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**VIRULENCE RESPONSE OF *ESCHERICHIA COLI* O157:H7 TO
BILE SALTS AND SHORT CHAIN FATTY ACIDS**

by

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A thesis
presented to Ryerson University

in partial fulfillment of the
requirements for the degree of
Master of Science

in the Program of
Molecular Science

Toronto, Ontario, Canada, 2008

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Abstract

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a gastrointestinal pathogen which causes hemorrhagic colitis and can lead to neurological damage, acute kidney failure (hemolytic uremic syndrome) and vascular lesions. During intestinal colonization EHEC is exposed to a variety of stresses including bile salts (BS) in the small intestine and short chain fatty acids (SCFA) in the large intestine; little is known about how these stresses affect this pathogen's virulence properties. The goal of this study was to investigate the impact of exposure of *E. coli* O157:H7 to physiologically relevant concentrations of BS and SCFA alone and in mixtures on bacterial survival, verotoxin production and adhesion to human epithelial cells. The results indicated that BS treatments significantly enhanced several virulence properties including survival and adhesion to human epithelial cell lines including colonic epithelial cells. Verotoxin production was not affected by any of the BS treatments. Bacterial pretreatment with erythromycin at a sub-minimal inhibitory concentration eliminated the adhesion enhancement after BS treatment, suggesting that protein synthesis was required for enhanced adhesion of BS treated organisms. Using the isogenic mutant of the known adhesins, intimin and iha it was established that there was no role for intimin or iha in the BS-induced adhesion enhancement. SCFA treatments reduced bacterial viability but significantly enhanced both adhesion to human epithelial cells and verotoxin production. The results of this research indicate that ingestion stresses such as BS and SCFA, which are part of the host's natural chemical assault on foreign organisms, may actually enhance the virulence properties of this pathogen and contribute to, rather than, prevent infection. Furthermore, they suggest that this pathogen may use these ingestion stresses to cue the expression of numerous virulence factors for successful infection of local microenvironments.

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Table of Contents

1	Introduction.....	1
1.1	Enterohemorrhagic <i>Escherichia coli</i>	1
1.2	EHEC Virulence Factors	3
1.2.1	Shiga Toxins/Verotoxins	5
1.2.2	Adherence to Host Cells	9
1.2.3	LEE Pathogenicity Island	11
1.2.4	Type Three Secretion System	12
1.2.5	TTSS Effectors	13
1.2.6	Non LEE Encoded Effectors	13
1.2.7	EHEC Adherence Factors.....	15
1.2.7.1	Fimbrial Adhesins.....	15
1.2.7.2	Nonfimbrial Adhesins.....	16
1.2.8	pO157:H7 Plasmid.....	18
1.2.9	Regulation of the EHEC Virulence Genes	19
1.3	Modulation of Host Cell Function.....	20
1.4	Disruption of the Barrier Function	20
1.4.1	Factors Contributing to Diarrhea	21
1.4.2	Host Cell Cytoskeletal Rearrangements	21
1.5	Impact of Ingestion Stress on Virulence.....	24
1.6	Bacterial Response to Bile Salts	24
1.6.1	Bile Composition and Function	24
1.6.2	Antimicrobial Actions of Bile	26
1.6.3	Bile Tolerance.....	26
1.6.4	Genetics of Bacterial Bile Tolerance	27
1.6.5	Bile Sensing and Regulation of Bile Stress Response.....	28
1.6.6	Bile and Pathogenesis.....	28
1.7	Short Chain Fatty Acid Stress Response	30
1.7.1	Short Chain Fatty Acid Composition and Function	30
1.7.2	Short Chain Fatty Acid Tolerance and Pathogenesis	31

1.8	Stress and EHEC Virulence.....	32
1.9	Purpose of Investigation and Hypothesis	33
1.9.1	Research Rationale	33
1.9.2	Hypothesis	34
1.9.3	The Main Objectives of this Thesis	34
2	Materials and Methods.....	35
2.1	Bacterial Cultivation.....	35
2.2	Tissue Cell Culturing.....	36
2.3	Bile Salt Treatment of <i>E. coli</i> O157:H7	36
2.4	Short Chain Fatty Acid Stress of <i>E. coli</i> O157:H7	38
2.5	Percent Survival of Treated <i>E. coli</i> O157:H7	39
2.6	Bacteria- Host Cell Adhesion Assay: Colony Plate Count Method	39
2.7	Impact of Erythromycin-Mediated Inhibition of Protein Synthesis on Bacteria- Host Adhesion	41
2.8	Bile Salt/Short Chain Fatty Acids Treated <i>E. coli</i> O157:H7 86-24 Growth during Infection Time	41
2.9	Qualitative Microscopic HEP-2 cell Adhesion Assay after Exposure to Bile Salts/Short Chain Fatty Acids.....	42
2.10	Vero Cell Cytotoxicity Assays	43
2.11	Statistical Analysis.....	44
3	Results.....	45
3.1	Bile Salt Treatment Enhances <i>E. coli</i> O157:H7 Survival.....	45
3.2	Bile Salt Treatment Enhances <i>E.coli</i> O157:H7 Adhesion to Human Epithelial Cells	46
3.3	Erythromycin Treatment Abrogates EHEC O157:H7 Adhesion Enhancement..	48
3.4	No Role of Intimin in Enhanced Adhesion of BS Treated EHEC O157:H7.....	49
3.5	No Role of Iha in Enhanced Adhesion of Bile Salt Treated EHEC O157:H7 ...	52
3.6	Short Chain Fatty Acids Decrease <i>E. coli</i> O157:H7 Viability	55
3.7	Short Chain Fatty Acids Stress Enhances <i>E. coli</i> O157:H7 Adhesion to Human Epithelial Cells.....	56

