCHERICHIA COLI* O157:H7

1. **Abstract**

Previous research has shown that animals co-colonized with both *E. coli* O157:H7 and probiotics including various *Lactobacillus* species, had less severe courses of disease with decreased concentrations of Stx-2. Likewise, related studies have shown that other *E. coli* O157:H7 virulence factors such as attaching and effacing lesions were reduced by co-cultivation with various lactobacilli, and that this reduction was not simply due to secreted molecules by the lactobacilli. In the present study, it was hypothesized that growth of the *E. coli* O157:H7 in co-culture with *Lactobacillus casei* or *Lactobacillus plantarum* would reduce Stx-2 production and *stx2* expression through direct cell-to-cell contact of the pathogen with the probiotic. It was thought that this reduction in virulence would be seen only when the *E. coli* and the lactobacilli were present in co-culture, and not when the pathogen was exposed to cell-free supernates from lactobacilli cultures. Results have indicated that indeed, Stx-2 production is reduced by direct co-culture with lactobacilli, but that reduction in Stx-2 was also caused in part by the production of organic acids by the lactobacilli.

2. Introduction

Due to the lack of conventional antimicrobial therapies for *E. coli* O157:H7 infections, scientists and physicians have looked to novel therapeutic treatments, including probiotic microbes. Probiotics are beneficial microbes that are ingested as homeopathic medicines or through particular food products, such as yogurts, kefir, and cheese (28, 45, 143, 150). The role of normal microbial flora (NMF) and probiotic microbes in the fight against pathogens is a rapidly-expanding chapter of microbiology and infectious diseases. Beneficial gut microbes aid in digestion, produce vitamins, and stimulate the immune system. Development of lymphocytes, particularly in the gut-associated lymphoid tissue (GALT), relies heavily on the presence of NMF (143, 135). Gnotobiotic animals, animals that possess no microorganisms whatsoever, display a much reduced immune system when left unchallenged by NMF (143). *L. casei* specifically has been shown to increase mucus-associated innate immunity in gnotobiotic mice (90). Probiotics have also been shown to promote gastrointestinal health by regulating cytokines, increasing the production of immunoglobulin A (IgA), and tightening intracellular junctions to inhibit pathogenic bacterial invasion. In this manner, probiotics act in a passive way to provide defense against pathogenic organisms by nutrient and space competition (143).

Probiotics can also play a more active role in human intestinal health by reducing the numbers of pathogenic organisms, and through attenuation of virulence of these harmful organisms (31). Sherman, et. al in 2005 showed that *Lactobacillus acidophilus*, *L. casei*, and *L. plantarum* reduced the shedding of enterohemorrhagic *E. coli* (EHEC) by cattle, sheep, and rabbits (142). The authors proposed that this reduction in pathogen

shedding was due to a lowered pH in the guts of these animals because of lactic acid production by the probiotic organisms. However, upon closer inspection it was seen that the lactobacilli appeared to prevent virulence factor production in these animal models, with the result of reducing the amount of attaching and effacing lesions noted on the intestinal surfaces. This reduction required cell-to-cell contact by the lactobacilli, and was not merely a passive, pH-related response (142).

In a study by Ogawa et. al (110), cultures of *L. casei* were fed to infant rabbits on the day of their birth. After allowing three days for colonization, the infant rabbits were fed shiga toxin-producing *E. coli* (STEC). The severity of disease was rated as “severe” in 77% of rabbits that were not pre-colonized with *L. casei*, whereas it was rated “severe” only in 16% of those rabbits that did receive *L. casei* on the day of their birth. Likewise, in the untreated group only 9% were rated as having no symptoms or “slight” disease, whereas in the *L. casei*-treated group, 68% had no or “slight” disease. This indicated that *L. casei* reduced the severity of STEC disease. The concentration of Stx-2 was reduced by more than 50% in the cecum and colon of rabbits that received the *L. casei* treatment, and histopathology showed less vacuolation, exfoliation and necrosis of gut epithelial cells (110).

In the present study, *E. coli* O157:H7 strain 90-2380 was grown in the presence of either *L. casei* or *L. plantarum* as probiotics prepared in ratios of 100:1, 1:1, and 1:100 of *E. coli* to probiotic. Control co-cultures contained only *E. coli* O157:H7 with sterile media substituting for the corresponding volume of probiotic. The *stx2* gene was measured for activity with and without the lactobacilli co-culture using q-PCR. Stx-2 production was measured using ELISA and MTT cytotoxicity studies in vero cells.

Growth competition assays were employed to determine the extent that *E. coli* O157:H7 numbers are affected by co-culture with the two species of *Lactobacillus*.

3. **Hypothesis**

It was hypothesized that co-cultivation of *E. coli* O157:H7 with *L. plantarum* or *L. casei* would reduce the expression of the *stx2* gene, and would also reduce the production of Stx-2. These reductions were thought to be due to direct cell-to-cell contact of the pathogen with the probiotic, and that no reduction in *stx2* expression or Stx-2 production would be seen if the *E. coli* O157:H7 were grown with cell-free supernatant fluid collected from the probiotic cultures.

4. **Materials and Methods**

Media and reagent preparations are provided in detail in Appendix A.

4.1. **Bacterial Cultivation**

The *E. coli* O157:H7 strain 90-2380 stock culture was maintained as described in Appendix B. *Lactobacillus plantarum* and *Lactobacillus casei* were acquired from Robert Mooney at the University of New Hampshire culture collection. Lactobacilli cultures were maintained similarly to *E. coli* cultures but were grown and stored on deMan-Rogosa-Sharpe (MRS) media. *E. coli* and lactobacilli cultures for co-culture experiments were grown in brain-heart infusion broth (BHIB) under static conditions at 37°C.

4.2. Vero Cell Growth and Maintenance

African green monkey kidney (vero) cells were acquired and maintained as described in Appendix B. Vero cells were prepared for use in cytotoxicity studies as outlined in Chapter 2, section 4.2. In brief, vero cells were grown to log-phase confluency in a T₂₅ flask and were then harvested and re-seeded into a 96-well plate at a concentration of 10,000 cells/well.

4.3. MTT Cytotoxicity Assay Standards

A standard curve for the MTT assay was constructed as outlined in Chapter 2, section 4.5. This standardization process allowed for unknown concentrations of vero cells to be enumerated based on a correlation to this standard.

4.4. Growth of Lactobacilli and *E. coli* O157:H7 Co-cultures for Growth Competitions

E. coli O157:H7 strain 90-2380 and *L. plantarum* and *L. casei* cultures were grown at 37°C in static culture in individual tubes overnight in BHIB. Cultures were standardized to an OD₆₀₀ of 0.2 in pre-warmed BHIB. These were then grown for 1 h to stimulate the cells into log phase growth. Cultures were centrifuged at 4,000 rpm for 5 min (IEC Centra) and the pellets resuspended in similar media and volume as before centrifugation. The cultures of *E. coli* and each of the lactobacilli were combined in the specific ratios outlined in Table 3.1, and the mixtures were incubated at 37°C for 24 h. Separate cultures of each of the lactobacilli and the *E. coli* were inoculated with pre-warmed media and incubated to serve as controls. Following incubation, all cultures were plated in triplicate onto eosin methylene blue (EMB) agar plates to select for the

Table 3.1. Co-culture Ratios of Lactobacilli to *E. coli* O157:H7

Sample	<i>E. coli</i> O157:H7	<i>L. casei</i>	Media
100:1 <i>E. coli</i> : <i>L. casei</i>	1980μL	20μL	--
1:1 <i>E. coli</i> : <i>L. casei</i>	1000μL	1000μL	--
1:100 <i>E. coli</i> : <i>L. casei</i>	20μL	1980μL	--
100:1 <i>E. coli</i> control	1980μL	--	20μL
1:1 <i>E. coli</i> control	1000μL	--	1000μL
1:100 <i>E. coli</i> control	20μL	--	1980μL
Sample	<i>E. coli</i> O157:H7	<i>L. plantarum</i>	Media
100:1 <i>E. coli</i> : <i>L. plantarum</i>	1980μL	20μL	--
1:1 <i>E. coli</i> : <i>L. plantarum</i>	1000μL	1000μL	--
1:100 <i>E. coli</i> : <i>L. plantarum</i>	20μL	1980μL	--
100:1 <i>E. coli</i> control	1980μL	--	20μL
1:1 <i>E. coli</i> control	1000μL	--	1000μL
1:100 <i>E. coli</i> control	20μL	--	1980μL

E. coli O157:H7. Colonies were enumerated to compare the number of colony forming units (CFU) of *E. coli* O157:H7 that were present after growth in the presence or absence of the lactobacilli.

4.5. Growth of Lactobacilli and *E. coli* O157:H7 Co-cultures for MTT Assay

E. coli O157:H7 strain 90-2380 and *L. plantarum* and *L. casei* cultures were grown and prepared identically to that outlined in section 4.4 above and combined in the ratios shown in Table 3.1 (previous page). Co-cultures were incubated at 37°C for 24 h, and following incubation the cultures were centrifuged at 4,000 rpm for 10 min at 4°C to pellet the cells. The supernates were removed and sterile filtered through a low-protein binding 0.2µm filter (Pall). Sterile cell-free supernates were stored at -20°C for use in MTT assays.

4.6. Co-cultures MTT Assay

Supernates (10µL) from probiotic and *E. coli* co-cultures were added in triplicate to a confluent vero cell monolayer in 96-well plates. Controls of lactobacilli-only and *E. coli*-only cell-free culture filtrates were also added to the vero cell monolayers. Plates were incubated at 37°C in 7% CO₂ for 72 h. Following the 3 day incubation, an MTT assay was performed on these vero cells as described in Chapter 2, section 4.7. This assay determined the extent of vero cell killing when subjected to Stx-2-containing supernate.

4.7. Growth of *Lactobacillus* and *E. coli* O157:H7 Co-cultures for Real Time PCR and ELISA

Co-cultures of *E. coli* O157:H7 with each lactobacilli species were prepared exactly as described in the previous section and in the same ratios as seen in Table 3.1. Immediately following the 3 h co-culture incubation period, 100µL of each co-culture was removed to a sterile microfuge tube and retained for immediate RNA extraction. The remaining 2 mL cultures were incubated at 37°C for a total of 24 hours, after which time they were centrifuged at 4°C at 4,000rpm for 10 min to pellet the cells. The supernates were removed and sterile filtered through a low-protein binding 0.2µm filter (Pall). Sterile cell-free supernates were stored at -20°C for use in ELISA assays.

4.8. Growth of *E. coli* O157:H7 with Lactobacilli Supernates, Organic and Inorganic Acids

Cultures of *E. coli* O157:H7 strain 90-2380, *L. casei*, and *L. plantarum* were grown overnight in BHIB as described in sections 4.5-4.7. The lactobacilli cultures were centrifuged at 7,500 rpm (Beckman) to pellet the cells. The supernatant fluids were collected and filtered through a low protein binding membrane (0.2 µm) and retained. In this experiment, unlike previous experiments in these studies, the lactobacilli cells were discarded. The pH of each lactobacilli supernate was measured and recorded to be pH 4.5. BHIB aliquots were lowered to pH 4.5 using either lactic acid, acetic acid or hydrochloric acid. The *E. coli* O157:H7 culture was subsequently standardized to an OD₆₀₀ of 0.2 and incubated at 37°C for an additional 1 h. The standardized culture of *E. coli* O157:H7 was combined 1:1 with either lactobacilli supernate or an acidified medium, as defined in Table 3.2, and incubated at 37°C for 24 h. Following incubation,

Table 3.2. *E. coli* O157:H7 Induction by Lactobacilli Supernates

Sample	<i>E. coli</i> O157:H7 Induced With:
A	<i>L. plantarum</i> cell-free supernate, pH 4.5 (in BHIB)
B	<i>L. casei</i> cell-free supernate, pH 4.5 (in BHIB)
C	BHIB control
D	BHIB, pH 4.5 with acetic acid
E	BHIB, pH 4.5 with lactic acid
F	BHIB, pH 4.5 with HCl

all the *E. coli* cultures were sterile filtered and the supernates retained for analysis by ELISA.

4.9. RNA Extraction and cDNA Synthesis

Directly following induction of the cultures, Bacterial RNA protect (Qiagen) was added to each aliquot of co-culture to prevent further expression of genes, and to prevent degradation of the mRNA present. RNA was isolated in the same manner as described in Chapter 2, section 4.9. The RNA was checked for DNA contamination by PCR as described, and samples containing DNA were discarded.

cDNA synthesis was performed using the SuperScript III cDNA synthesis kit (Invitrogen) exactly as described in Chapter 2, section 4.9. cDNA samples which amplified in a PCR reaction were deemed acceptable and were stored at -20°C until their use in Real Time PCR.

4.10. Quantification of cDNA

cDNA samples were quantified using the Qubit (Invitrogen) High Sensitivity (HS) DNA quantification kit™, according to the manufacturer's instructions. A brief description of this process is outlined in Chapter 2, section 4.10.

4.11. cDNA standardization

Co-culture-induced cDNA samples were standardized to a 50 ng/mL concentration using nuclease-free water prior to use in Real Time PCR experiments. Standardized cDNA samples were stored at -20°C.

4.12. Real Time Polymerase Chain Reaction

Real Time PCR was performed using an Applied Biosystems 7300 Thermocycler and software, as described in Chapter 2, section 4.12. Primers and dual-labeled fluorescent probes were designed to specifically amplify the target gene, *stx2*, and the housekeeping gene, *serC*, within *E. coli* O157:H7 without any nonspecific amplification of lactobacilli genes. These primers and probes are listed in Appendix C. Primers and probes were tested before this experimentation to be sure that no cross-amplification occurred when lactobacilli DNA was used as the template for a PCR reaction.

4.13. Enzyme-linked Immunosorbent Assay

The ELISA was designed based on a protocol by Krishna Moody (102), and was conducted as described in Chapter 2, section 4.13.

5. Results

Statistical significance was determined as described in Appendix B, Section 5.

5.1. *E. coli* O157:H7 and Lactobacilli Co-culture Growth Competitions

E. coli O157:H7 strain 90-2380 was cultured together with either *L. plantarum* or *L. casei* in a ratio of 100:1, 1:1 or 1:100 to determine the effect of co-culture with lactobacilli on the growth of *E. coli* O157:H7.

A dose-dependent response was seen in the recovery of *E. coli* O157:H7 when grown with varying amounts of *L. casei*. An approximately 2.5-log reduction in *E. coli* O157:H7 numbers were seen when grown in a ratio of 1:100 *L. casei*, and a 2-log

reduction was seen when grown in a 1:1 ratio. A 1-log reduction in the amount of *E. coli* O157:H7 was seen when grown in a 100:1 ratio with *L. casei*. These data are represented graphically in Figure 3.1.

Similar inhibition of *E. coli* O157:H7 recovery was noted with *L. plantarum* co-cultures (Figure 3.2). A slightly greater than 2-fold reduction in *E. coli* O157:H7 was seen when grown in a 1:100 ratio with *L. plantarum*, and a 2-fold reduction was seen with a 1:1 ratio. This reduction was not as pronounced with approximately a 0.75-log reduction in *E. coli* O157:H7, when it outnumbered the *L. plantarum* in a ratio of 100:1.

5.2. MTT Cytotoxicity Assay

An MTT cytotoxicity assay was employed to measure the effect of Stx-2-containing supernates on vero cells. The MTT assay was standardized by seeding a 96-well plate with a set number of vero cells (without Stx-2) and calculating the cell viability. A standard curve was established, as shown in Chapter 2, Figure 2.4.

Vero cells were subjected to various Stx-2-containing supernates collected from *E. coli* O157:H7 grown in co-culture with either *L. casei* or *L. plantarum*. As depicted in Figures 3.3 and 3.4, co-culture of the *E. coli* O157:H7 with *L. casei* or *L. plantarum* in a ratio of 1:100 or 1:1 significantly increased vero cell survival compared to the controls (supernates from *E. coli* O157:H7 grown without the lactobacilli). When *E. coli* O157:H7 outnumbered the lactobacilli in a ratio of 100:1, there was not a significant difference in vero cell survival with this supernate compared to its corresponding control of *E. coli* only.

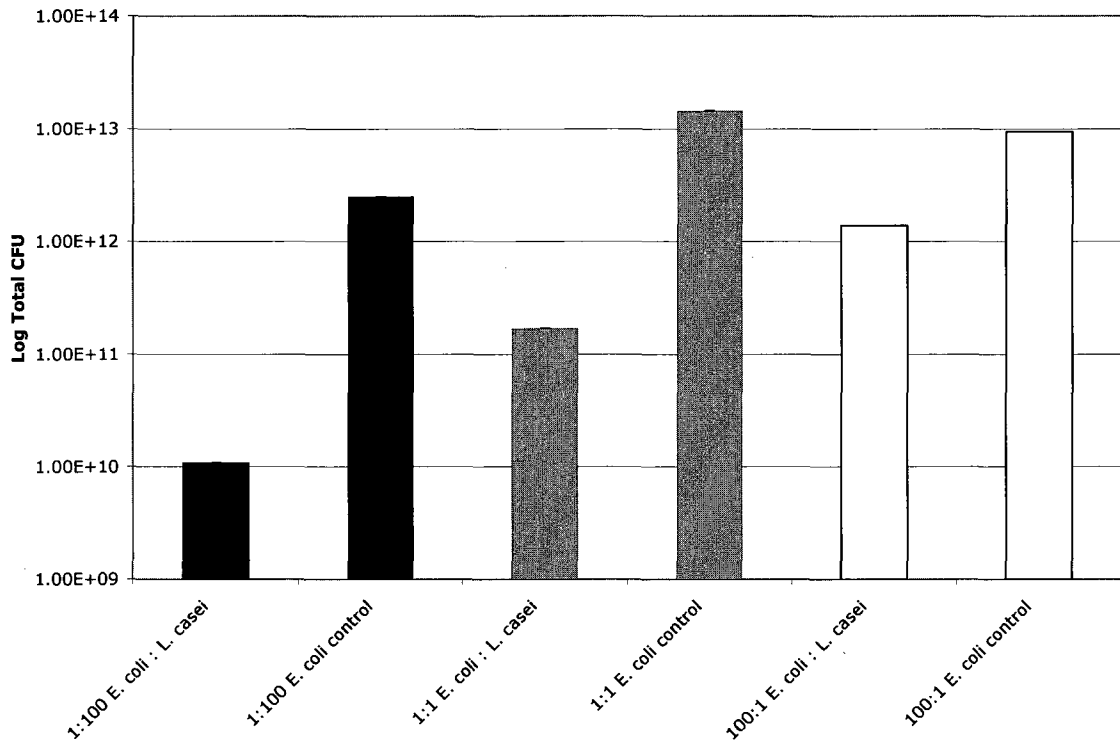


Figure 3.1. The Effect of *L. casei* on the Growth of *E. coli* O157:H7.

■ Black bars = 1:100 *E. coli* to *L. casei* and corresponding control

▒ Gray bars = 1:1 *E. coli* to *L. casei* and corresponding control

□ White bars = 100:1 *E. coli* to *L. casei* and corresponding control

Note that less *E. coli* was recovered from those cultures grown with *L. casei* than those grown in pure culture, and this reduction was dose-dependent on the amount of *L. casei* present in each co-culture.

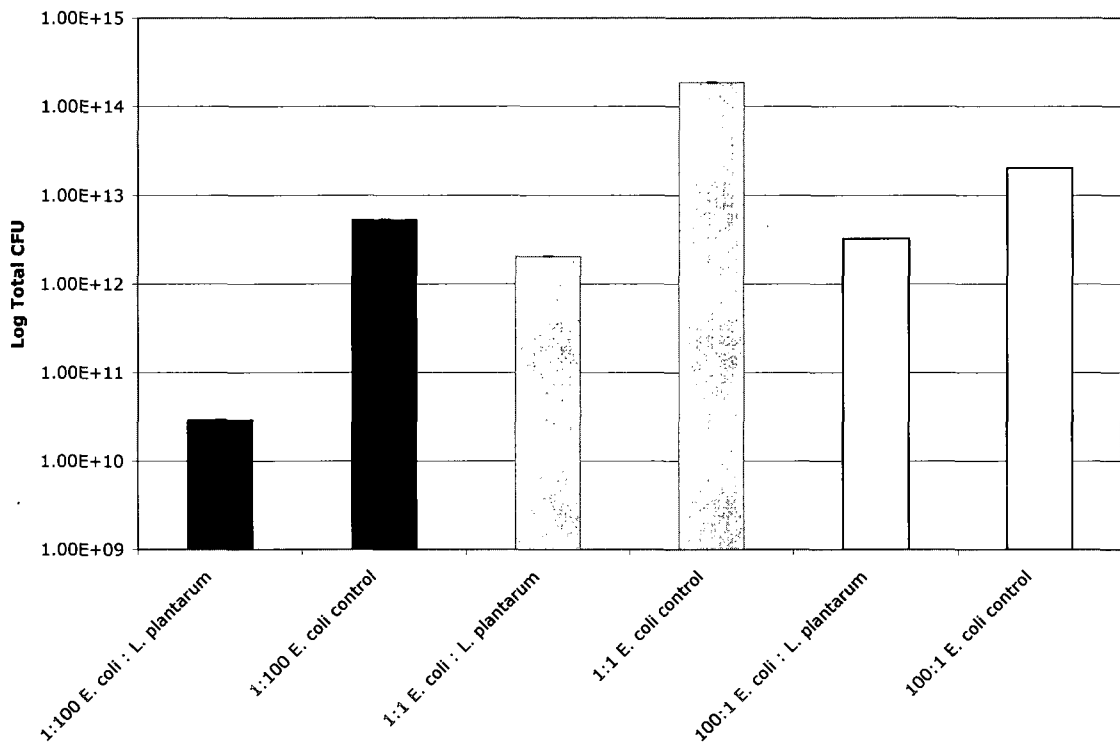


Figure 3.2. The Effect of *L. plantarum* on the Growth of *E. coli* O157:H7.

- Black bars = 1:100 *E. coli* to *L. plantarum* and corresponding control
- ▒ Gray bars = 1:1 *E. coli* to *L. plantarum* and corresponding control
- White bars = 100: 1 *E. coli* to *L. plantarum* and corresponding control

As with the *L. casei*, less *E. coli* was recovered from those cultures grown with *L. plantarum* than those grown in pure culture. The dose-dependent fashion of this growth inhibition was seen with both species of *Lactobacillus*.

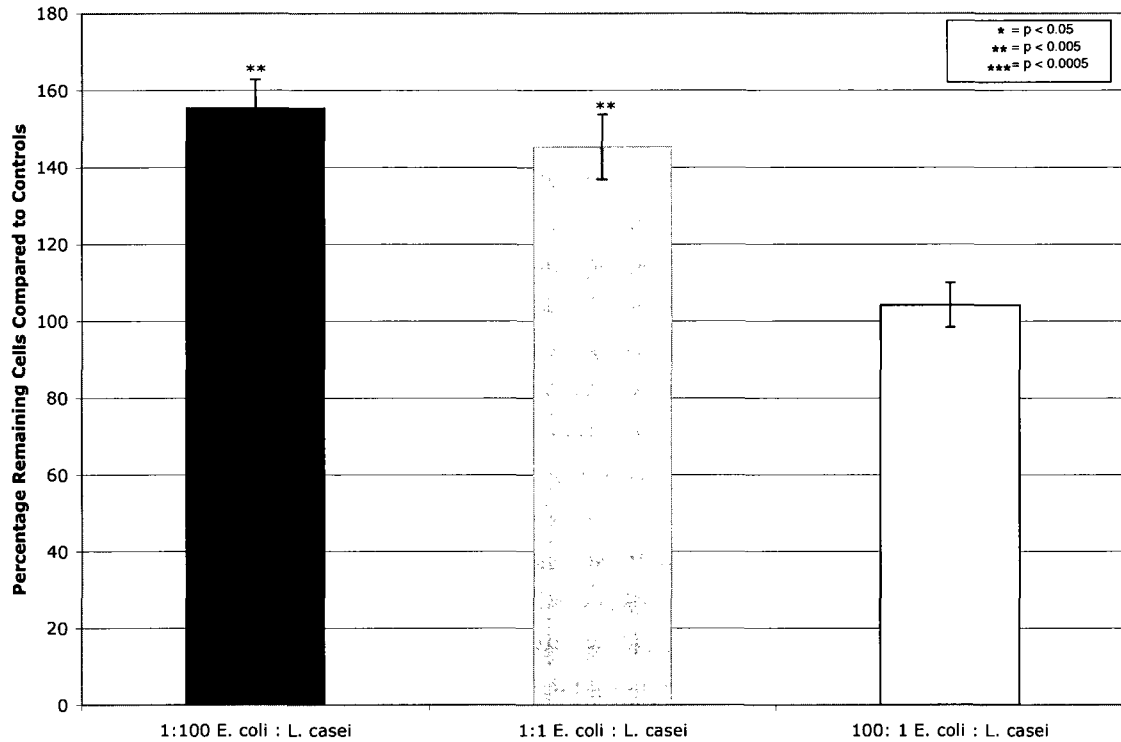


Figure 3.3. MTT Cytotoxicity Results: Co-Culture Induction with *L. casei*.

- Black bars = 1:100 *E. coli* to *L. casei* compared to corresponding control
- ▒ Gray bars = 1:1 *E. coli* to *L. casei* compared to corresponding control
- White bars = 100: 1 *E. coli* to *L. casei* compared to corresponding control

Results are displayed as a percentage of remaining vero cells when subjected to supernates from co-cultures compared to supernates from the controls (no *L. casei*). In a ratio of 1:100 or 1:1 of *E. coli* to *L. casei*, the supernates from *L. casei*-containing cultures significantly increased vero cell survival compared to the supernates from *E. coli* control cultures.

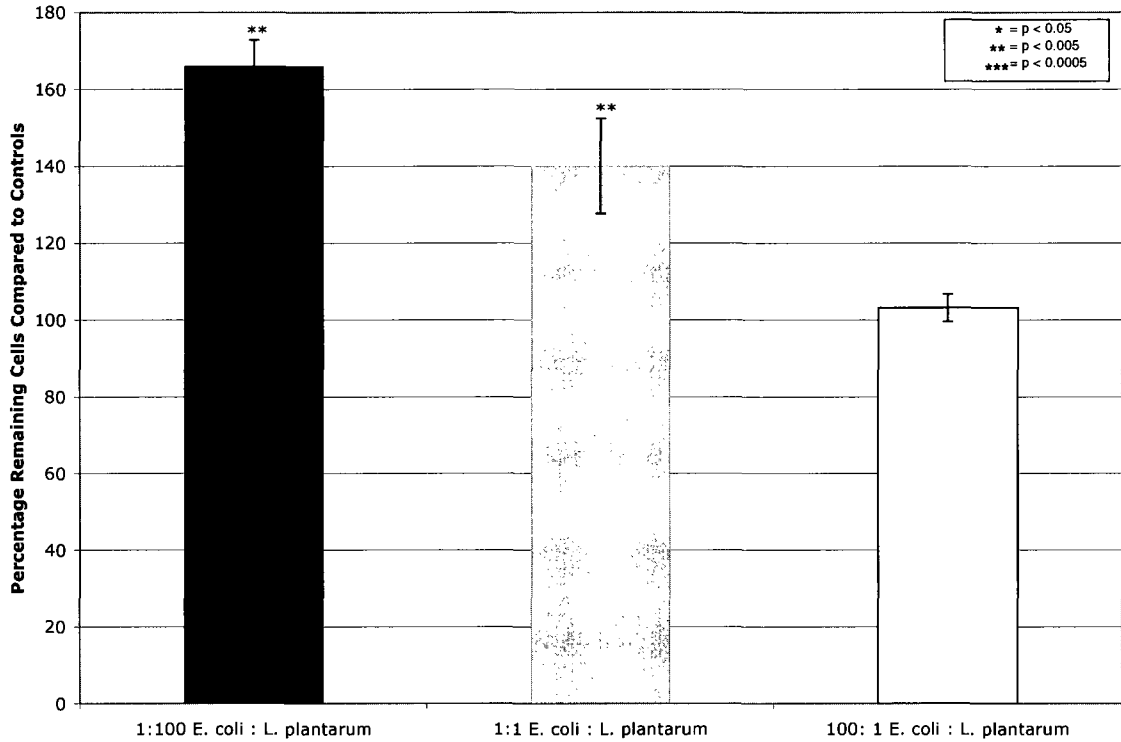


Figure 3.4. MTT Cytotoxicity Results: Co-Culture Induction with *L. plantarum*.

- Black bars = 1:100 *E. coli* to *L. plantarum* compared to corresponding control
- ▒ Gray bars = 1:1 *E. coli* to *L. plantarum* compared to corresponding control
- White bars = 100: 1 *E. coli* to *L. plantarum* compared to corresponding control

In this figure, results are displayed as a percentage of remaining vero cells when subjected to supernates from co-cultures compared to supernates from the controls (no *L. plantarum*). As with *L. casei*, in a ratio of 1:100 or 1:1 of *E. coli* to *L. plantarum*, the supernates from *L. plantarum*-containing cultures significantly increased vero cell survival compared to the supernates from *E. coli* control cultures.

5.3. ELISA Analysis of *E. coli* O157:H7 and Lactobacilli Co-Cultures.

Dilutions of commercially prepared Stx-2 were used to construct a standard curve for comparison in ELISA analysis, as seen in Chapter 2, Figure 2.6. This standard curve provided a linear relationship between μg of Stx-2 and absorbance.

Supernates were collected from co-cultures of *E. coli* O157:H7 grown with either *L. casei* or *L. plantarum* and assayed for Stx-2 concentration by ELISA. In a ratio of 1:100 (*E. coli* : *L. casei*), *L. casei* significantly reduced the production of Stx-2 by *E. coli* O157:H7 when compared to the control. Approximately 5 μg of Stx-2 was produced when co-cultured with the *L. casei*, compared to 14 μg in the control culture. In a 1:1 ratio, a smaller, but still significant, reduction of Stx-2 production was seen. When the pathogen outnumbered the *L. casei* in a ratio of 100:1, there was not a significant difference in Stx-2 production compared to the control. Figure 3.5 depicts the absolute concentrations of Stx-2 (μg) produced for each of the co-cultures and their controls. Figure 3.6 represents the same data, but portrays the percentage of Stx-2 in the co-cultures as compared to the controls. In this figure, it is clear that the reduction of Stx-2 by the various amounts of *L. casei* occur in a dose-dependent manner. The concentration of Stx-2 was reduced to 37%, 67%, and 87% (not significant) of the corresponding controls for 1:100, 1:1 and 100:1 ratios, respectively.

L. plantarum exerted similar pressure on *E. coli* O157:H7 and its production of Stx-2, as seen in Figure 3.7. The 1:100 ratio co-culture of *L. plantarum* reduced Stx-2 concentration from 16 μg to 3 μg . The 1:1 ratio also significantly reduced Stx-2 production, from 17 μg to 8.5 μg . When the *E. coli* was in a 100:1 ratio with the *L. plantarum*, no change in Stx-2 production was detectable. Figure 3.8 depicts the

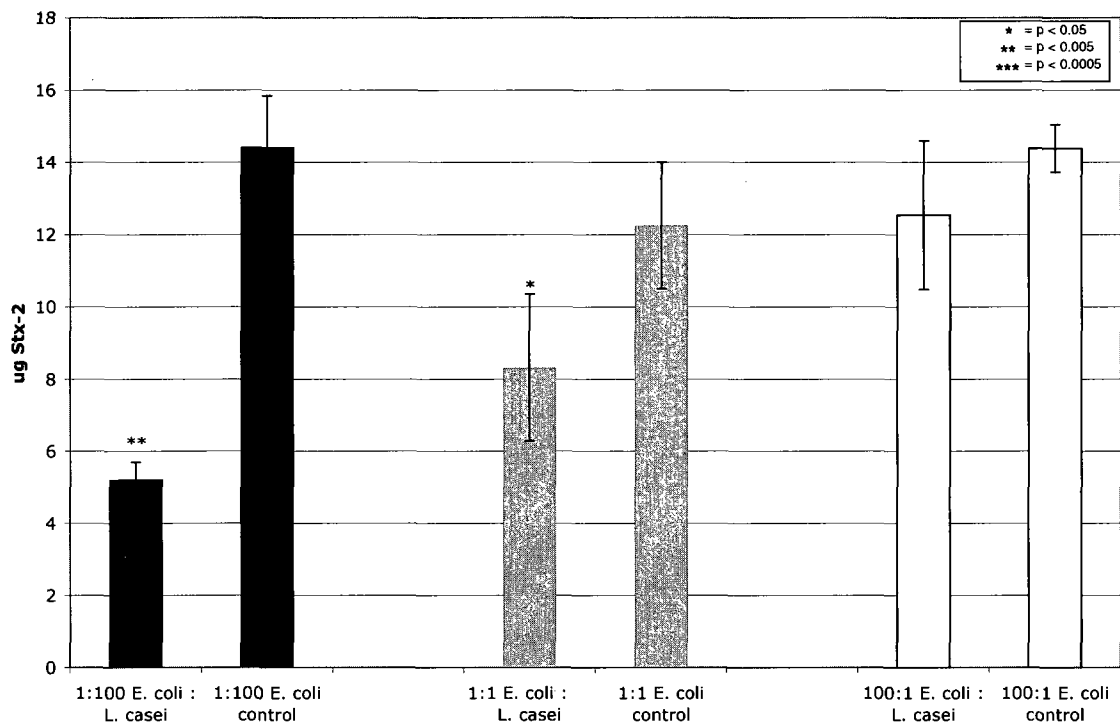


Figure 3.5. ELISA: The Effect of *L. casei* on the Production of Stx-2 by *E. coli* O157:H7. In this figure, Stx-2 concentration from co-cultures are compared to pure culture *E. coli* Stx-2 production. Note that *L. casei* exerted a dose-dependent effect on the production of Stx-2 by *E. coli* O157:H7.

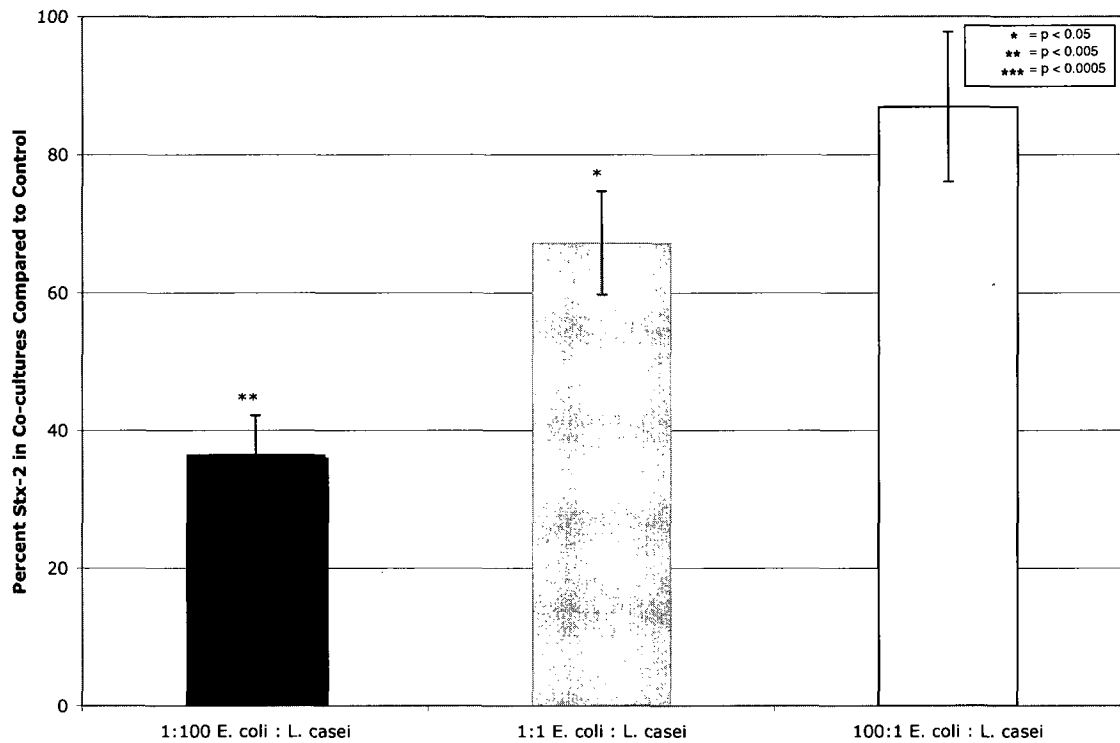


Figure 3.6. ELISA: The Effect of *L. casei* on the Production of Stx-2 by *E. coli* O157:H7. Co-culture concentrations of Stx-2 are normalized against their corresponding controls. Results are displayed as a percentage of Stx-2 in co-cultures compared to the controls (no *L. casei*). In a ratio of 1:100 or 1:1 of *E. coli* to *L. casei*, the *L. casei* significantly reduced Stx-2 production by *E. coli* O157:H7. No significant change was detected at a ratio of 100:1.