3.8	Erythromycin Treatment Reduces Short Chain Fatty Acids Host Adhesion Enhancement.....	58
3.9	The Effect of Bile Salts and Short Chain Fatty Acids Stress on <i>E. coli</i> O157:H7 86-24 Growth during Infection Time	59
3.10	No Change in Secreted and Periplasmic VT Production after BS Treatment	61
3.11	VT Levels Increased after SCFA Mixture.....	66
4	Discussion	70
4.1	Effect of Intestinal Stress on <i>E. coli</i> O157:H7 Survival: Summary	70
4.1.1	Bile Salt Mix Treatment Enhances <i>E. coli</i> O157:H7 Survival	70
4.1.2	Short Chain Fatty Acids Treatment Reduces <i>E. coli</i> O157:H7 Survival...72	
4.2	BS Mix Treatment Enhances <i>E. coli</i> O157:H7 Adhesion to Epithelial Cells	73
4.3	Short Chain Fatty Acid Stress Enhances Adhesion of <i>E. coli</i> O157:H7 to Epithelial Cells.....	77
4.4	The Effect of Bile Salts and Short Chain Fatty Acids Treatments on <i>E. coli</i> O157:H7 Growth during Infection Time	78
4.5	The Effect of Bile Salts and Short Chain Fatty Acids Treatment on <i>E. coli</i> O157:H7 86-24 Verotoxin Production:	79
4.5.1	No Change in Secreted and Periplasmic VT Production after Bile Salt Treatment.....	80
4.5.2	Increased VT Levels after Short Chain Fatty Acids Mixture Stress	81
5	Summary and Conclusions	82
5.1	Summary of Results.....	82
5.2	Future Work.....	84
5.3	Significance and Conclusions.....	85
6	Supplementary Data	87

List of Tables

Table 1-1 Bile Salts in Duodenum	25
Table 2-1 Bacterial Strains Used in this Study	35
Table 2-2 Cell Lines Used in This Study	36
Table 2-3 Bile Salt Experimental Treatments	38
Table 2-4 Short Chain Fatty Acid Stress Experimental Treatments	39
Table 6-1 Supplemental Data 1	87
Table 6-2 Supplemental Data 2	88

List of Figures

Figure 1.2-1 EHEC genes involved in pathogenesis.	4
Figure 1.2-2 Ribbon crystal structure of <i>E. coli</i> O157:H7 Stx2.	6
Figure 1.2-3 Simplified cartoon of the mode of action of Stx. B.	8
Figure 1.2-4 Model for EHEC Adhesion to Host Cells.	10
Figure 1.2-5 Genetic organization of LEE genes in EHEC.	12
Figure 1.2-6 Simplified cartoon model for EHEC translocation of LEE encoded and other proteins into the host cell cytosol.	14
Figure 1.4-1 EPEC and EHEC translocate effector proteins into the host cell cytosol.	23
Figure 3.1-1 BS Treatment Enhances EHEC O157:H7 Survival	45
Figure 3.2-1 BS Treated <i>E. coli</i> O157:H7 86-24 Show Enhanced Adhesion to HEp-2 Cells	46
Figure 3.2-2 Qualitative Comparison of BS Treated <i>E. coli</i> O157:H7 86-24 Adhesion to HEp-2 Cells	47
Figure 3.3-1 Erythromycin Abrogates <i>E. coli</i> O157:H7 86-24 Adhesion Enhancement to Caco-2 Cells.	49
Figure 3.4-1 BS Treated <i>E. coli</i> O157:H7 86-24 <i>eae</i> Δ 10 Show Enhanced Adhesion to HEp-2 Cells	51
Figure 3.4-2 BS Treated <i>E. coli</i> O157:H7 8624 <i>eae</i> Δ (pEB310) Show Enhanced Adhesion to HEp-2 Cells.	52
Figure 3.5-1 BS Treated <i>E. coli</i> O157:H7 86-24 Δ <i>iha</i> Show Enhanced Adhesion to HEp-2 Cells	53
Figure 3.5-2 BS Treated <i>E. coli</i> ORN 172(pIHA) and (pSK+) Adhesion to HEp-2 Cells	55
Figure 3.6-1 SCFA Treatment Decreases EHEC O157:H7 86-24 Survival	56
Figure 3.7-1 SCFA Shocked <i>E. coli</i> O157:H7 86-24 Show Enhanced Adhesion to HEp-2 Cells	58
Figure 3.9-1 The Effect of BS Treatment on <i>E. coli</i> O157:H7 86-24 Viability during 4 h Incubation	60

Figure 3.9-2 The Effect of SCFA Stress on <i>E. coli</i> O157:H7 86-24 Viability during 4 h Incubation	61
Figure 3.10-1 Secreted Verotoxin Production of <i>E. coli</i> O157:H7 86-24 after Exposure to BS	63
Figure 3.10-2 Periplasmic Verotoxin Production of <i>E. coli</i> O157:H7 86-24 after Exposure to BS	64
Figure 3.10-3 Initial and Final Bacteria Concentrations of BS Treated <i>E. coli</i> O157:H7 86-24 during Verotoxin Production	65
Figure 3.11-1 Secreted Verotoxin Production of SCFA Stressed <i>E. coli</i> O157:H7 86-2467	
Figure 3.11-2 Secreted Verotoxin Production of SCFA Stressed <i>E. coli</i> O157:H7 86-2468	
Figure 3.11-3 Initial and Final Bacteria Concentrations of SCFA Stressed <i>E. coli</i> O157:H7 86-24 during Verotoxin Production	69

Abbreviations

A/E	Attaching and effacing lesions
AcrAB pump	Drug efflux pumps
ATCC	American Type Culture Collection
BS	Bile salts
CaCo-2	Colonic adenocarcinoma cells
CFU	Colony forming units
CT	Cholera toxin
Da	Daltons
dd H ₂ O	Distilled deionized H ₂ O
DMEM	Dulbecco's MEM
EDTA	Ethylenediaminetetraacetic acid
Efa-1	EHEC factor for adherence
EHEC	Enterohemorrhagic Escherichia coli
FBS	Fetal bovine serum
Gb-3	Glycolipid globotriaosylceramide-3 receptor
GI	Gastrointestinal tract
HC	Hemorrhagic colitis
HEp-2	Laryngeal carcinoma cells
HUS	Hemolytic-uremic syndrome
HUVEC	Human umbilical vein endothelial cells
Iha	IrgA homologue adhesion
LB	Luria-Bertani
LEE	Locus of enterocytes effacement LEE
Ler	LEE-encoded regulator
LPS	Lipopolysaccharide
MEM	Minimal essential media
MLC	Myosin light chain
NaCDC	Chenodeoxycholate

NaDOC	Deoxycholate
NaGIC	Glycholate
NaUDC	Ursodeoxycholate
N-WASP	Neuronal Wiskott-Aldrich syndrome protein
OmpA	Outer membrane protein A
OmpF	OmpC Porin proteins
PAI	Large pathogenicity island
PBS	Phosphate buffered saline
perC	Plasmid encoded regulator
PKC	Protein kinase C
RpoS	Stress response regulon
Saa	Autoagglutinating adhesion
SCFA	Short chain fatty acids
SFP	Sorbitol fermenting EHEC O157 fimbriae plasmid
STEC	Shiga toxin producing E. coli
Stx	Shiga toxin
TccP	Tir-cytoskeleton coupling protein
TCP	Production and toxin-coregulated pilus
TCSs	Two-component system
Tir	Transmembrane intimin receptor
Tir	Translocated intimin receptor
ToxT	Transcription factor
TTSS	Type III secretion system
Vero cells	African Green Monkey Kidney cells
VT	Verotoxin
wt	Wild type
σ B	Sigma B transcriptional regulator
σ S	RpoS transcriptional regulator

1 Introduction

1.1 Enterohemorrhagic *Escherichia coli*

Enterohemorrhagic *Escherichia coli* (EHEC) are food-borne pathogens which primarily colonize the human intestinal tract. They are Gram-negative bacteria that belong to the Shiga toxin producing *E. coli* (STEC) family. EHEC can cause a wide range of illnesses ranging from mild diarrhea to hemorrhagic colitis (HC) which can progress to several life threatening diseases including hemolytic-uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (Wells *et al.*, 2008). *E. coli* strain O157:H7, designated by the somatic antigen O and the flagellar antigen H, was first recognized as a pathogen in 1982. It is the most well studied serotype of the group since it is most commonly associated with severe EHEC infections and also has been linked to outbreaks of illness worldwide. EHEC infections lead to a high proportion of deaths due to HUS (with mortality rate of this complication of 3-17%), and cause a great deal of morbidity with many surviving individuals experiencing chronic renal insufficiency and neurological damage (Nataro & Kaper, 1998).

The production of Shiga toxin (Stx) and the presence of a pathogenicity island that encodes the locus of enterocytes effacement (LEE) are virulence factors that enable *E. coli* O157:H7, to cause the severe gastrointestinal infections with the potential life-threatening complications (Wells *et al.*, 2008). Nonpathogenic *E. coli* strains do not possess these genetic characteristics that may contribute to specific virulence traits or to successful survival and fitness of the bacteria in the host (Hacker & Kaper, 2000). Cattle have been identified as the most important reservoir of EHEC/STEC (Naylor *et al.*, 2003; Low *et al.*, 2006). Common routes of transmission include fecal transmission and contaminated food or water (Caprioli *et al.*, 2005; Ceponis *et al.*, 2005). Although most outbreaks are associated with undercooked ground beef, recently many of sources of infection have originated from vegetables including lettuce, spinach, cabbage, sprouts or tomatoes (Borczyk *et al.*, 1987). Other outbreaks have also been reported after the consumption of contaminated fruits, water or among persons visiting farms, petting zoos