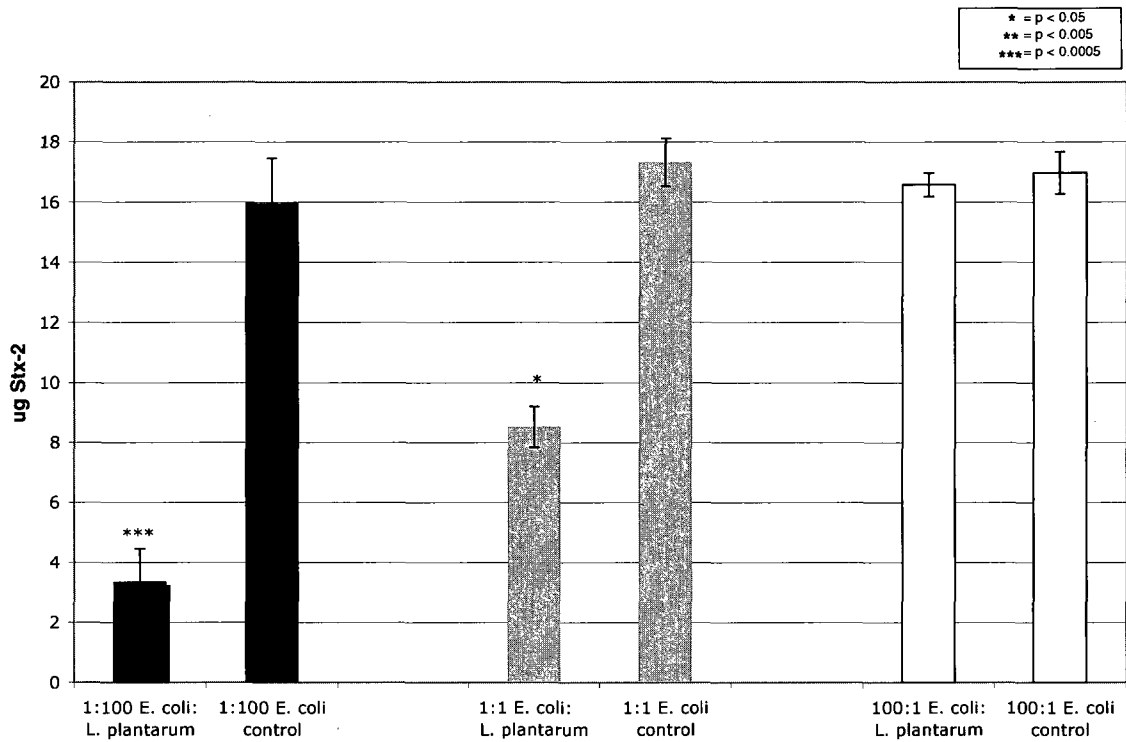


Figure 3.7. ELISA: The Effect of *L. plantarum* on the Production of Stx-2 by *E. coli* O157:H7. In this figure, Stx-2 concentration from co-cultures is compared to pure culture *E. coli* Stx-2 production. Note that *L. plantarum* exerted a dose-dependent effect on the production of Stx-2 by *E. coli* O157:H7.

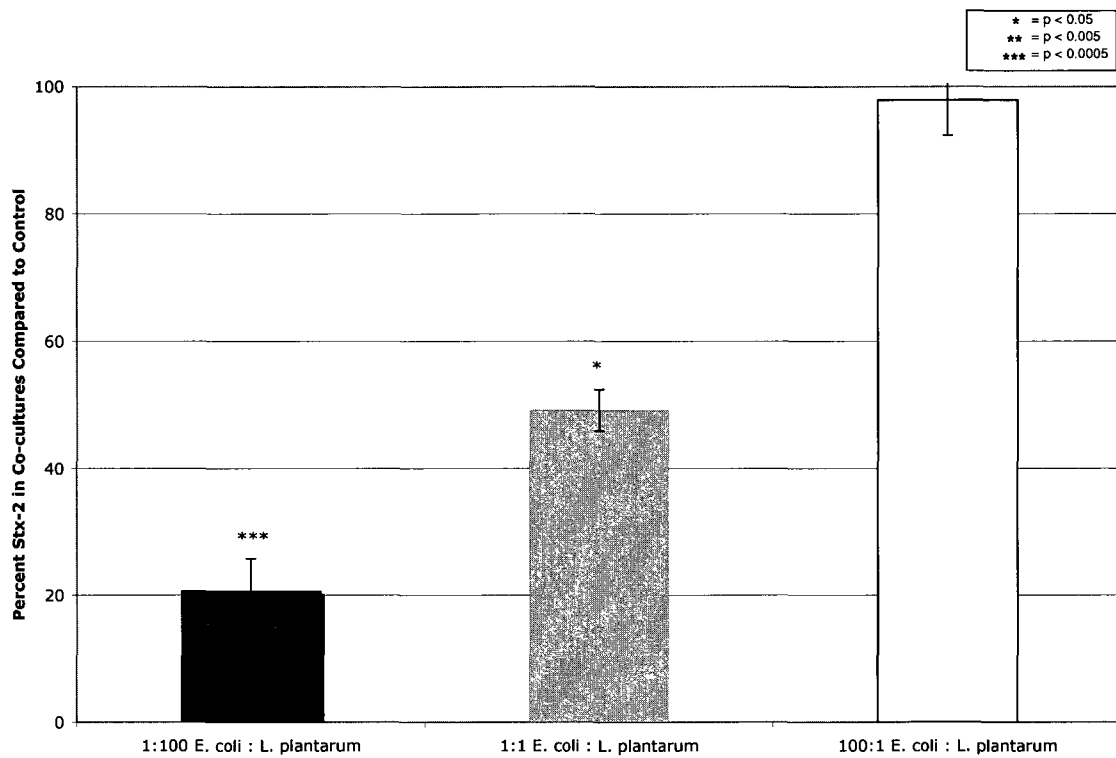


Figure 3.8. ELISA: The Effect of *L. plantarum* on the Production of Stx-2 by *E. coli* O157:H7. Results are displayed as a percentage of Stx-2 in co-cultures compared to the controls (no *L. plantarum*). As seen with *L. casei*, a ratio of 1:100 or 1:1 of *E. coli* to *L. plantarum*, the *L. plantarum* significantly reduced Stx-2 production by *E. coli* O157:H7.

percentage of Stx-2 in the co-cultures compared to the control in these ELISA analyses. Stx-2 was reduced by 21% in the 1:100 co-culture, 49% in the 1:1 co-culture, and 98% in the 100:1 co-culture. As with the *L. casei*, the reduction in Stx-2 was dependent on the concentration of *L. plantarum* present in each co-culture.

5.4. ELISA Analysis of *E. coli* O157:H7 Supernates from Cultures Grown with Lactobacilli Supernates or Acidified Media

Lactobacilli supernates and media that was acidified to pH 4.5 by an organic acid (acetic or lactic acid) significantly reduced the production of Stx-2 by *E. coli* O157:H7. However, media that was acidified to the same pH but with hydrochloric acid, an inorganic acid, caused no significant change in Stx-2 production. Absolute concentrations of Stx-2 are shown in Figure 3.9. The control culture of *E. coli* O157:H7 produced just fewer than 16µg of Stx-2. The cultures of *E. coli* O157:H7 when grown with *L. plantarum* supernate, *L. casei* supernate, acetic acid-containing media, and lactic acid-containing media all produced approximately 12µg of Stx-2. Figure 3.10 depicts the concentration of each test sample as a percentage compared against the control. A significant reduction to 70-80% of the control was seen for all conditions except hydrochloric acid-containing media.

5.5. Real Time PCR Analysis of *E. coli* O157:H7 Transcripts.

The expression of the *stx2* gene in *E. coli* O157:H7 was significantly reduced when grown in co-culture with *L. casei* and *L. plantarum* in a ratio of 1:100 or 1:1. Growth of the pathogen with either lactobacilli species in a ratio of 100:1 did not significantly alter *stx2* expression. As shown in Figure 3.11, the 1:100 ratio of *E. coli* to

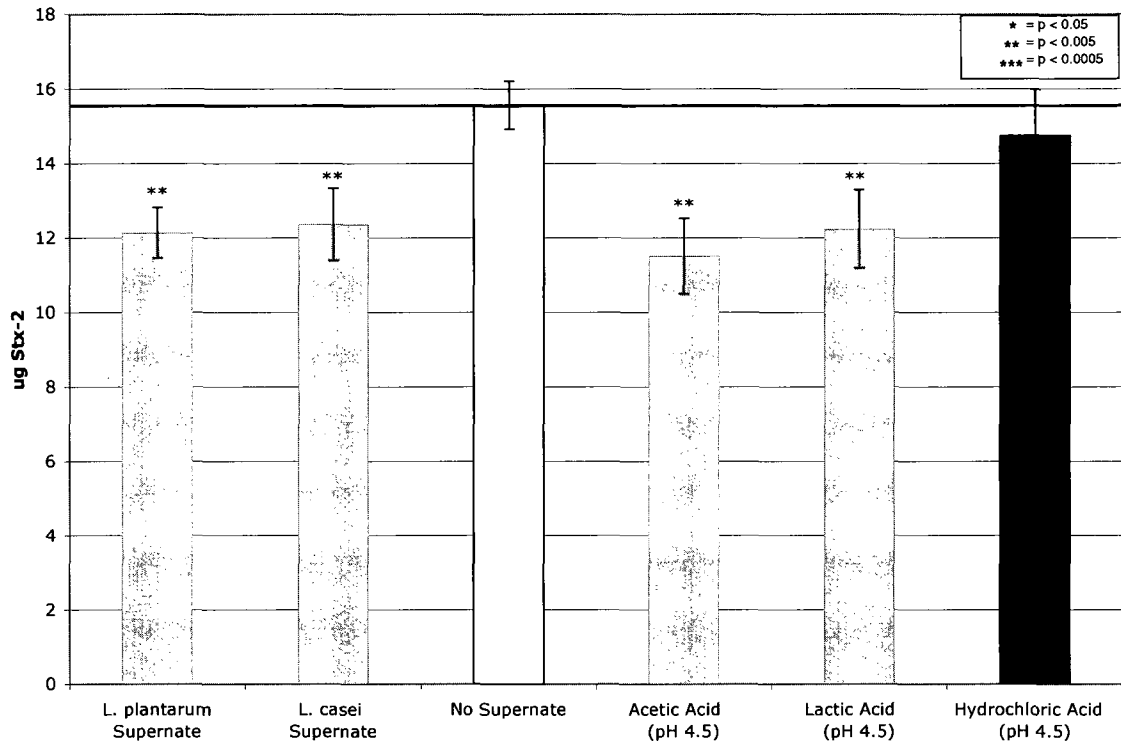


Figure 3.9. ELISA: The Effect of Lactobacilli Supernates or Acidified Media on the Production of Stx-2 by *E. coli* O157:H7. *E. coli* O157:H7 was grown with lactobacilli supernate or acidified BHIB, and Stx-2 concentration was assayed by ELISA. Both lactobacilli supernates and both organic acids (acetic acid and lactic acid) significantly reduced Stx-2 production by *E. coli* O157:H7. However, hydrochloric acid, an inorganic acid, did not affect the production of Stx-2.

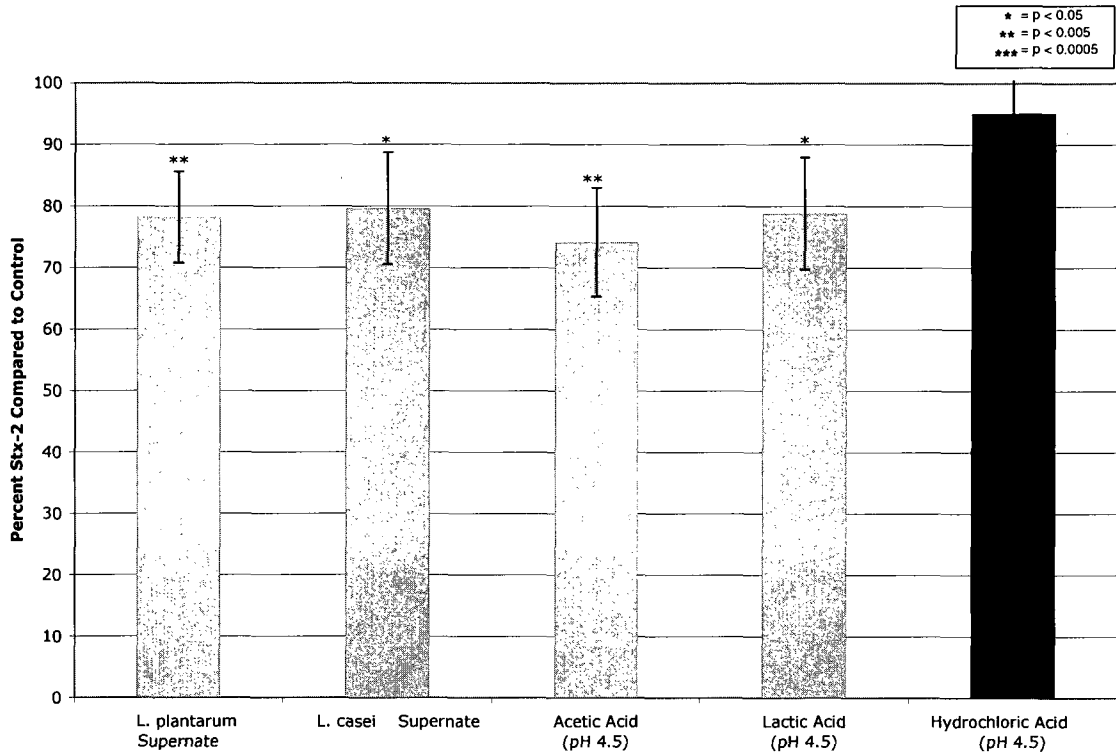


Figure 3.10. ELISA: The Effect of Lactobacilli Supernates or Acidified Media on the Production of Stx-2 by *E. coli* O157:H7. Data are depicted as a percent Stx-2 compared to the control (*E. coli* grown without supernate or acidified media). Growth of *E. coli* O157:H7 with lactobacilli supernate, acetic acid, or lactic acid led to a 70-80% reduction in Stx-2 production compared to the control.

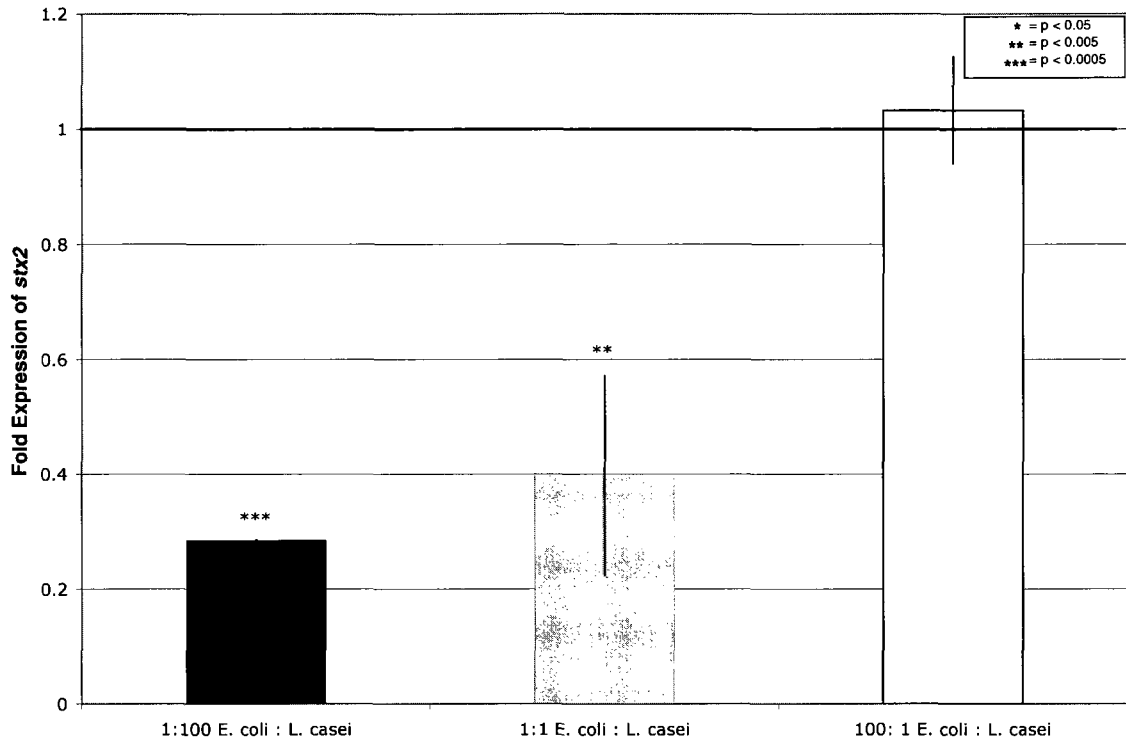


Figure 3.11. Real Time PCR Analysis of *E. coli* O157:H7 and *L. casei* Co-Cultures. Note that both the 1:100 and 1:1 *E. coli* to *L. casei* ratio and the 1:100 *E. coli* to *L. casei* culture (solid gray bar, right) significantly reduced *stx2* transcripts compared to the *E. coli* only controls.

L. casei decreased *stx2* expression to 29% of the control. The 1:1 ratio reduced *stx2* expression to 40% of the control. *L. plantarum* co-cultures with *E. coli* O157:H7 also reduced *stx2* expression, though not to the degree that *L. casei* did. As seen in Figure 3.12, *L. plantarum* co-cultures reduced *stx2* expression to 55% and 75% of the control, for the 1:100 and 1:1 ratios, respectively.

6. Discussion

The study of probiotics, microbes that are beneficial for human consumption, is a rapidly-expanding subdiscipline of microbiology. Probiotics have been shown to reduce the shedding and severity of disease in various animal models of *E. coli* O157:H7 infections (23, 25, 142, 160, 175, 176). The reduction in disease can in part be attributed to the effects the probiotics have on the production of virulence factors by the pathogen. Animal model studies have shown that lactobacilli inhibit the formation of attaching and effacing lesions in the gut and that this response is due to direct cell-to-cell contact of lactobacilli and *E. coli* O157:H7 (25, 142). Likewise, studies have shown that the *in vivo* production of Stx-2 by *E. coli* O157:H7 is reduced in animals co-colonized with a probiotic organism (4, 30, 110, 141, 160, 176).