and via direct contact in hospital and daycare settings (Louie *et al.*, 2000). EHEC is a highly efficient pathogen in humans since the infective dose is estimated to be only approximately 50-100 organisms (Griffin and Miner, 1995). The ability of EHEC to persistently colonize the gastrointestinal tract (GI) relies on its capability to sense and respond to many stressful environments (Maroncle *et al.*, 2006). *E. coli* O157:H7 attachment to the epithelial cells is the first critical step in colonization and is mediated by bacterial adhesins. In order to colonize the human colon, EHEC initially adheres non-intimately, followed by intimate attachment to the host cell (Torres *et al.*, 2005b).

It has been proposed that initial attachment of EHEC to the epithelial cells is mediated by EspA filaments as well as fimbrial adhesions, including LP fimbriae (Kenny, 1999). The Esp effector proteins and transmembrane intimin receptor (Tir) are translocated via the multicomponent apparatus type III secretion system (TTSS) into the human host. This initial adherence of the bacteria to the target cells is followed by the intimate attachment which is associated with the binding of the bacterial adhesin intimin to the Tir (Kenny, 1999).

The ability of *E. coli* O157:H7 to promote the development of attaching and effacing (A/E) lesions has been attributed to the presence of a pathogenicity island on the bacterial genome. The LEE pathogenicity island encodes the genes products responsible for the generation of A/E lesions on intestinal epithelial cells (Elhanafi *et al.*, 2004). Apart from the LEE encoded proteins, several adhesins including adherence conferring molecule (Iha) (Tarr *et al.*, 2000), long polar fimbriae (lpf) (Torres *et al.*, 2002), curli (Uhlich *et al.*, 2001) and F9 fimbriae (Dziva *et al.*, 2004; Low *et al.*, 2006) are associated with adherence to mammalian cells. The production of Stx (verocytotoxin) is one the major virulence characteristics of EHEC since the toxins are associated with development of HC and HUS due to EHEC infections (Law, 2000; Caprioli *et al.*, 2005). HUS arises as a consequence of microvascular disease triggered by the toxins entering the bloodstream and binding to the receptor Gb3 of kidney and brain cells (Spear *et al.*, 2006).

In spite of significant advances made in understanding EHEC pathogenesis, we still do not fully appreciate the impact of environmental stresses on virulence factor production and the influence this may exert on pathogenesis. Following consumption, the

acidity of the stomach, is a major barrier encountered by bacteria. The EHEC that survive the extreme low pH then transit on to the intestines, where they must overcome a different set of biological barriers including bile salts in the small intestine and short chain fatty acids in the large intestine. As a consequence of these extreme environmental changes, the bacteria must adapt physiologically and morphologically in order to survive. Questions on whether these changes influence major virulence properties, including host cell adhesion and verotoxin production, remain to be answered. To properly address these questions a multidisciplinary approach that includes molecular, cellular and microscopic techniques to identify pathogen-induced changes in infected cells will be needed. **The research presented in this thesis will focus on the impact of bile salt and short chain fatty acid stress on the virulence of EHEC.** The literature review will provide details of the pathogenic mechanism as well as known and postulated virulence factors, followed by a review of the effect of BS and SCFA stress on virulence of other related gastrointestinal pathogens.

1.2 EHEC Virulence Factors

The ability of *E. coli* O157:H7 to cause a spectrum of human illness, including HC and HUS has been attributed to the production of Shiga toxins (Stx) and the presence of a large pathogenicity island (PAI) (Wells *et al.*, 2008). Genes involved in EHEC pathogenesis are presented in Figure 1.2-1. The pathogenicity island, termed the locus for enterocyte effacement (LEE), is a large multi-gene segment which encodes intimin (bacterial adhesin), its receptor Tir (translocated intimin receptor), a type III secretion machine and secreted proteins (EspA, EspB, EspD) necessary for the formation of attaching-and-effacing (A/E) lesions on intestinal epithelial cells (Elhanafi *et al.*, 2004). The two primary classes of Stx (Stx1 and Stx2) are encoded by two type λ lysogenic phages inserted in the bacterial genome (Elhanafi *et al.*, 2004). In addition to Stx, the pO157 plasmid is known to encode the enterohemolysin (ehx) as well as a catalase peroxidase (katP), a zinc metallo protease (StcE), a serine protease (Esp) and a large toxin (ToxB) that contribute to EHEC pathogenesis (Herold *et al.*, 2004).

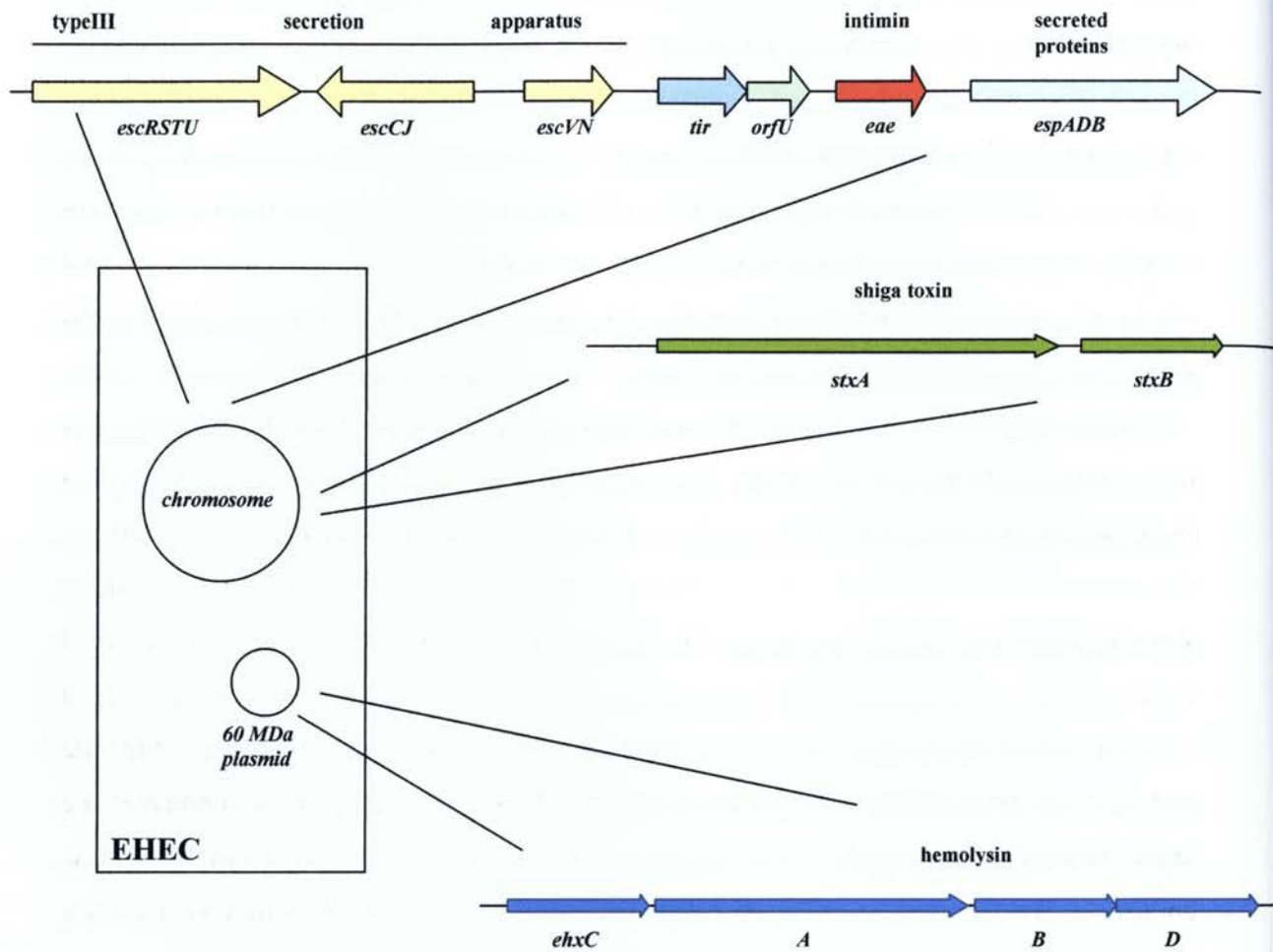


Figure 1.2-1 EHEC genes involved in pathogenesis.

Genes involved in EHEC pathogenesis are similar to those implicated in EPEC, except for the presence of the Stx-encoding phage on the EHEC chromosome and the presence of a EHEC plasmid which is known to encode enterohemolysin and other proteins involved in EHEC pathogenesis. Figure adapted from Nataro & Kaper, 1998.

1.2.1 Shiga Toxins/Verotoxins

One of the major pathogenicity factors of EHEC is the production of Shiga toxins (Stx), which are responsible for the major symptoms of HC and a sequela called hemolytic uremic syndrome (HUS) (Kaper *et al.*, 1998). Stxs act to inhibit the synthesis of host cell proteins and contribute to vascular complications of HC. HUS arises as a consequence of microvascular disease triggered by the toxins entering the bloodstream by clathrin-dependent endocytosis and binding to a cell surface receptor of kidney and brain cells (Hoey *et al.*, 2003). HUS is characterized by thrombocytopenia, hemolytic anemia and kidney lesions which can lead to a chronic renal failure and even death (Spear *et al.*, 2006).

EHEC strains may produce two major toxins Stx1 and Stx2 which are related to Stx from *Shigella dysenteriae* serotype 1 (O'Loughlin & Robins-Browne, 2001; Garmendia *et al.*, 2005), however, Stx2 is more frequently associated with HUS. The Stx1 form is highly conserved and homologous to Stx with only one amino acid difference in the A subunit. The Stx1 and Stx2 A and B subunits are 68 and 73% similar at the amino acid level, respectively, and the crystal structures of Stx and Stx2 are remarkably similar. In contrast, Stx2 has numerous variants including Stx2c, Stx2d, Stx2e, and Stx2f which are distinguished by the receptor to which they bind, thus contributing to the ability of the toxin to target different cells (Fraser *et al.*, 2004). The Stx/Stx1 and Stx2 differ from one another by the fact they are serologically distinct. The polyclonal antiserum to one toxin group shows no cross neutralization of the cytotoxicity of the other toxin (Strockbine *et al.*, 1986; Wen *et al.*, 2006).