In the present study, *E. coli* O157:H7 was grown in co-culture with two probiotics, *L. casei* and *L. plantarum*, to determine the effect of the lactobacilli on the overall growth of *E. coli* O157:H7 as well as on the expression of the *stx2* gene, and the production of Stx-2. It was hypothesized that the lactobacilli would reduce *stx2* expression and Stx-2 production by the *E. coli*. It was similarly thought that co-culture of the *E. coli* with the probiotics would reduce the overall numbers of *E. coli* O157:H7 cells

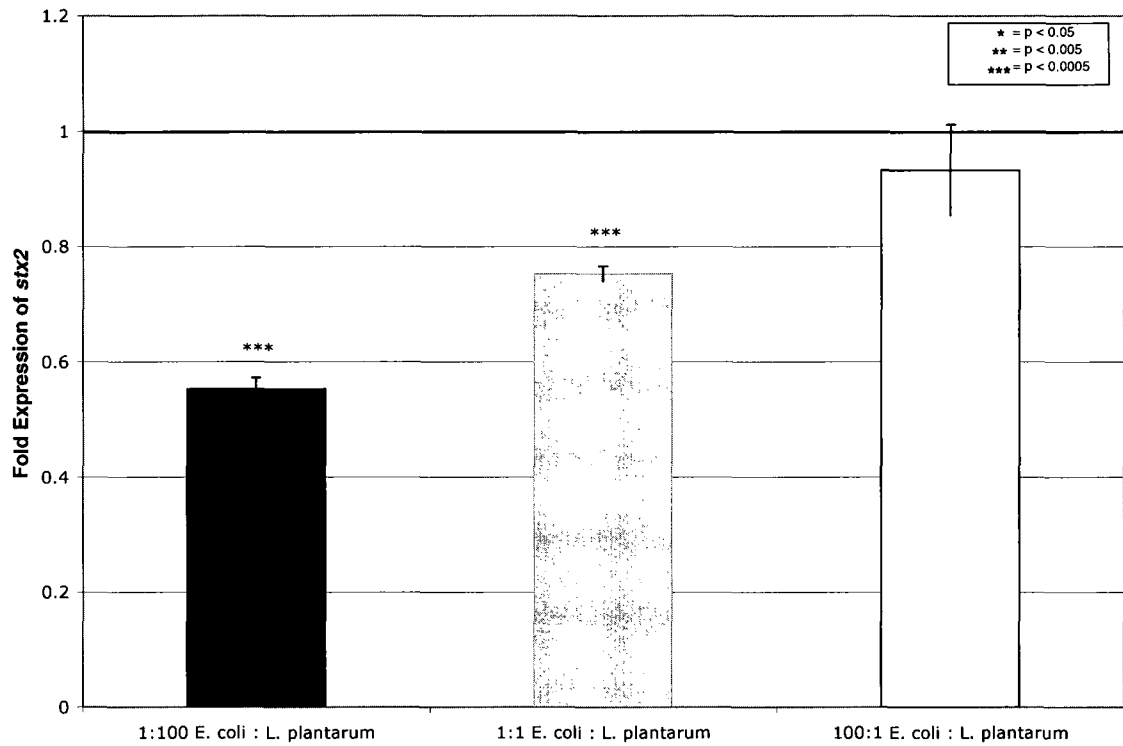


Figure 3.12. Real Time PCR Analysis of *E. coli* O157:H7 and *L. plantarum* Co-Cultures. Both the 1:100 and 1:1 *E. coli* to *L. plantarum* ratio significantly reduced *stx2* transcripts compared to the *E. coli* only controls. These results were similar to what was seen with *L. casei* co-cultures.

in culture when compared to growth of the pathogen in culture alone. It was indeed determined that growth of the *E. coli* O157:H7 was reduced in co-culture with the lactobacilli, and this growth inhibition was related to the starting concentrations of both organisms. ELISA analysis confirmed that when the *E. coli* O157:H7 started as either 1% (1:100) or 50% (1:1) of the total number of cells in culture, then Stx-2 production was significantly reduced. When the *E. coli* outnumbered lactobacilli 100:1, no significant change in Stx-2 production was noted when compared to the control. Real Time PCR showed similar results for the toxin gene expression at these organism concentrations. It was clear that the mode of action of cell-to-cell contact of the lactobacilli on the virulence of *E. coli* O157:H7 was complex, and involved a reduction in pathogen growth, *stx2* expression, and toxin production. However, subsequent ELISA experiments with cell-free lactobacilli supernate and acidified media indicated that this reduction in Stx-2 was also due to secreted compounds from the lactobacilli.

Cell-free supernates from the lactobacilli, and both organic acids, acetic acid and lactic acid, reduced Stx-2 production to 75-80% that normal (no supernate, not acidified) medium. This reduction in part accounts for the reduction of Stx-2 seen in co-culture models, but does not account for the full decrease in Stx-2 seen in these studies (as low as 20% Stx-2 concentration compared to the pure culture supernate). The hypothesis that lactobacilli reduce Stx-2 production by cell-to-cell contact was not disproven by these studies. However, it was determined that the production of organic acids by these probiotics may also play a role. Interestingly, growth of the pathogen in media acidified to the same pH, but using an inorganic acid (HCl) showed no affect on the bacterial production of Stx-2. It has been shown that *E. coli* has various overlapping means of

inorganic acid resistance, but that these resistance mechanisms are not always as potent against organic acids (9, 51). Unlike inorganic acids, organic acids can enter the *E. coli* cell in an undissociated state and then dissociate. This results in a lowering of cytoplasmic pH, which can kill the *E. coli* cell, or alter its membrane composition (9, 34, 51, 131). Indeed, Stx secretion is reduced when the lipid composition of the *E. coli* membrane is altered (60, 173). It appears likely that this mode of action of organic acids on *E. coli* O157:H7 contributes to the reduction in Stx-2 seen when the pathogen was grown in lactobacilli supernates, or when it was grown in organic acid-containing media.

Real Time PCR analysis of the *stx2* gene provided additional insight into the mechanisms surrounding the effect of lactobacilli on Stx-2 production. These studies showed that the lactobacilli were able to alter the expression of *stx2* gene in *E. coli* when they were in equal or greater numbers to the pathogen. It is possible that the presence of the lactobacilli disrupted *E. coli* cell-to-cell signaling, and therefore reduced *stx2* expression. Regulation of virulence by quorum sensing has been noted in numerous pathogens (98), and specifically studies have shown that quorum sensing molecules produced by both the pathogenic and benign strains of *E. coli* are able to upregulate *stx2* expression (47, 48, 147, 148). In a study by Medellin-Pena et. Al (94), probiotics were demonstrated to “quorum-quiet” *E. coli* cell-to-cell communication, resulting in a reduction in the virulence of the organism. Stx-2 production was not significantly affected by the quorum-quieting in that study, however a different species of *Lactobacillus* was used than in the present study, which could account for the difference in observed results.

Probiotics have been demonstrated to reduce virulence of *E. coli* O157:H7 in numerous other cases (4, 30, 73, 88, 142, 175, 176). *Saccharomyces boulardii* was shown to reduce signaling in T84 cells that allows enterohemorrhagic *E. coli* to maintain adherence to the cells. Although *S. boulardii* did not affect the number of *E. coli* cells, it did reduce the virulence of the pathogen by interfering with adhesion (30). Lactobacilli have specifically been shown to affect adherence of pathogenic strains of *E. coli* as well; in one study, the production of attaching and effacing lesions was significantly reduced in the presence of *L. rhamnosus* and *L. acidophilus* (142). Probiotics have even been suggested to cross-domain signal with intestinal cells, causing those cells to increase their production of mucus, which is inhibitory to pathogen adherence and persistence (88).

Within the course of an infection in the human gastrointestinal tract, *E. coli* O157:H7 is exposed to numerous signaling molecules, all with the possibility of modulating virulence in some manner. Even human hormones such as epinephrine and norepinephrine have been shown to upregulate the virulence genes in enterohemorrhagic *E. coli* (7). It is possible that probiotics such as lactobacilli contribute to the mass of signaling that occurs in this complex environment, and that they are able to alter cell-to-cell communication of the *E. coli* O157:H7 and thus reduce its ability to regulate the production of its virulence factors, including Stx-2.

CHAPTER 4

IMPACTS OF GLUCOSE, GLYCEROL AND CYCLIC ADENOSINE MONOPHOSPHATE ON STX-2 PRODUCTION BY *ESCHERICHIA COLI* O157:H7

1. Abstract

Previous research has shown that growth of lambda-infected non-pathogenic *E. coli* in the presence of glucose led to a significantly higher rate of induction of the lytic phase of lambda, whereas growth in glycerol resulted in phage lysogeny. In the present study, it was hypothesized that growth of *E. coli* O157:H7 in glycerol would likewise maintain 933-W (a lambda-like phage harboring the *stx2* gene) in a lysogenic state and therefore reduce the production of Stx-2. Likewise, increasing the availability of cAMP to the organism would decrease Stx-2 production by simulating intracellular low-glucose availability. *E. coli* O157:H7 was grown in glucose or glycerol-supplemented minimal media, or in Luria Bertani (LB) broth supplemented with cAMP. The *stx2* gene was assessed using q-PCR, and the production of Stx-2 toxin was measured using a capture ELISA as well as by MTT cytotoxicity assay. It was determined that Stx-2 production increased with increasing availability of glucose; indeed, cultures of the organism in minimal media with 1.0% glucose yielded 2-times higher concentrations of Stx-2 compared to those grown in 0.04% glucose. Varying concentrations of glycerol had no effect on Stx-2 production. As predicted, increasing the concentrations of cAMP decreased the amount of Stx-2 produced. These studies suggest that glucose but not

glycerol stimulates Stx-2 expression by lambda-like phage 933-W carrying strains of *E. coli* O157:H7.

2. Introduction

The *stx2* gene is located on a lambda-like bacteriophage inserted as a prophage into the chromosome of *E. coli* O157:H7. When the organism is metabolically stable, the prophage remains in a lysogenic state. However, if the *E. coli* host is subjected to stress, the prophage converts to a lytic state and is excised from the chromosome. During excision, the *stx2* gene is co-expressed with phage genes, leading to an increase in toxin production (78, 104, 120, 164). Upregulation of *stx2* gene expression is a consequence of activation of the previously-dormant prophage and the switch from a lysogenic state to a lytic state. Numerous factors have been cited as inducers of the bacteriophage (that is, factors that encourage the phage to convert from lysogeny to a lysis) (47, 57, 74, 71).

In 2001 Czyz et. al (29) conducted a study on the rates of lysis and lysogeny by a lambda phage in a non-pathogenic *E. coli* strain. The purpose of the research was to determine whether the growth medium influenced the lytic cycle of lambda bacteriophages within *E. coli*. The authors concluded that lysis occurred more frequently in cultures of *E. coli* grown in minimal media (M9) with glucose than in cultures of *E. coli* grown in M9 with glycerol. Concentrations of glucose are inversely proportional to the concentrations of cyclic adenosine monophosphate (cAMP) in bacterial host cells. It was reasoned that because cAMP indirectly inhibits proteases that breakdown the cII protein, and because the protein cII is an integral part of the regulatory system that

encourages lambda lysogeny, by increasing glucose in the cell, and therefore lowering cAMP, lysis of the phage was promoted through the degradation of cII (29).

It is possible that such conditions apply to the pathogenic *E. coli* O157:H7 (EHEC strains) and the lambda-like 933-W prophage it carries. Indeed, the genetic system controlling lysogeny of the *E. coli* O157:H7 BP-933W bacteriophage is similar to that of lambda in that it is a complex, self-repressing, and self-regulating system. When the regulatory proteins cII and cIII are expressed at low levels they activate the repressor cI. cI in turn binds and represses the promoters, Pro_L and Pro_R, to stop the transcription of the majority of the phage genome, including cII and cIII and thus the toxin gene, *stx2* (78, 86, 164). In the case of reduced levels of cAMP (due to high levels of glucose), more cellular proteases are present, and the rate of cII degradation is increased. Without the cII protein, the expression of cI repressor is arrested, and the late phage genes are expressed including *stx2*. Because the *stx2* gene is located between the late phage promoter and phage-excision genes, it is co-transcribed when these genes become activated (86, 164). Therefore, it is possible that *stx2* is upregulated under conditions of high glucose availability. In conditions of low glucose availability and high cAMP concentrations, *stx2* may be downregulated.

In the current study, the production of the toxin Stx-2 and the activity of the *stx2* gene were measured when the organism *E. coli* O157:H7 was grown in M9 containing either 0.04%, 0.2% or 1.0% glucose, or 0.04%, 0.2% or 1.0% glycerol. Likewise, *E. coli* O157:H7 was grown in LB medium in the absence of exogenous cAMP or containing 1mM or 10mM exogenous cAMP. Stx-2 production was measured by both cytotoxicity assays and by enzyme-linked immunosorbent assay (ELISA). The activity of the *stx2*

gene was measured by quantitative Real Time PCR, and for these investigations, the *serC* (serine synthase) was used as a housekeeping gene; it is constitutively transcribed despite growth conditions. All results were expressed as a function of *serC* activity to allow for differences in experimental techniques (efficacy of RNA extraction, cDNA synthesis, etc.).

3. Hypothesis

It was hypothesized that growth of the pathogenic *E. coli* O157:H7 in glucose would lead to an increase in *stx2* expression and Stx-2 production, compared to growth of the organism in glycerol. This upregulation in *stx2* was believed to be linked to induction of the lytic phase of the 933-W phage that resides within the *E. coli* O157:H7 chromosome. Conversely, supplementation of a growth medium with exogenous cAMP was believed to inhibit the lytic phase of the 933-W phage, and therefore reduce both *stx2* expression and Stx-2 production.

4. Materials and Methods

Media and reagent preparations are provided in detail in Appendix A.

4.1. Bacterial Cultivation

The *E. coli* O157:H7 strains 90-2380 and 43888 stock cultures were acquired and maintained as described in Appendix B. Overnight and experimental cultures used in these experiments were grown in Mueller-Hinton broths (MHB), or M9 minimal media supplemented with glucose or glycerol. All cultures were grown statically at 37°C.

4.2. Growth Curves of *E. coli* O157:H7 in Glucose or Glycerol Supplemented Minimal Media

E. coli O157:H7 strain 90-2380 was inoculated into six separate broths: three cultures of M9 supplemented with 0.04%, 0.2%, or 1.0% glucose, and three cultures of M9 supplemented with 0.04%, 0.2%, or 1.0% glycerol, and incubated at 37°C overnight under static conditions. The following day the cultures were adjusted with pre-warmed media containing the same carbon source concentrations as they had been grown in to give an optical density of 0.2 at 600 nm. These cultures were grown for one additional hour to stimulate the cells into log phase growth and were centrifuged at 4,000 rpm for 5 min (IEC Centra). Pellets were resuspended in the identical type and volume of media as before centrifugation.

Three 200µL aliquots from each of these six tubes were immediately removed and pipetted in triplicate into a sterile 96-well plate (Corning). The plate was placed in a microplate reader (Tecan), and incubated at 37°C under static for 6 h. Absorbance readings at 600 nm were recorded every 15 min. These data were plotted to determine the growth curves for *E. coli* O157:H7 when grown M9 media in the presence of the various concentrations of each of the two carbon sources.

4.3. Growth Curves of *E. coli* O157:H7 in cAMP Supplemented LB Media

E. coli O157:H7 was grown and prepared as described in the previous section. The culture was then divided into three equal 2-mL aliquots. Exogenous cAMP was added to the second and third aliquots of culture to yield 1 mM and 10 mM final concentrations, respectively.

Three 200 μ L aliquots from each of these three tubes were immediately removed and pipetted in triplicate into a sterile 96-well plate (Corning), and growth density was recorded over time, exactly as described in section 4.2. These data were plotted to determine a growth curve for *E. coli* O157:H7 when grown in 0 mM cAMP-, 1 mM cAMP-, and 10 mM cAMP-supplemented LB.

4.4. Vero Cell Growth and Maintenance

African green monkey kidney (vero) cells were acquired and maintained as described in Appendix B. In preparation for cytotoxicity assays, log-phase vero cells were passaged into a 96-well microtiter plate (Corning) seeded into the 96-well plate at a concentration of 10,000 cells/well, as described in Chapter 2, section 4.2.

4.5. MTT Cytotoxicity Assay Standards

A MTT assay standard curve was prepared as described in Chapter 2, section 4.5. This standard curve was used in the analysis of subsequent MTT assays to determine the number of live vero cells remaining after treatment with Stx-2 containing media.

4.6. Growth of *E. coli* O157:H7 in M9 with Glucose or Glycerol for MTT Assay

E. coli O157:H7 strain 90-2380 was inoculated into six broths containing 0.04%, 0.2% or 1.0% glucose, or 0.04%, 0.2% or 1.0% glycerol. Cultures were then grown overnight at 37°C under static conditions. The following day these cultures were adjusted to an optical density of 0.2 at 595 nm using pre-warmed media containing the same carbon source concentrations as they had been grown in previously. An additional one

hour incubation was used to stimulate the cells into log phase growth. These cultures were centrifuged at 4,000 rpm for 5 min (IEC Centra) and the pellets were resuspended in the identical type and volume of media as before centrifugation. This served to remove residual toxin formed during the overnight incubation so that only newly-formed toxin would be assayed. These cultures were incubated at 37°C for 24 h. Immediately following incubation, the cultures were centrifuged at 4,000 rpm at 4°C for 10 min to pellet the cells. The supernates were removed and sterile filtered through a low-protein binding 0.2 µm filter (Pall). Sterile cell-free supernates were stored at -20°C for use in MTT assays.

4.7. Growth of *E. coli* O157:H7 in cAMP-supplemented LB for MTT Assay

E. coli O157:H7 was grown and prepared as described in the previous section, except LB broth was used in place of M9 medium. The culture was then divided into 3 equal 2-mL aliquots. cAMP was added to the second and third aliquots of culture to give 1 mM and 10 mM final concentrations. Cultures were incubated at 37°C for 24 h at which time they were centrifuged at 4,000 rpm at 4°C for 10 min to pellet the cells. The supernates were removed and sterile filtered through a low-protein binding 0.2 µm filter (Pall). Sterile cell-free supernates were stored at -20°C for use in MTT assays.

4.8. *E. coli* O157:H7 Growth in M9 with Glucose or Glycerol MTT Assay

Ten µL each of supernates collected from the growth of *E. coli* O157:H7 in glucose or glycerol containing media were added in triplicate to confluent vero cells in a 96-well plate. The plate was incubated at 37°C in 7% CO₂ (Airgas) for 72 h. Following

the 3-day incubation, an MTT assay was performed on the vero cells, as described in Chapter 2, section 4.7. This assay was used to determine the cytotoxicity supernates produced from *E. coli* O157:H7 cultures grown in the various concentrations of either glucose or glycerol.

4.9. *E. coli* O157:H7 Growth in cAMP-Supplemented LB MTT Assay

A similar MTT assay to that described in the previous section was performed on the supernatant fluid collected from the growth of *E. coli* O157:H7 in cAMP-supplemented LB medium. The purpose of this experiment was to determine the amount of Stx-2 in these supernates, to determine if cAMP has an effect on the production of the toxin by the pathogen.

4.10. Growth of *E. coli* O157:H7 in M9 with Glucose or Glycerol for Real Time PCR and ELISA

E. coli O157:H7 strain 90-2380 was grown in glucose- or glycerol-containing medium and prepared exactly as described in section 4.2. Immediately following incubation, 100 μ L of each culture was removed to a sterile microfuge tube and retained for RNA extraction, which was performed immediately. The remaining 2-mL cultures were incubated for a total of 24 h, followed by centrifugation and collection of the cell-free supernate, as previously described. The supernates were sterile filtered through a low-protein binding 0.2 μ m filter (Pall), and stored at -20°C for use in ELISA assays.