Other studies have also shown that more severe infections are often associated with the expression of additional factors encoded on the LEE island and the plasmid pO157 (Nataro & Kaper, 1998). Stx toxins are encoded by *stxA* and *stxB* genes which are found on the lysogenic bacteriophages and expressed at neutral pH conditions similar to those of the intestinal tract as VT could be detected in faeces in natural and experimental infections (Karmali *et al.*, 1983; Ramotar *et al.*, 1995; Rahn *et al.*, 1996), cattle (Montenegro *et al.*, 1990; Ball *et al.*, 1994; Hyatt *et al.*, 2001), pigs (Cornick *et al.*, 2000), and mice (Isogai *et al.*, 2000; Zhang *et al.*, 2000).

The expression of the *stx* genes is thought to be under the control of the *E. coli* SOS response which is induced by elevated level of intracellular single stranded DNA (Fraser *et al.*, 2004).

Stx is an holotoxin (approximately 70 kilodaltons) formed by five identical B subunits that surround a single A subunit. A model of Stx2 is presented in Figure 1.2-2. Once produced in the lower intestine, Stx translocates across the intestinal epithelium, reaches the blood and targets cellular receptors (Paton & Paton, 1998).

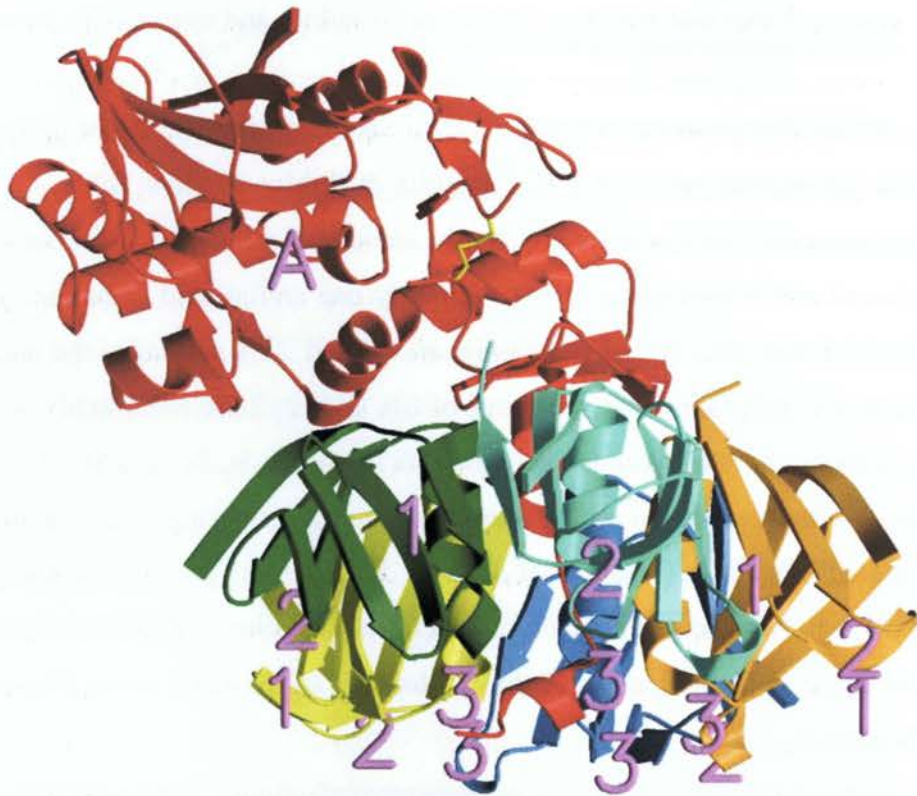


Figure 1.2-2 Ribbon crystal structure of *E. coli* O157:H7 Stx2.

The A-subunit is red, whereas the B-subunits are orange (chain B), cyan (chain C), green (chain D), yellow (chain E), and blue (chain F). The active site in the A-subunit is marked by the magenta letter A. The side chains of the cysteine residues that link A1 and A2 are depicted in yellow. The sites equivalent to the Gb3-binding sites on the B-pentamer of Stx1 are shown by magenta numbers that distinguish the type of binding site. Reproduced from Fraser *et al.*, 2004.

A simplified model which highlights the mode of action of Stx is presented in Figure 1.2-3. The B subunit (7.7 kDa) is implicated in the binding to the glycolipid globotriaosylceramide-3 (Gb-3) receptor of endothelial cells referred as CD77. Following internalization, the toxin is transported to the trans Golgi network (Schmitt *et al.*, 1999). Retrograde transport of toxin from the Golgi to the endoplasmic reticulum may also be required for the action of toxin to the cells (Sandvig *et al.*, 1992; Sandvig *et al.*, 1994). The A subunit of the toxins (32 kDa) is implicated in the inhibition of host protein synthesis and also activation and secretion of chemokines, leading to activation of cells of the immune system (Hoey *et al.*, 2003). As result, the integrity of the epithelial barrier is altered and the translocation of Stx across the mucosa to underlying tissues is facilitated (Gobert *et al.*, 2007). Shiga toxins also induce a cascade of responses including elevated levels of pro-apoptotic proteins, mitochondrial dysfunction and activation of apoptosis caspase cascades that lead to apoptosis in endothelial cells (Hoey *et al.*, 2003). After translocation through the gut mucosa, Stxs first enter the bloodstream to reach the endothelial cells particularly those in the gut, kidney and occasionally other organs (Caprioli *et al.*, 2005). Once the endothelial cells of the target organs are compromised, the toxins act on glomerular and brain microvascular endothelial cells and activate prothrombic and pro-inflammatory cascades, which lead to HUS and complications of the central nervous system (Caprioli *et al.*, 2005). Shiga toxins are cytotoxic to human renal endothelial cells. The characteristic renal histopathology associated with Stx includes swollen glomerular endothelial cells and platelet and fibrin deposition within the glomeruli (Caprioli *et al.*, 2005). It is a decrease in the rate of glomerular filtration that causes the acute renal failure associated with HUS (Paton & Paton, 1998). One report in the literature showed that Stxs contribute to increased adherence to epithelial cells by increased surface expression of nucleolin and $\beta 1$ integrin which interact with intimin (Robinson *et al.*, 2006). Their findings indicate that these eucaryotic proteins are present on the luminal surface of intestinal epithelia and are potentially accessible as receptors for intimin during EHEC O157:H7 infection (Robinson *et al.*, 2006). These data are consistent with another study by Barnett Foster *et al.* (2000) which reported that Stx upregulates the level of phosphatidyletanolamine (PE) outer membrane on the host cell which they identified as a candidate host receptor previously reported to bind EHEC.

Recently, another study reported that Stx subverts the inflammatory response of human enterocytes by decreasing NF- κ B (Gobert *et al.*, 2007). These results highlight the possibility that Stx is an unrecognized inhibitor of the epithelial cell innate immune response (Gobert *et al.*, 2007). Together these findings underline an essential role for Stx in the initial step of colonization of the intestinal mucosa. Some studies support the notion that most probably, the production of toxin alone is most likely insufficient to cause disease. Stx (both Stx1 and Stx2) when added to colonic epithelial cells, were shown to move across the cell layer without any cytotoxic effects, however when STEC strains were incubated with the cell monolayer, toxin(s) were transported through the cells at the same time and the cell monolayer was destroyed (Law, 2000). These observations suggest that STEC strains may produce other potent toxic products and/or there are possibly other bacterial factors that enhance Shiga toxin activity (Law, 2000).

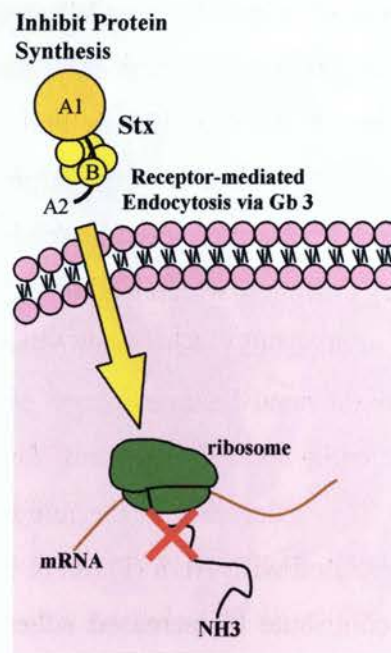


Figure 1.2-3 Simplified cartoon of the mode of action of Stx. B.

Inhibition of protein synthesis by Shiga toxins (Stx). Holotoxin, which consists of an enzymatically active (A) subunit and five binding (B) subunits, enters cells through the globotriacylglyceride (Gb3) receptor. The N-glycosidase activity of the A subunit then cleaves an adenosine residue from 28S ribosomal RNA, which halts protein synthesis. Figure adapted from Schmitt *et al.*, 1999.

1.2.2 Adherence to Host Cells

For successful establishment of infection, *E. coli* O157:H7 use some remarkable strategies to adhere to host epithelial cells, survive peristaltic clearance and obtain access to derived nutrients of the distal ileum, cecum and colon. *E. coli* O157:H7 adhesion to the epithelial cells is an essential step in the diarrheal infection. EHEC adheres initially non-intimately to the intestinal epithelium surface and then intimately to enhance its attachment (Torres *et al.*, 2005a). It has been proposed that initial attachment of EHEC to the epithelial cells is mediated by EspA filaments and fimbrial adhesions including long polar fimbria (Lpf) (Kenny, 1999). Following initial adherence, EHEC translocates various Esp effector proteins and Tir into the host via TTSS (Kenny, 1999). The initial adherence of the bacteria to the host cells is followed by the intimate attachment which is associated with the binding of the bacterial non-fimbrial adhesin (intimin) to its own injected receptor Tir (Kenny, 1999). After export to the surface, intimin is then integrated into the bacterial outer membrane with the amino-terminal region and the carboxy-terminal region of the polypeptide exposed to the surface (Adams *et al.*, 2005). From the bacterial surface, intimin projects a cell binding region which is positioned to contact the Tir (Adams *et al.*, 2005). Apart from its interaction with Tir, intimin also recognizes and binds to host cell receptors such as integrin and nucleolin (Donnenberg *et al.*, 1993; McKee *et al.*, 1995; Robinson *et al.*, 2006).