4.11. Growth of *E. coli* O157:H7 in cAMP-Supplemented LB for Real Time PCR and ELISA

E. coli O157:H7 was grown in cAMP-supplemented LB medium as described in section 4.3. Immediately following incubation, 100 μ L of each culture was removed to a sterile microfuge tube and RNA extraction was performed immediately. The remaining 2-mL cultures were incubated for a total of 24 h, after which cell-free supernates were collected and stored at -20°C for use in ELISA assays.

4.12. RNA Extraction and cDNA Synthesis

Bacterial RNA protect (Qiagen) was added to each aliquot of culture to prevent degradation of mRNA and to halt all transcription within the *E. coli* cells. RNA was isolated in the same manner as described in Chapter 2, section 4.9. The RNA was assayed for contaminating genomic DNA by PCR, as described in Chapter 2, and samples containing DNA were discarded and the RNA extraction protocol was repeated on a freshly prepared culture.

cDNA synthesis was performed using the SuperScript III cDNA synthesis kit (Invitrogen) exactly as described in Chapter 2, section 4.9, and samples were tested in a standard PCR assay to ensure that the cDNA synthesis was successful. cDNA samples were stored at -20°C until their use in Real Time PCR studies.

4.13. Quantification of cDNA

cDNA samples were quantified using the Qubit (Invitrogen) High Sensitivity (HS) DNA quantification kit™, according to the manufacturer's instructions. A brief

explanation of the Qubit fluorometer quantification protocol is described in Chapter 2, section 4.10.

4.14. cDNA standardization

Carbon source-induced and cAMP-induced cDNA samples were standardized using nuclease-free water to give a concentration of 100 ng/mL prior to use in Real Time PCR experiments. Standardized cDNA samples were stored at -20°C.

4.15. Real Time Polymerase Chain Reaction

Real Time PCR was performed using an Applied Biosystems 7300 Thermocycler and software, as described in Chapter 2, section 4.12. The primers and dual-labeled probes used in this assay are described in Appendix C.

4.16. Enzyme-linked Immunosorbent Assay

Enzyme-linked Immunosorbent Assay (ELISA) was designed based a protocol by Krishna Moody (102), and is described in detail in Chapter 2, section 4.13. A dilution of purified Stx-2 (Toxin Technologies) was measured concurrently with the test samples for accurate quantification of Stx-2 in the *E. coli* O157:H7 supernates. The microtiter plate was read spectrophotometrically at 405 nm and the data were recorded.

5. Results

Statistical significance was determined as described in Appendix B, Section 5.

5.1. Growth Curves of *E. coli* O157:H7 in Glucose or Glycerol Supplemented Minimal Media

Several growth curves were constructed to show the growth *E. coli* O157:H7 in glucose- or glycerol-supplemented M9 medium. These curves are shown in Figure 4.1 and clearly show that the growth rate of *E. coli* O157:H7 is not affected by the concentration or type of carbon-source used.

5.2. Growth Curves of *E. coli* O157:H7 in cAMP Supplemented LB

A similar set of growth curves were constructed for the growth of *E. coli* O157:H7 in cAMP-supplemented LB medium (Figure 4.2). No significant change in the growth rate of the organism was seen, regardless of the concentration of exogenous cAMP.

5.3. MTT Cytotoxicity Assay

A MTT cytotoxicity was employed to measure the effect of Stx-2-containing supernates on vero cells. Supernates obtained from *E. coli* O157:H7 grown in glucose-containing media killed significantly more vero cells than those supernates obtained from *E. coli* O157:H7 grown in glycerol-containing media (Figure 4.3). The amount of vero cells killed was proportional to the amount of glucose present in the growth medium used to grow the *E. coli* O157:H7; higher concentrations of glucose yielded higher kill rates of vero cells. There was no significant change between varying concentrations of glycerol.

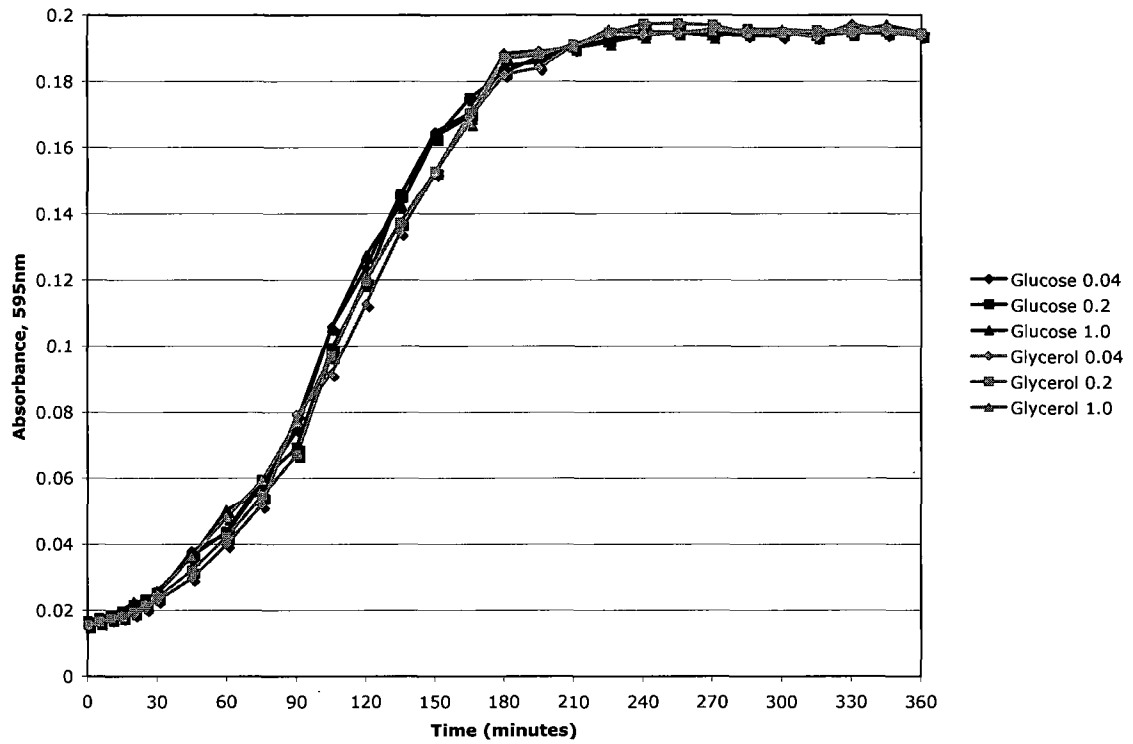


Figure 4.1. Growth Curve of *E. coli* O157:H7 in M9 with Glucose or Glycerol. *E. coli* was grown in either glucose or glycerol supplemented M9 media. Note that growth rate of the organism was not impacted by the type or concentration of the medium.

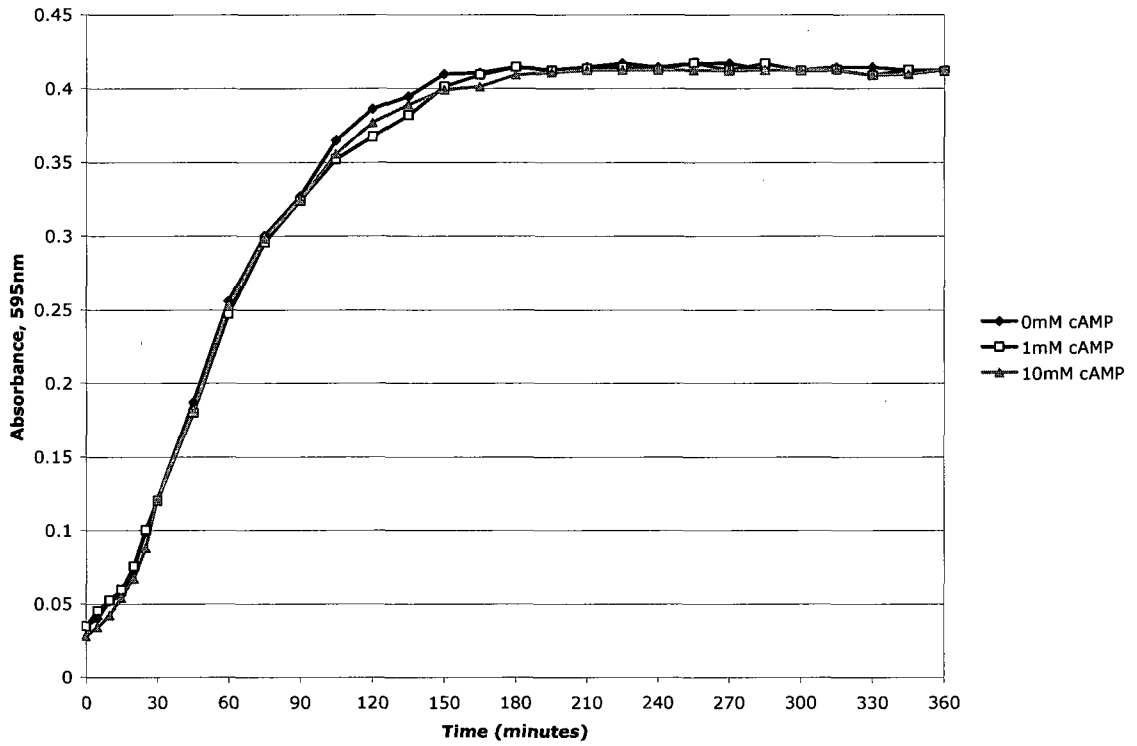


Figure 4.2. Growth Curve of *E. coli* O157:H7 in cAMP Supplemented LB. *E. coli* was grown in LB medium with either 0mM, 1mM or 10mM cAMP. No difference was seen in growth rate for those cultures grown with or without cAMP.

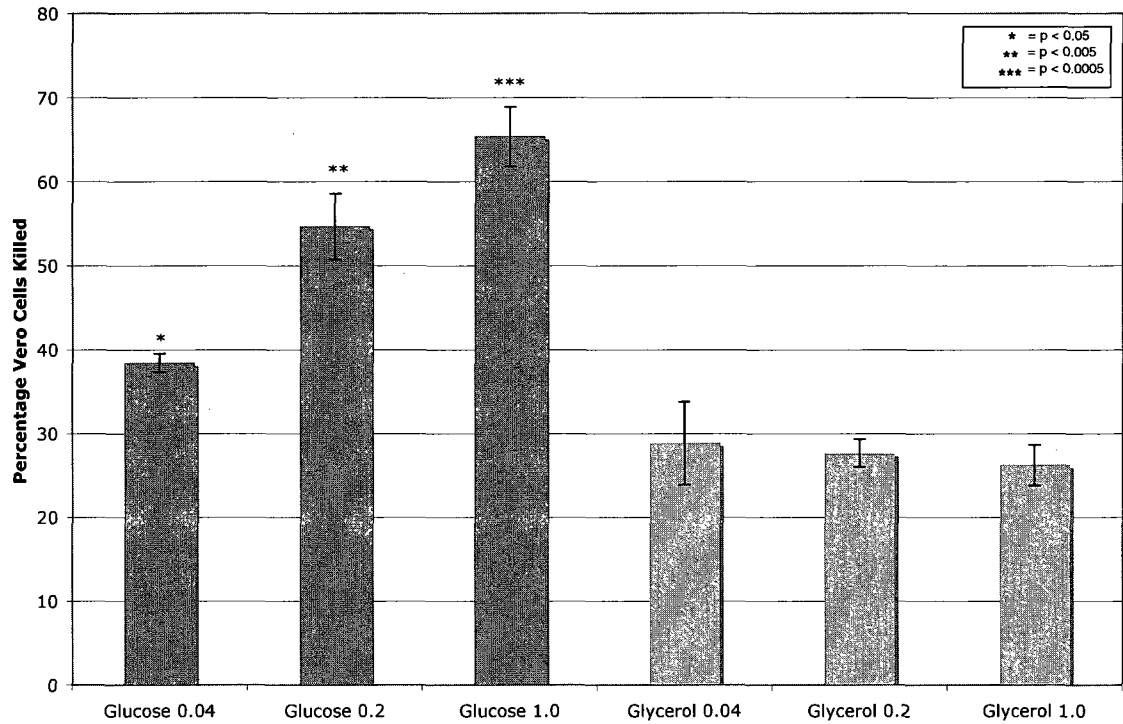


Figure 4.3. MTT Cytotoxicity Assay: The Effect of Glucose and Glycerol on Stx-2 Production by *E. coli* O157:H7. When grown in glucose-containing medium, the *E. coli* O157:H7 produces more cytotoxic compounds (likely Stx-2) than when grown in glycerol-containing media. Note that the cytotoxic affects increase with the concentration of glucose, yet no significant difference is seen between the different concentrations of glycerol.

The concentration of cAMP appeared to exert an inversely proportional reaction on the cytotoxicity of the *E. coli* O157:H7 supernates, as seen in Figure 4.4. When cultured without cAMP, *E. coli* O157:H7 supernate killed approximately 80% of the vero cell monolayer. The addition of 1 mM cAMP reduced the percentage of vero cell death to 70%, though this was not found to be statistically significant. However, *E. coli* O157:H7 grown with 10 mM cAMP reduced vero cell death to 37%. This was a considerable reduction in cytotoxicity.

5.4. ELISA Analysis of *E. coli* O157:H7 Grown with Carbon Source Variation.

Supernates were collected from cultures of *E. coli* O157:H7 grown in either M9 with glucose, M9 with glycerol, or LB supplemented with cAMP. These supernates were assayed for Stx-2 concentration by ELISA, and compared to the previously-constructed standard curve (Figure 2.6).

As depicted in Figure 4.5, when grown in 0.04% glucose, *E. coli* O157:H7 produced 1.9 μg of Stx-2. This increased to 3.1 μg in 0.2% glucose, and to 3.8 μg in 1.0% glucose. In 0.04%, 0.2% or 1.0% glycerol, *E. coli* O157:H7 produced approximately 0.5 to 0.8 μg of Stx-2. Growth in glucose-containing media produced significantly more Stx-2 than in the same concentration of glycerol. Likewise, production of Stx-2 was dose-dependent on glucose concentration; more Stx-2 was produced when more glucose was available to the microorganism during growth. The concentration of glycerol, however, did not significantly change the production of Stx-2.

Production of Stx-2 by *E. coli* O157:H7 was also affected by the concentration of exogenous cAMP added to the growth medium. When no exogenous cAMP was added,

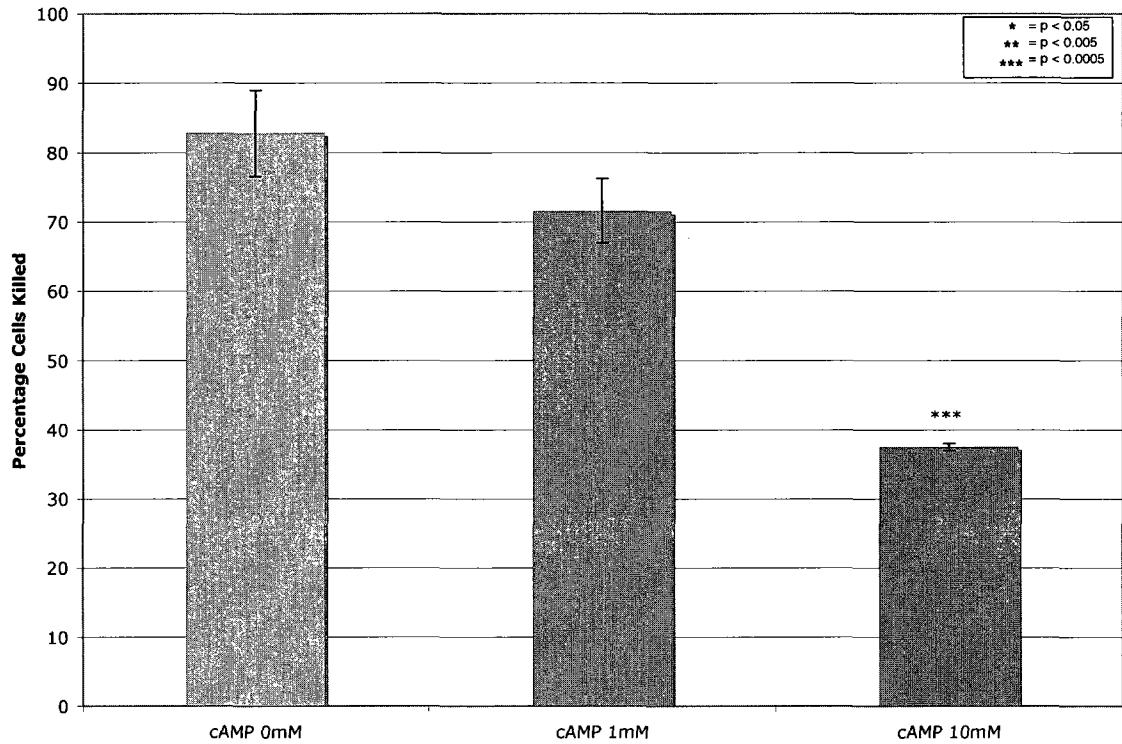


Figure 4.4. MTT Cytotoxicity Assay: The Effect of cAMP on Stx-2 Production by *E. coli* O157:H7. Note that the supernates obtained from *E. coli* O157:H7 grown in 10mM cAMP killed significantly less vero cells than those supernates obtained from *E. coli* O157:H7 grown in either 0mM or 1mM cAMP.

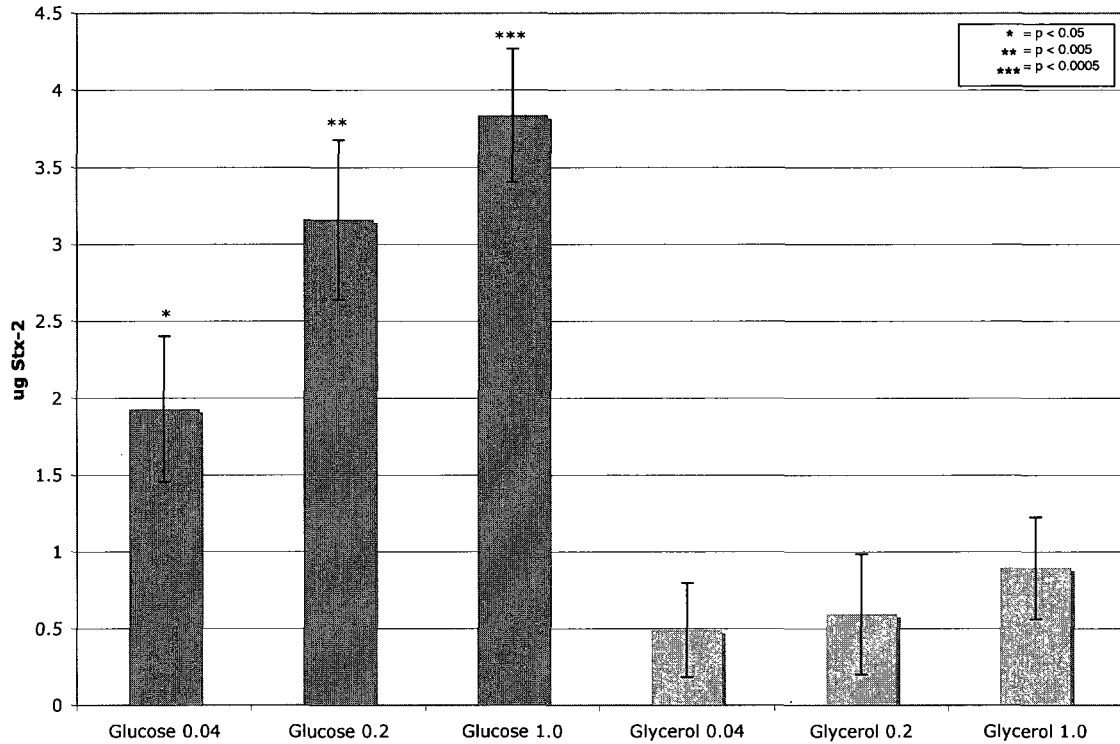


Figure 4.5. ELISA Analysis of the Effect of Glucose and Glycerol on the Production of Stx-2 by *E. coli* O157:H7. Cultures grown in glucose-containing M9 produced significantly more Stx-2 than those grown in glycerol-containing M9. This response was dose-dependent for glucose but not for glycerol. Increased concentrations of glucose produced increased concentrations of Stx-2, however, varying the concentration of glycerol did not effect the production of Stx-2 in those cultures.

E. coli produced 10 µg of Stx-2. The addition of 1mM cAMP reduced this to 9 µg of Stx-2, which was not statistically significant. However, the addition of 10 mM of cAMP reduced the production of Stx-2 to 6.5 µg. This data is represented in Figure 4.6.

5.5. Real Time PCR Analysis of *E. coli* O157:H7 Transcripts.

The cDNA collected from glucose and glycerol-induced cultures was used in Real Time PCR analysis. Two genes were assayed: *serC* and *stx2*. All data for *stx2* was first normalized against *serC*. Once normalized, each glucose sample was compared directly to its glycerol counterpart for a change *stx2* expression. As shown in Figure 4.7, all three concentrations of glucose resulted in increased *stx2* expression when compared to the equal concentration of glycerol. Growth in 0.04% glucose resulted in 5.5-fold higher *stx2* expression than 0.04% glycerol. Likewise, 0.2% glucose and 1.0% glucose yielded 4.8-fold and 4.5-fold higher *stx2* expression than their glycerol counterparts. There was not, however, a significant difference in *stx2* expression between the three concentrations of glucose (5.5-fold, 4.8-fold and 4.5-fold).

Figure 4.8 depicts how cAMP appears to exert an opposite effect on *stx2* expression than did growth in glucose. In this analysis, the expression of *stx2* in 0 mM cAMP is normalized to one, and the samples grown in 1 mM cAMP and 10 mM cAMP are compared against that control sample. These results indicate that both 1 mM and 10 mM cAMP reduced *stx2* expression to approximately 0.45- and 0.6-fold of the expression in the absence of cAMP. However, the concentration of cAMP (either 1 mM or 10 mM) was not statistically significant.

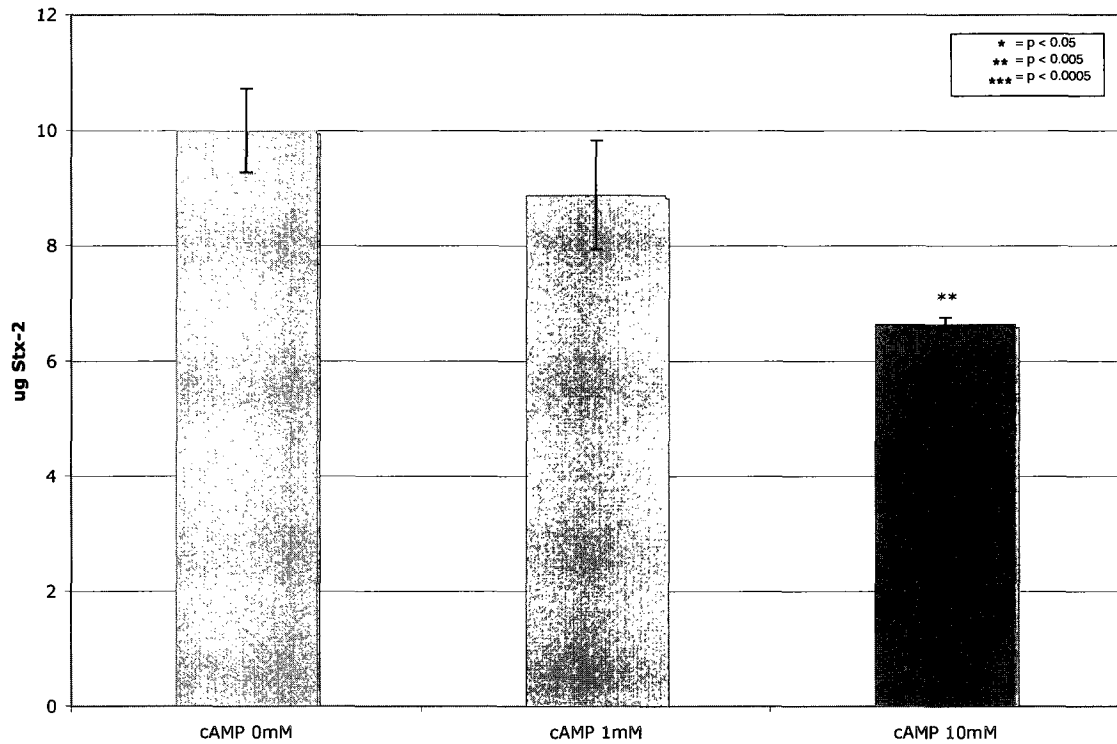


Figure 4.6. ELISA Analysis of the Effect of cAMP on the Production of Stx-2 by *E. coli* O157:H7. Note that cultures grown in 10mM cAMP produced significantly less Stx-2 than those grown in 0mM cAMP. No significant change in Stx-2 production was seen when the pathogen was grown in 1mM compared to 0mM cAMP.

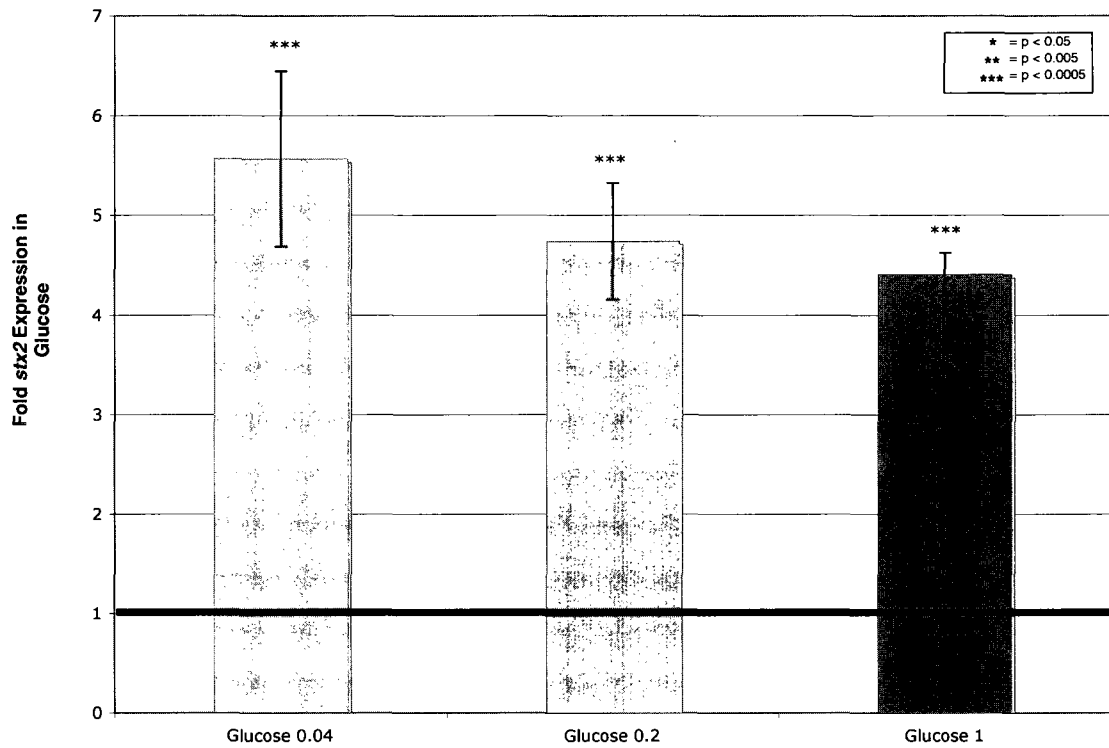


Figure 4.7. Real Time PCR Analysis of *stx2* in *E. coli* O157:H7 Grown in Glucose- or Glycerol-Containing Media. Real Time PCR analysis was performed on the cDNA of samples grown in glucose or glycerol. Samples from glucose-containing media were compared to their glycerol counterparts for *stx2* expression. Note that all cultures grown in glucose had significantly higher expression of *stx2* than those grown in glycerol. However, no significant difference was noted between the various concentrations of glucose.

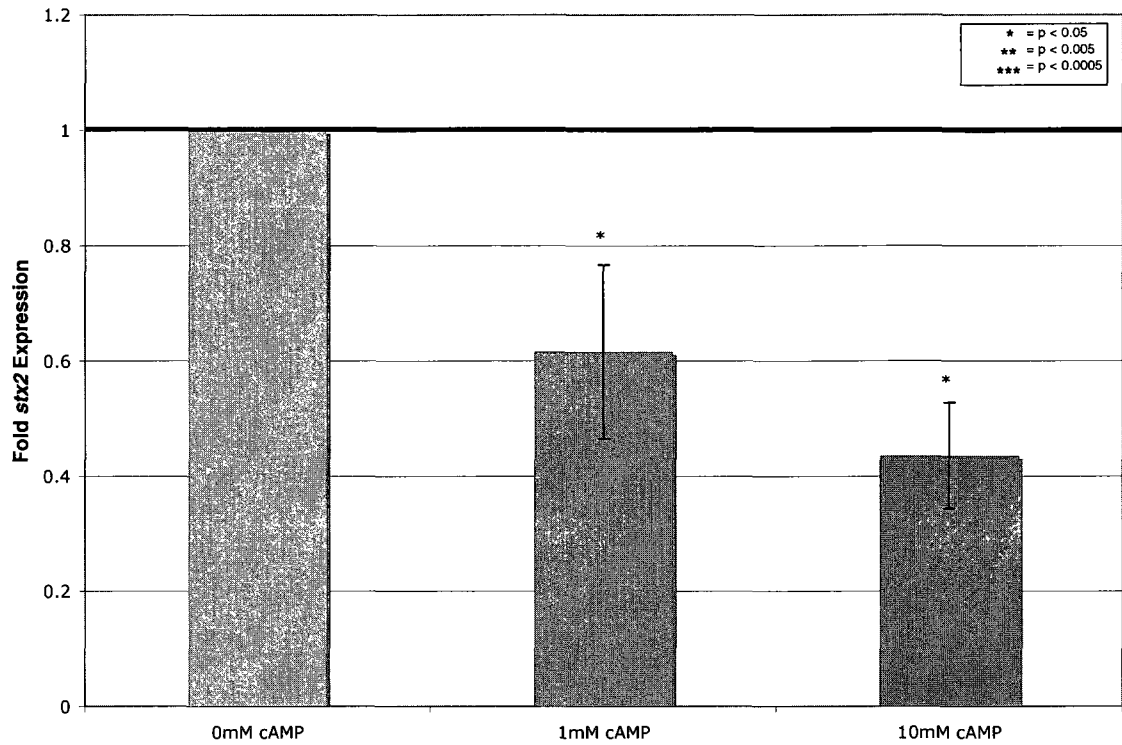


Figure 4.8. Real Time PCR Analysis of *stx2* in *E. coli* O157:H7 Grown in cAMP-containing LB. Note that both 1mM and 10mM cAMP decreased the amount of *stx2* transcripts produced by *E. coli* O157:H7 when compared to growth in the absence of exogenous cAMP.

6. Discussion

Czyz et al (29) showed that in non-pathogenic *E. coli*, a lambda phage was more likely to stay in a lysogenic state when the *E. coli* was grown in glycerol compared to glucose. These findings were applied in the present study to the pathogenic *E. coli* O157:H7, which harbors a lambda-like phage, 933-W. In *E. coli* O157:H7, the *stx2* gene is located within the 933-W phage structural genes (49). It is expressed at low, baseline levels unless the phage switches from a lysogenic to a lytic state. When this switch occurs, the phage structural genes are transcribed, and the *stx2* gene is consequently co-transcribed (78, 104, 120, 164). It was hypothesized that this switch from lysogeny to lysis would occur more readily when the *E. coli* O157:H7 was grown in glucose-supplemented media compared to glycerol-supplemented media, and that this would be demonstrated by an increase in *stx2* expression and Stx-2 production. Similarly, it was thought that this increase in *stx2* expression and Stx-2 production in the presence of glucose would be inversely linked to a decrease in cAMP, and that increasing concentrations of cAMP could cause the lambda-like phage to remain lysogenic, thereby decreasing *stx2* expression and Stx-2 production.

These studies showed that indeed, growth of the *E. coli* O157:H7 in glucose-supplemented media increased *stx2* expression and Stx-2 production compared to growth in glycerol-supplemented media. It is likely that high levels of glucose reduced the intracellular concentration of cAMP in *E. coli* O157:H7. The presence of cAMP reduces the concentration of intracellular proteases, including those that degrade cII. The degradation of cII causes a reduction in the production of the repressor cI, which is responsible for repressing expression of the phage lysis genes, including *stx2* (78). This

hypothesis is confirmed by the upregulation of *stx2* seen in the presence of glucose, and the opposite downregulation of *stx2* seen in the presence of cAMP. Higher concentrations of cAMP decreased overall Stx-2 production, and increasing concentrations of glucose caused the *E. coli* O157:H7 to produce increasingly more Stx-2, and also led to greater vero cell death in cytotoxicity assays.

Interestingly, this dose-dependent response to glucose was not mimicked in *stx2* expression studies; all concentrations of glucose led to higher *stx2* expression than when grown in glycerol, but this upregulation was not significantly different between the various concentrations of glucose. It is possible that because the Real Time PCR studies were performed after only 1 h of growth that the differences between glucose concentrations were not as defined as after 24 h of growth, as in the ELISA and MTT assays. A similar pattern was detected in the cAMP-supplemented media studies. In Real Time PCR studies, cAMP decreased *stx2* expression, yet this decrease was not dependent on cAMP concentration (both 1mM and 10mM cAMP decreased *stx2* expression), whereas in ELISA and MTT studies, the 1mM cAMP concentration was not sufficient to cause a significant change from the 0mM cAMP culture. It required 10mM cAMP to cause a significant decrease in Stx-2 production and a significant decrease in vero cell killing.

The information obtained from these studies may be applied in a practical manner to the problem of *E. coli* O157:H7 carriage in dairy and beef cattle. It has been demonstrated that the diet of feedlot cattle can also greatly affect the microbial ecology of the rumen and colon (22, 155, 154). Numerous studies have shown that the microbial makeup of the bovine digestive tract is drastically different when fed a grain (corn) diet

compared to a forage (hay, grass) diet (22, 33, 36, 77, 154, 155). Cattle are naturally accustomed to a forage diet, and have few of the pancreatic amylases that are essential for the proper digestion of the high concentration of starch found in grain diets (61). As such, undigested starch is often passed from the rumen through the small intestines into the colon of these cattle where it is fermented by microorganisms such as *E. coli* (61). This kind of diet has been correlated with increased *E. coli* O157:H7 carriage and shedding by these feedlot cattle (6). In fact, 100-fold higher concentrations of *E. coli* populations (both benign and human pathogen strains) were recovered when the cattle were fed a corn and soybean (grain) diet compared to a high-quality hay diet (33). The fermentation of starch in the colon creates an increasingly acidic environment that allows these *E. coli* to become more acid-resistant (33, 82). In a study by Diez-Gonzalez et. al (33), grain-feeding of cattle increased the ability of *E. coli* to survive an artificial gastric shock (mimicking the conditions of the human stomach) by 1000-fold. However, when the researchers switched the cattle back to a hay diet, *E. coli* populations declined 1000-fold, and the number of acid-resistant *E. coli* decreased by 100,000-fold in 5 days (33, 22). It should be noted that in this study, no differentiation was made between populations of non-pathogenic *E. coli* and enterohemorrhagic *E. coli*. However, these nonpathogenic strains of *E. coli* have been shown to increase *stx2* expression through quorum sensing, and thus may be significant (47, 48). Likewise, studies have shown that Stx-harboring phages released from enterohemorrhagic *E. coli* are able to infect nonpathogenic strains of *E. coli*, which subsequently become Stx-producing strains (46, 48).

The microbial population of other microorganisms in the cattle digestive tract is also affected by a diet change (36, 77, 154, 155). In 2004, Elias (36) showed that cows fed an 85% molasses diet (extremely high in sucrose and glucose) had no populations of lactobacilli present. As previously demonstrated, lactobacilli may aid in the attenuation of virulence of some pathogens. Likewise, Gregory et. al (53) showed that a diet of hay increased enterococci populations in the cattle rumen, and that these enterococci were inhibitory to *E. coli* (53). It is possible that a high-glucose diet not only increases the pathogenicity and numbers of enterohemorrhagic strains of *E. coli*, but also decreases the probiotic organisms that are capable of diminishing its virulence.

In the present study, glucose, present in much higher quantities in grain diets, was shown to increase the expression of *stx2*, and therefore increased the production of 933-W phage. Cattle that are fed a high glucose diet have increased numbers of both pathogenic and nonpathogenic *E. coli*, and it is possible that this can lead to increased virulence in enterohemorrhagic *E. coli* strains (through upregulation of Stx-2 production and increased acid resistance) (33, 46, 48). Likewise, these grain-based diets diminish populations of beneficial microbes (36, 53), furthering the colonization of pathogenic organisms. A strong argument can be made that a grain diet leads to a greater likelihood of *E. coli* O157:H7 contamination in human food products and should be evaluated further.

CHAPTER 5

GENERAL DISCUSSION AND FUTURE STUDIES

1. Discussion

E. coli O157:H7 is a food and water borne pathogen that causes symptoms ranging from mild diarrhea to severe hemorrhagic colitis (127). These infections can result in devastating sequelae and deadly complications such as HUS and TTP (13, 71, 101, 123, 172). The severity of disease is linked intimately with the production of its principle virulence factor, Stx-2. Stx-2 is an AB₅ toxin that inhibits cellular protein synthesis in affected cells (14). Currently, treatment for *E. coli* O157:H7 is palliative; antibiotic treatment to reduce pathogen numbers is controversial, as it is believed that antibiotics may increase the release of Stx-2 from *E. coli* O157:H7 (57, 74, 132).

The purpose of this study was three-fold: i) to assess the effects of various classes of antibiotics on *stx2* expression and Stx-2 production; ii) to assess the probiotic microorganisms *L. casei* and *L. plantarum* for their effects on the reduction of *stx2* expression and Stx-2 production; and iii) to analyze *stx2* expression and Stx-2 production when *E. coli* O157:H7 is grown with either glucose, glycerol, or in the presence of exogenous cAMP. These three facets were examined by MTT cytotoxicity assay, ELISA analysis and Real Time PCR.

The present study elucidated a number of factors that regulate *stx2* expression, including the impact of several classes of antibiotics, the effect of co-culture with lactobacilli, and the nature of growth substrates used for pathogen growth. These three seemingly disparate factors, however, appear to be more closely linked than previously appreciated. It was determined that DNA-affecting antibiotics increase *stx2* expression and Stx-2 production, and this is linked to an upregulation in the SOS DNA repair response. Likewise, a connection was also observed between the upregulation of *stx2* and cell membrane integrity disrupting antibiotics, gentamicin and polymyxin B, though this was not tied to DNA damage, as the *dinD* gene was not upregulated concurrently with *stx2*. Ultimately, these antibiotics did not increase the overall Stx-2 released from *E. coli* O157:H7. Interestingly, growth of *E. coli* O157:H7 in organic acid-containing media (either artificially acidified, or acidified through the growth of lactobacilli) also inhibited the release of Stx-2. As with gentamicin and polymyxin B, organic acids are thought to disrupt the lipid composition and structure of bacterial cell membranes, which may interfere with the assembly or release of Stx-2 toxin molecules (66, 67, 131, 137, 173). It was clear that disruption of the bacterial cell membrane, either through the action of antibiotics or organic acid, interferes with the secretion of Stx-2.

The reduction of Stx-2 seen by co-culture with probiotics was in part attributable to the production of organic acids, but was in part a response to mechanisms as yet unknown resulting from the lactobacilli. It appears from this research that cell-to-cell contact is required for full reduction in Stx-2 production; growth of the *E. coli* O157:H7 in co-culture with the probiotic microorganisms *L. casei* and *L. plantarum* were found to decrease Stx-2 release to a greater extent than organic acids alone. It is possible that

these probiotics interfere with the quorum sensing between *E. coli* O157:H7 cells (94). Quorum sensing has been shown to play a role in the virulence of this and other pathogens (23, 31, 47, 98) and if the lactobacilli are quorum-quenching the *E. coli* signaling molecules, this could explain the reduction in *stx2* expression and Stx-2 release.

Interestingly, the microbial population (and therefore the quorum-sensing potential) in the bovine gut is greatly influenced by the diet of the animal (33, 36, 53, 154, 155). Animals fed high-starch grain diets have increased populations of both benign and pathogenic *E. coli*, and decreased populations of probiotic microbes (33, 36, 77). Conversely, cattle fed a more natural hay-based diet had higher populations of microorganisms that are proven to inhibit *E. coli* populations (53). These grain-based diets contain more starch than the cattle are capable of digesting in the rumen, and therefore some undigested starch is passed to the colon of the animal where it aids in the proliferation of *E. coli* populations (53, 61). The present study has shown that concentrations of glucose increase the production of Stx-2, and likely the production of the 933-W phage. These Stx-carrying phage have the ability to infect other nonpathogenic strains of *E. coli* and confer Stx production upon them (48). It is possible that a higher glucose, starch-based diet for cattle may contribute to the pathogenicity of *E. coli* O157:H7, and increase the likelihood of contamination in human food products.

The regulation of *stx2* expression and the production of Stx-2 has been shown to be complex, involving a range of factors from the diet of the host animal, to the microbial population in that niche, to the treatment options used in human patients infected with this pathogen. In this research three unique factors, antibiotics, probiotics, and carbon source, have each been shown to play a role in this network of *stx2* regulation, and further

research is needed to fully understand their connection to each other and the disease that results from *E. coli* O157:H7 and Stx-2.

2. **Future Studies**

A multifaceted approach to understanding the regulation of *stx2* in *E. coli* O157:H7 was implemented in this study. Numerous factors that either increase or decrease the expression of the *stx2* gene, and alter the production of Stx-2 by the organism have been uncovered. Many interesting lines of work have developed from this work and warrant further investigation.

2.1. **Synergistic Treatment of *E. coli* O157:H7 Infections with Rifampin and a Cephalosporin**

This research has shown that rifampin could be a viable treatment option for patients infected with *E. coli* O157:H7, as it has been found to decrease *stx2* expression. However, due to ease of resistance to rifampin, this drug is often used in synergy with other antibiotics. In particular, cephalosporins and rifampin have been used together in synergy for treatment of other infections (24), including difficult-to-treat infections, such as those caused by *P. aeruginosa* (41) and MRSA (15). In the current study, cefotaxime (a third-generation cephalosporin) was shown to not increase *stx2* expression or Stx-2 production more than the uninduced control. Future studies could examine the effect of a synergistic combination of rifampin and cefotaxime (or another cephalosporin) on the expression of *stx2* and release of Stx-2 from *E. coli* O157:H7 cells. This could lead to a viable treatment option for an infection that currently has no accepted curative treatment.

2.2. Cell Membrane Disrupting Compounds and Stx-2 Production

Polymyxin B and gentamicin both have the ability to disrupt cell membranes (66, 67, 137). In these studies, both of these antibiotics decreased Stx-2 production compared to the controls. Interestingly, organic acids were also demonstrated to reduce the production of Stx-2. Polymyxin B, gentamicin, and organic acids all alter the cell membrane of these microbes, and this is possible that disruption of the lipid composition of bacterial membranes affects secretion of the toxin, as it does in the case of pertussis toxin (131, 173). One study showed that the twin arginine translocation (TAT) system contributed to the secretion of Stx-2 (121), however, further research on the effect of membrane-altering compounds on the TAT system is necessary to completely understand how these compounds interfere with Stx-2 release from enterohemorrhagic *E. coli*.

2.3. The Role of Quorum-sensing on *stx2* Regulation

Microbiology has realized the great effect that co-colonizing microbes play on one another through quorum sensing (98). Studies have shown that quorum sensing molecules produced by nonpathogenic *E. coli* can upregulate *stx2* expression in *E. coli* O157:H7 (48). Conversely, *in vivo* co-culture of the pathogen with probiotic organisms, such as lactobacilli and species of *Bifidobacterium*, has been shown to decrease Stx-2 expression in animal models (4, 110). This research demonstrated that cell-to-cell contact of lactobacilli with *E. coli* O157:H7 was necessary for the full reduction in Stx-2 production. It is possible that the probiotics actively released quorum sensing molecules that downregulated *stx2* expression. Perhaps the lactobacilli played a more passive role; it is possible that the probiotics absorbed the quorum sensing molecules released by the

other *E. coli*, which in turn reduced the amount of molecules available for signaling the *E. coli* to upregulate its *stx2* expression. Studies aimed at elucidating the role that quorum sensing of these lactobacilli play on the expression of *stx2* would be beneficial to understanding how probiotics attenuate pathogen virulence.

2.4. STEC in the Gut of Grain-Fed Cattle: *stx2* Transduction

Previous studies have implicated that nonpathogenic *E. coli* contribute to the virulence of enterohemorrhagic *E. coli* by upregulating *stx2* expression (48). These nonpathogenic strains also have the ability to be infected by the Stx-carrying phage released by the pathogenic strains, and thereby become Stx-producing strains themselves (48). Grain-fed cattle have been shown to have increased populations of *E. coli*, and decreased concentrations of probiotic organisms (33, 36, 53, 77). Likewise, the present study has concluded that elevated concentrations of glucose lead to an increased release of Stx-2, and likely to an increased release in the 933-W phage. A study on the rate of *stx2* transduction into previously-nonpathogenic *E. coli* within the gut of grain-fed cattle would provide valuable information as to the role of cattle diet on the prevalence of enterohemorrhagic strains of this pathogen.

APPENDIX A
MEDIA AND REAGENTS

1. **Bacteriologic media**

1.1. **Trypticase soy agar (TSA) plates**

Trypticase soy agar (TSA) plates were made for general growth purposes. This medium was purchased in dehydrated form from Difco and prepared according to the manufacturer's instructions (40.0g dehydrated media into 1.0L distilled H₂O). The solution was autoclaved at 121.0°C for 20 min and dispensed into sterile Petri dishes. TSA plates were stored at 4°C.

1.2. **Trypticase soy broth (TSB)**

Trypticase soy broth (TSB) was made for general growth purposes. This medium was purchased in dehydrated form from Difco and prepared according to the manufacturer's instructions (30.0g dehydrated media into 1.0L distilled H₂O). Fifty milliliters of the solution was dispensed into 250mL Erlenmeyer flasks and autoclaved at 121.0°C for 20 min. Broths were stored at room temperature.

1.3. Luria-Bertani (LB) broth

Luria-Bertani (LB) broth was made for general growth purposes. This medium was prepared according to the recipe below. Fifty milliliters of the solution was dispensed into 250mL Erlenmeyer flasks and autoclaved at 121.0°C for 20 min. Broths were stored at room temperature.

Formulation for LB:

20 g tryptone (Difco)
10g yeast extract (Difco)
10g NaCl (Sigma)
2000mL d.H₂O

1.4. Trypticase soy broth (TSB) with 15% glycerol

Trypticase soy broth (TSB) with 15% glycerol was made for the cryopreservation of bacterial strains. The base of this medium (TSB) was purchased in dehydrated form from Difco and prepared according to the manufacturer's instructions (30.0g dehydrated media into 1.0L distilled H₂O). Fifteen milliliters of glycerol (EM) was also added. The solution was transferred to a glass bottle and autoclaved at 121.0°C for 20 min. The broth was stored at room temperature.

1.5. Mueller-Hinton (MH) agar plates

Mueller-Hinton (MH) plates were made for general growth purposes and use in antibiotic studies. This medium was purchased in dehydrated form from Difco and prepared according to the manufacturer's instructions (30.0g dehydrated media into 1.0L distilled H₂O). The solution was autoclaved at 121.0°C for 20 min and dispensed into sterile Petri dishes. MH plates were stored at 4°C.

1.6. Mueller-Hinton broth (MHB)

Mueller-Hinton broth (MHB) was made for general growth purposes and for antibiotic studies. This medium was purchased in dehydrated form from Difco and prepared according to the manufacturer's instructions (22.0g dehydrated media into 1.0L distilled H₂O). Fifty milliliters of the solution was dispensed into 250mL Erlenmeyer flasks and autoclaved at 121.0°C for 20 min. Broths were stored at room temperature.

1.7. Brain-heart infusion (BHI) broth

Brain-heart infusion (BHI) was made for growth of lactobacilli and lactobacilli co-cultures. This medium was purchased in dehydrated form from Difco and prepared according to the manufacturer's instructions (37.0g dehydrated media into 1.0L distilled H₂O). Fifty milliliters of the solution was dispensed into 250mL Erlenmeyer flasks and autoclaved at 121.0°C for 20 min. Broths were stored at room temperature.

1.8. deMan-Rogosa-Sharpe (MRS) broth

The deMan-Rogosa-Sharpe (MRS) broth was purchased in dehydrated form from Acumedia. It was prepared according to the manufacturer's instructions: 55g was added into 1 liter of d. H₂O. It was autoclaved at 121°C for 15 min to sterilize. The broth was stored at room temperature.

1.9. M9 10x salts

M9 minimal media base 10x salts were made according to the formulation below.

The solution was autoclaved at 121°C for 20 min.

Formulation for M9 10x Salts:

60 g Na₂HPO₄ (Sigma)
30 g KH₂PO₄ (Sigma)
5 g NaCl (VWR)
10 g NH₄Cl (Sigma)
1000 mL d.H₂O

1.10. M9 Base

M9 base was made according to the formulation below.

Formulation for M9 10x Salts:

100 mL 10x Salts
10 mL 10mM CaCl₂
2 mL 1M MgSO₄

2. Reagents

2.1. 0.5M ethylene diamine tetraacetic acid (EDTA), pH 8.0

Ethylene diamine tetraacetic acid (EDTA) was made at a concentration of 0.5M.

The buffer was prepared according to the formulation below and adjusted to pH 8.0 with approximately 5 g of NaOH pellets (Sigma). The solution was autoclaved for 20 min at 121.0°C and stored at room temperature.

Formulation for 0.5M EDTA, pH 8.0

46.5 g NaEDTA (Sigma)
200 mL distilled H₂O

2.2. Tris-acetate-ethylene diamine tetraacetic acid (TAE) buffer, 50x

Tris-acetate-ethylene diamine tetraacetic acid (TAE) buffer, 50x, was made as a concentrated buffer for agarose electrophoresis gels. The buffer was prepared according to the formulation below. The buffer stored at room temperature, and diluted to 1x with distilled H₂O for use.

Formulation for TAE, 50x:

242 g Tris base (Biorad)
100 mL 0.5M EDTA
57.1 mL Glacial acetic acid (Mallinckrodt)

The solution was heated and mixed to dissolve the Tris base. The pH was adjusted to 8.5 with a few drops of glacial acetic acid.

2.3. 1M Tris-hydrochloride (Tris-HCl), pH 6.8

Tris-hydrochloride (Tris-HCl) was made at a concentration of 1M according to the formulation below. The pH was adjusted to 6.8 by hydrochloric acid (VWR). It was stored at room temperature.

Formulation for 1M Tris-HCl:

15.76 g Tris-HCl (Research Organics)
100 mL distilled H₂O

2.4. 10% (w/v) Sodium dodecyl sulfate (SDS)

A 10% solution of sodium dodecyl sulfate (SDS) was prepared by the recipe below. This solution was stored at room temperature.

Formulation for 10% SDS:

10 g SDS (Biorad)
100 mL d.H₂O

2.5. Tris-ethylene diamine tetraacetic acid (TE) buffer, 1mM EDTA, 10mM Tris

RNase-free TE buffer was prepared for use in RNA extractions. All weighing materials (weigh boat, spatulas) were soaked overnight in a 1% solution of diethylpyrocarbonate (DEPC) to inactivate any RNases. These materials were autoclaved to inactivate the DEPC. A 2mM EDTA solution, and a 20mM Tris solution were prepared separately according to the formulations below. The EDTA solution was autoclaved at 121°C for 15 min to inactivate the DEPC. The Tris solution was made with certified RNase-free Tris and DEPC-treated (inactivated) water, as Tris is sensitive to DEPC. They were combined in a ratio of 1:1 under sterile conditions to yield the final concentrations of 1mM EDTA and 10mM Tris in the finished TE buffer.

Formulation for 2mM EDTA solution:

0.149 g EDTA (Sigma)
200 mL d. H₂O
2 mL DEPC (active)

Formulation for 20mM Tris (Sigma)

0.48 g Tris
200 mL DEPC-treated d.H₂O

2.6. 10N Sodium hydroxide (NaOH)

A 10N solution of NaOH was made according to the recipe below. It was stored at room temperature.

Formulation for 10N NaOH:

50.0 g NaOH pellets (J.T. Baker)
125 mL d. H₂O

2.7. 1% (w/v) Sodium dodecyl sulfate (SDS)

A 1% solution of sodium dodecyl sulfate (SDS) was prepared by the recipe below. This solution was stored at room temperature.

Formulation for 1% SDS:

1 g SDS (Biorad)
100 mL d.H₂O

2.8. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) 5 mg/ML

A solution of MTT was made at a concentration of 5 mg/mL, according to the formulation below. The solution was sterile filtered through a 0.2µm filter (Pall) and stored at 4°C, protected from light.

Formulation for MTT, 5 mg/mL:

50 mg MTT (Sigma)
10 mL d.H₂O

2.9. MTT Lysing solution

MTT lysing solution was prepared according to the formulation below. The final concentrations of the components were 20% SDS and 50% Dimethylformamide (DMF). This solution was stored at room temperature.

Formulation for MTT lysing solution:

8 g SDS (Biorad)
20 mL DMF (Sigma)
20 mL d.H₂O

2.10. Lysis Buffer

Lysis buffer was formulated for the lysing of Gram negative bacteria using the recipe below. This buffer was stored at room temperature.

Formulation for lysis buffer:

250 μ L 10% SDS
50 μ L 10N NaOH
9.7 mL d. H₂O

2.11. Phosphate buffered saline (PBS)

Phosphate buffered saline (PBS) was made using the recipe below. The pH was adjusted to 7.4 using hydrochloric acid. The solution was transferred to a glass bottle and autoclaved at 121.0°C for 20 min. The PBS was stored at room temperature.

Formulation for 1x PBS:

1.44 g Sodium phosphate, dibasic (Na₂HPO₄)
8 g Sodium chloride (NaCl)
0.2 g Potassium Chloride (KCl)
0.24 g Potassium Phosphate, Monobasic (KH₂PO₄)
1.0 L d.H₂O

2.12. Antibiotic preparations

Antibiotics used in these studies were prepared to a stock concentration of 20 mg/mL in an appropriate solvent, as seen in Table B1 below. Each antibiotic stock solution was sterile filtered using a 0.2 μ m (Pall), then serially diluted 1:10 in sterile d.H₂O to 2 μ g/mL. All dilutions of each antibiotic were stored at -20°C.

Table B1. Antibiotic Preparations

Antibiotics	Manufacturer	Solvent
Cefotaxime	Sigma	Water
Ciprofloxacin	Cellgro	0.1M NaOH to dissolve
Chloramphenicol	Calbiochem	95% ethanol
Gentamicin	Sigma	Water
Polymyxin B	Sigma	Water
Rifampicin	Sigma	Methanol
Norfloxacin	Sigma	0.1M NaOH to dissolve
Trimethoprim	Sigma	0.1M HCl to dissolve

2.13. 100mM Magnesium sulfate (MgSO₄)

A 100mM solution of magnesium sulfate was made according to the formulation below. It was sterile filtered through a 0.2µm filter (Pall) and stored at room temperature.

Formulation for 100mM MgSO₄:

1.204 g MgSO₄ (Mallinckrodt)
100 mL d.H₂O

2.14. 10mM Calcium chloride (CaCl₂)

A 10mM solution of calcium chloride was made according to the formulation below. It was autoclaved at 121°C for 20 min and stored at room temperature.

Formulation for 10mM CaCl₂:

0.111 g CaCl₂ (J.T. Baker)
100 mL d.H₂O

2.15. 1M Magnesium sulfate (MgSO₄)

A 1M solution of magnesium sulfate was made according to the formulation below. It was sterile filtered through a 0.2µm filter (Pall) and stored at room temperature.

Formulation for 1M MgSO₄:

12.04 g MgSO₄ (Mallinckrodt)
100 mL d.H₂O

2.16. 20% (w/v) Glucose

A 20% glucose solution was prepared by adding 20 g of glucose (Sigma) to 100 mL of d.H₂O. The solution was autoclaved at 121°C for 20 min.

2.17. 20% (v/v) Glycerol

A 20% glycerol solution was prepared by adding 20 mL of glycerol (EM) to 100 mL of d.H₂O. The solution was autoclaved at 121°C for 20 min.

2.18. ELISA Primary antibody (11E10)

The murine primary antibody, STX2-11E10, was purchased from Toxin Technologies. It is specific to the A subunit of the Stx-2 toxin. It was diluted to 1mg/mL in 1x PBS for the stock solution, and a working solution was also made as a 1:250 dilution.

2.19. ELISA Blocking buffer

Blocking buffer was made using a 5% dehydrated skim milk solution. 5.0 g of dehydrated milk (Difco) was added to 100 mL of 1x PBS and mixed thoroughly. This solution was made fresh for each ELISA experiment to prevent contamination of the buffer.

2.20. ELISA Secondary antibody

The secondary antibody was STXPC-1, a pooled monoclonal IgG antibody specific to Stx. It was conjugated to horseradish peroxidase. This antibody was supplied from Toxin Technologies in liquid form. It was diluted 1:300 in blocking buffer for a working stock. It was stored at 4°C.

2.21. ELISA ABTS Peroxidase Substrate Buffer and Stop Solution

The ELISA substrate buffer was purchase from Pierce. The concentrated substrate solution (10x) was diluted in autoclaved d. H₂O to 1x stock. One tablet of the substrate was dissolved in the substrate buffer. A stop solution was also prepared as 1% SDS.

3. Cell culture media and reagents

3.1. Dulbecco's modified Eagle's minimal essential media (DMEM) with 3% bovine calf serum (BCS)

Dulbecco's modified Eagle's minimal essential media (DMEM) was prepared for use in vero cell culture. The media was prepared using the formulation below. After each of the components were added, the media was adjusted to pH 7.2 with 5M HCl (VWR). The liquid was sterile filtered under vacuum using a 0.2µm filter (Nalgene). Two 1-liter bottles were autoclaved at 121°C for 20 min prior to the preparation of this media, and served to hold the filtered media. The medium was incubated at 37°C overnight to check for sterility. Following incubation, bovine calf serum (BCS) was added to a final concentration of 3%. A second overnight 37°C sterility check was performed before final storage at 4°C.

Formulation for DMEM:

2 bottles of pre-measured powdered DMEM-4.5, high glucose with L-glutamine,
25mM HEPES buffer, without sodium pyruvate (Atlanta Biologicals)
7.40 g sodium bicarbonate (Fisher)
20 mL non-essential amino acids, 100x (Sigma)
2 L d.H₂O
60 mL bovine calf serum (Sigma)

3.2. Hank's balanced salt solution-modified (HBSS-mod)

Hank's balanced salt solution-modified (HBSS-mod) was prepared for use in vero cell culture. The media was prepared using the formulation below. After each of the components were added, the media was adjusted to pH 7.2 with 5M HCl (VWR). The liquid was sterile filtered under vacuum using a 0.2µm filter (Nalgene). Two 1-liter bottles were autoclaved at 121°C for 20 min prior to the preparation of this media, and served to hold the filtered media. The medium was incubated at 37°C overnight to check for sterility before final storage at 4°C.

Formulation for HBSS-mod:

2 bottles of pre-measured powdered HBSS-modified (No Ca²⁺, no Mg²⁺) (Atlanta Biologicals)
0.70 g sodium bicarbonate (Fisher)
2 L d.H₂O

3.3. Trypsin- ethylene diamine tetraacetic acid (trypsin-EDTA)

A trypsin-ethylene diamine tetraacetic acid (trypsin-EDTA) solution was made according to the formulation below. The solution was sterile filtered using a low protein binding 0.2µm filter (Pall) and transferred into sterile 15-mL conical tubes. The aliquots were stored at -20°C.

Formulation for trypsin-EDTA:

0.5 g trypsin (Sigma)
0.2 g EDTA (Sigma)
1000 mL HBSS-mod

3.4. Trypan blue (0.4%)

A solution of trypan blue was made in 1x PBS according to the recipe below. The solution was sterile filtered using a low protein binding 0.2µm filter (Pall). The solution was stored at room temperature.

Formulation for trypan blue solution:

0.04g trypan blue (Sigma)
100 mL 1x PBS

3.5. Mitomycin C (0.5µg/mL)

A solution of mitomycin C was prepared to a concentration of 0.5µg/mL. The solution was made according to the formulation below, and stored at 4°C, protected from light.

APPENDIX B

ORGANISM ACQUISITION AND VERIFICATION

1. Maintenance of Bacterial Strains

Two fully virulent strains of *E. coli* O157:H7 were used throughout the course of these experiments, and were acquired from Robert Mooney (UNH-Durham). Strain 43888 originated from the American Type Culture Collection (ATCC) and does not contain the genes for *stx1* or *stx2*. Strain 90-2380 from the National Laboratories for Enteric Pathogens (NLEP) produces Stx-2 only (not Stx-1).

Upon receipt, *E. coli* O157:H7 strains 90-2380 and 43888 were streaked onto trypticase soy agar (TSA), and aliquots of bacteria were cryopreserved in trypticase soy broth (TSB) with 15% glycerol at -20°C and then transferred to -80°C for long term storage. After 1 week of storage, viability and purity were confirmed by macroscopic and microscopic examination of colonies grown on TSA from thawed vials.

Stock cultures were streaked onto TSA slants from cryopreservation vials every four months. Each month, the TSA stock slant was used to streak two TSA plates for general use. This procedure was used to limit the amount of genetic variability caused by excess passaging of the organism. Due to the virulence of the organism and its toxin's classification as a Center for Disease Control and Prevention (CDC) Select Agent (in quantities greater than 100 mg), its growth and use in the laboratory was closely monitored, and toxin levels did not exceed 100 mg.

2. Verification of Strains by Biochemical Media and Tests

E. coli strains 90-2380 and 43888 were plated onto various selective and differential mediums to ensure that they exhibited the appropriate biochemical profile of *E. coli*. Mediums used include: Eosin methylene blue (EMB) agar plates, MacConkey (MAC) agar plates, Hektoen enteric (HE) agar plates, triple sugar iron (TSI) agar slants, Simmon's citrate (CIT) agar slants, motility-indole-ornithine (MIO) tubes, urea (URE) broth. An oxidase (OX) test was performed using colonies from a TSA plate. All mediums were incubated at 37°C for 24 h and observed.

The observed biochemical profiles of *E. coli* strains 90-2380 and 43888 are shown in Table B1. The typical biochemical profile for *E. coli* is included as a reference.

3. Verification of *stx2* gene by Polymerase Chain Reaction

In addition, the strains were tested to ensure that the designated toxin genes were present by way of Polymerase Chain Reaction (PCR) and agarose gel electrophoresis. PCR cycles consisted of an initial denaturation at 94°C for five min followed by 30 cycles of 94°C for 30 sec, 54°C for 30 sec and 72°C for 30 sec. A final extension at 72°C for seven min completed the amplification. Primers previously described by Wang, et al. were utilized and are described in Table B2. Following amplification, a 2.5% agarose gel was run and amplicons were visualized by ethidium bromide staining.

Table B1. Biochemical Profiles of *E. coli* Strains

Medium/Test	<i>E. coli</i> 90-2380	<i>E. coli</i> 43888	Expected Result
EMB	Growth, Lac +	Growth, Lac +	Growth, Lac +
MAC	Growth, Lac +	Growth, Lac +	Growth, Lac +
HE	Growth, Lac/Suc/Sal +	Growth, Lac/Suc/Sal +	Growth, Lac/Suc/Sal +
TSI	A/A, Gas+, H ₂ S-	A/A, Gas+, H ₂ S-	A/A, Gas+, H ₂ S-
CIT	Cit -	Cit -	Cit -
MIO	Mot +, Ind +, Orn +/-	Mot +, Ind +, Orn +/-	Mot +, Ind +, Orn +/-
URE	Ure -	Ure -	Ure -
OX	Ox -	Ox -	Ox -

Table B2. Primers used for *stx* gene detection.

Gene to Amplify	Primer Set	Amplicon Size
<i>stx1</i>	Stx1a: TCTCAGTGGGCGTTCTTATG Stx1b: TACCCCCTCAACTGCTAATA	338 bp
<i>stx2</i>	Stx2a: GCGGTTTTATTTGCATTAGC Stx2b: TCCCGTCAACCTTCACTGTA	115 bp

3.1. Results of Verification of *stx2* gene by Polymerase Chain Reaction

Strain 90-2380 was verified to contain only the genes *for stx2*. Strain 43888 was shown to lack genes for either *stx1* or *stx2*. Results are shown in Figure B1.

4. Eukaryotic Cell Cultures: Vero (African Green Monkey Kidney) Cells

4.1. Vero Cell Growth and Cryopreservation

African green monkey kidney (vero) cells were obtained from the American Tissue Culture Collection (ATCC) and are designated by number CCL-81. Vero cells are adherent epithelial cells and are extremely susceptible to Stx. Cellular morphology was observed to be consistent with vero cell morphology.

Vero cells were maintained in Dulbecco's modified Eagle's modified minimal essential medium (DMEM) with high glucose, L-glutamine, 25mM hepes, sodium bicarbonate (3.7g/L), non-essential amino acids, 5% fetal bovine serum (FBS), and grown at 37°C in a 7% CO₂ open system. Vero cells were passaged for 5 rounds before a new frozen aliquot was seeded as the new stock culture.

Following initial propagation of the vero cells vero cells were diluted to 1x10⁶ cells/mL in DMEM with 5% FBS. Cells were combined with 5% dimethylsulfoxide (DMSO) and 1mL aliquots were cryopreserved in liquid nitrogen.

4.2. Verification of Stx-2-mediated Toxicity on Vero Cells

Verification of Stx-2 production was performed by observation of cytopathic effects on vero cells when subjected to Stx-2-containing supernate. *E. coli* O157:H7

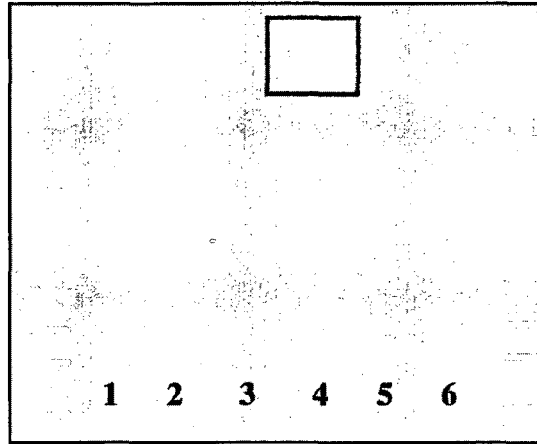


Figure B1. PCR Products of Stx Gene Amplification. 100 bp ladder, from 100 to 1000 bp. Wells 1-2, strain 43888 with *stx1* and *stx2* primers, respectively. Wells 3-4, strain 90-2380 with *stx1* and *stx2* primers, respectively. Wells 5-6, negative control with *stx1* and *stx2* primers, respectively. Note that *stx2* was amplified by the *stx2* primers in strain 90-2380. No amplification occurred in strain 43888.

90-2380 and *E. coli* O157:H7 43888 were grown overnight in TSB at 37°C while shaking. The cultures were centrifuged and the cells discarded. Supernates were sterile filtered (0.2µm low-protein binding filter, Pall) and applied to confluent vero cell monolayers in T₂₅ flasks and in 96-well plates. Cytopathic effects were monitored over the course of 24 h. Blind assays were conducted in which toxin-containing supernate was applied randomly to wells of a 96-well plate and was identified at 6, 12 and 24 h.

5. Statistics

Each experiment was run three times in triplicate (N=3). Statistical significance was determined using the T-test. The T-test is appropriate for use when comparing a control group to the test group. In the case of these experiments, induced cultures were compared to their uninduced controls as follows: Antibiotic-induced samples were compared to the no-antibiotic control; *E. coli* O157:H7 and lactobacilli co-culture were compared to the *E. coli*-only controls; Growth in glucose was compared to growth in glycerol; and growth with exogenous cAMP was compared to growth without additional cAMP. This test was employed for use in Real Time PCR assays, ELISA studies, MTT cytotoxicity assays, and the co-culture growth competition assays. Significance was rated on a one star (*) to three star (***) level (as indicated on each graph) with one star indicating a p value of less than 0.05, two stars indicating a p value of less than 0.005, and three stars indicating a p value of less than 0.0005.

APPENDIX C

PRIMERS AND PROBES

1. Maintenance of Oligonucleotide Primers

1.1. Design of Oligonucleotide Primers

Oligonucleotide primers and probes were designed using a combination of no-cost, online software. First, the DNA sequence of the gene of interest was found either by a BlastN search on the website <http://blast.ncbi.nlm.nih.gov/Blast.cgi>, or by a gene search on the website <http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi>. Secondly, the software “GeneFisher” on the website <http://bibiserv.techfak.uni-bielefeld.de/genefisher2/>, was used to find a preliminary set of possible primer sets. This software allows the user to input a DNA sequence and assign set parameters (primer length, amplicon length, melting temperature, etc.), and returns a list of possible primer sets. This program does not check for secondary structure of primers, so a third program was used to narrow the list of possible primers to the best set. Using the NetPrimer software on the website <http://www.premierbiosoft.com/netprimer/index.html>, the list of possible primers was checked for interfering structure until a satisfactory primer pair was discovered.

1.2. Acquisition of Oligonucleotide Primers

All primers were ordered through <http://www.idtdna.com>. Standard desalting purification method was ordered for all primer sets.

1.3. Resuspension of Oligonucleotide Primers

Once received, each oligonucleotide was resuspended in nuclease-free, sterile water to a final concentration of 100uM. This was deemed the main stock. That main stock was then diluted 1:5 in nuclease-free, sterile water to yield a 20uM working stock.

1.4. Storage of Oligonucleotide Primers

Resuspended oligonucleotides (both main and working stocks) were stored at -20°C between uses. The main stock was only defrosted to make new working stocks.

2. Primer Sets

2.1. Stx1A, Stx1B

Stx1A and Stx1B amplify a portion of the *stx1* gene in *E. coli* O157:H7. These primers were used to verify the presence of the *stx1* gene in newly-acquired *E. coli* O157:H7 strains.

Primer	Sequence 5' to 3'	Tm
Stx1A	TCT CAG TGG GCG TTC TTA TG	54.4°C
Stx1B	TAC CCC CTC AAC TGC TAA TA	52.4°C

2.2. Stx2A, Stx2B

Stx2A and Stx2B amplify a portion of the *stx2* gene in *E. coli* O157:H7. These primers were used to verify the presence of the *stx2* gene in newly-acquired *E. coli* O157:H7 strains. They were also used to check for genomic DNA contamination in extracted RNA samples.

Primer	Sequence 5' to 3'	Tm
Stx2A	GCG GTT TTA TTT GCA TTA GC	50.7°C
Stx2B	TCC CGT CAA CCT TCA CTG TA	52.6°C

2.3. Stx2operon-F, Stx2operon-R

Stx2operon-F and Stx2operon-R were designed based off primers by K. Moody (102). These primers were used to amplify a large portion (0.8kb) of the *stx2* operon, which was subsequently sequenced.

Primer	Sequence 5' to 3'	Tm
Stx2operon-F	AGA ATT CAG AGC GGG CGT TTT GAG CAG A	63.8°C
Stx2operon-R	AAG GAT CCC CGG CCG GGA TAA TAT TGT GAG TA	64.6°C

2.4. BigDinD-F, BigDinD-R

BigDinD-F and BigDinD-R were designed to amplify an approximately 0.8kb piece of DNA flanking the *dinD* gene in *E. coli* O157:H7. They were specifically

designed to not amplify in *L. plantarum* or *L. casei*. This amplicon was further purified and used as a standard in Real Time PCR experiments.

Primer	Sequence 5' to 3'	T_m
BigDinD-F	TGA ACG AAC ATC ATC AAC CTT T	52.7°C
BigDinD-R	AGC GAT TTG TGC GTA TTG TCA	55.2°C

2.5. SerCBigPiece1-R

SerCBigPiece1-F and SerCBigPiece2-R were designed to amplify an approximately 0.8kb piece of DNA flanking the *sercC* gene in *E. coli* O157:H7. They were specifically designed to not amplify in *L. plantarum* or *L. casei*. This amplicon was further purified and used as a standard in Real Time PCR experiments.

Primer	Sequence 5' to 3'	T_m
SerCBigPiece1-F	GGA CTA TGC CTG TAT TCG TT	52.0°C
SerCBigPiece2-R	AAT CGG ACG GGA AAG AAT G	52.6°C

3. Dual-labeled Probes

Dual-labeled probes were designed for use in Real Time PCR experiments. All probes were acquired, resuspended, and stored identically to oligonucleotide primers, as listed in this appendix. Probes were purchased already labeled with a 5' FAM fluorophore, and a 3' TAMRA quencher.

3.1. DinD Probe and Primers

The DinD probe and primers were used to quantify transcripts of *dinD* during Real Time PCR assays.

Probe	Sequence 5' to 3'	T _m
DinD-RT-Probe	FAM-CAA GCG TGC GAA GCC AGC AAT-TAMRA	61.4°C
DinD-F	GAA TTG GCA CCG TTA CTG GAT T	55.7°C
DinD-R	CGA AAT GGT CAG ATG CAG CTT	55.8°C

3.2. SerC Probe

The SerC probe and primers were used to quantify transcripts of *serC* during Real Time PCR assays.

Probe	Sequence 5' to 3'	T _m
SerC-RT-Probe	FAM-CGT GTC CGT CGA TTC TGG ATT-TAMRA	57.3°C
SerC-F	CGT CAT CGT CCG TGA AGA TTT	55.2°C
SerC-R	CAT GGA GCC GTT ATC GTT GAG	55.8°C

3.3. Stx2 Probe

The Stx2 probe and primers were used to quantify transcripts of *stx2* during Real Time PCR assays.

Probe	Sequence 5' to 3'	T_m
Stx2-RT-Probe	FAM-TGG TTC AAA TCC AGC AAG GGC CA-TAMRA	61.5°C
Stx2-F	AGA GCG AGC GAC TCA TAA TCG	56.6°C
Stx2-R	CTA ATG GCG GTA TGT GAT ATG G	53.0°C

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