By adhering intimately to intestinal epithelial cells, EHEC has the capacity to induce a histopathological feature known as A/E lesions. A/E lesions are distinguished by loss of intestinal microvilli, rearrangement of the cytoskeleton and formation of pedestal like structures that cup the bacterium (Campellone & Leong, 2003; Caprioli *et al.*, 2005). The proteins required for the formation of A/E lesions are encoded by genes located on the chromosomally-encoded LEE pathogenicity island (Hayashi *et al.*, 2001; Perna *et al.*, 2001; Torres *et al.*, 2005a). The LEE of EHEC consists of genes responsible for the expression of TTSS, intimin, Tir and secreted proteins such as EspA, EspB, EspD, EspG and Map (Torres & Kaper, 2001; Spear *et al.*, 2006). The TTSS is required for the translocation of various proteins (Esp) including Tir into the host cells. Once Tir has been translocated into the host cell, Tir moves to the plasma membrane where it forms a

hairpin loop structure (Garmendia *et al.*, 2005). While the amino- and carboxy-terminal domains are located within the cell, its central region is located at the surface of the host cell and allows binding of intimin (Garmendia *et al.*, 2005). The cytosolic domains of Tir interact with cytoskeleton proteins which lead to formation of actin pedestal structures (Garmendia *et al.*, 2005). A model for the early and later events associated with EHEC adherence to host cells is presented in Figure 1.2-4.

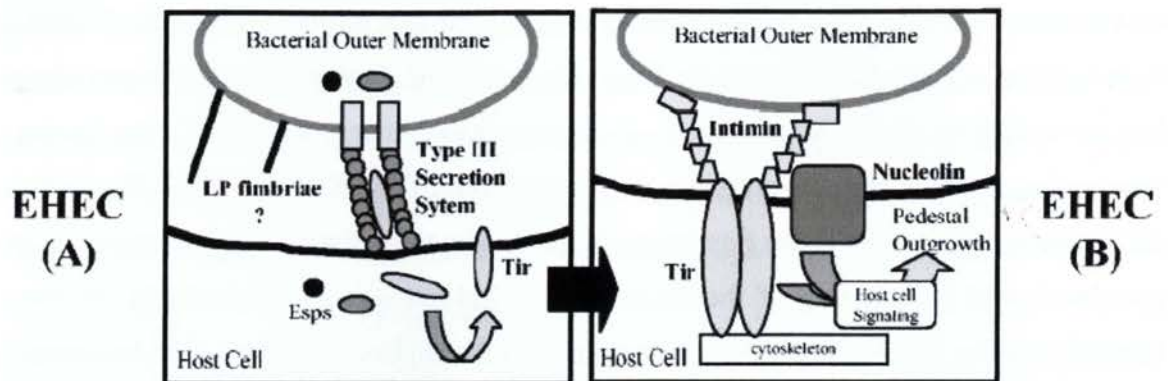


Figure 1.2-4 Model for EHEC Adhesion to Host Cells.

A: Early events in EHEC adhesion to host cells. EspA filaments of the TTSS and other possible adhesions such as LP fimbriae, mediate initial attachment of the bacteria to host cells. Various effector proteins (Esp), including Tir, are translocated into the host cell via the TTSS. Tir localizes to host plasma membrane and takes on a hairpin loop structure. B: Later events in EHEC adhesion to host cells. Intimate adhesion of the bacteria to the host cell is mediated by the binding of the bacterial adhesin intimin to its receptor Tir. Intimin also binds to a host cell receptor, nucleolin. Translocated effector proteins, including Tir, trigger cytoskeleton rearrangements, disruption of the epithelial barrier, cytotoxicity and are responsible for attaching and effacing (A/E) lesion formation, activating and inhibiting signal transduction pathways in host cells which can ultimately lead to disease. Reproduced from Torres *et al.*, 2005b with the permission of *Infection and Immunity*, American Society for Microbiology.

1.2.3 LEE Pathogenicity Island

Most of the genetic elements responsible for the formation of A/E lesions are encoded within a 43 kb pathogenicity island in the O157:H7 chromosome, named the locus of enterocyte effacement (LEE) (Nataro & Kaper, 1998; Caprioli *et al.*, 2005; Garmendia *et al.*, 2005). EHEC are a subgroup of the Shiga-toxigenic *E. coli* (STEC) pathogroup which includes strains that produce Stx but may contain the LEE region or not. LEE negative EHEC strains have been associated with some cases of HUS, indicating that the LEE region is not essential for pathogenicity and that additional factors are involved in causing disease (Paton *et al.*, 2001). The LEE region includes 5 major operons: *LEE1*, *LEE2*, *LEE3*, *tir* (*LEE5*), and *LEE4* (Garmendia *et al.*, 2005). A model of the LEE region that highlights the organization of the genes is presented in Figure 1.2-5. The 41 genes of LEE encode structural components of the TTSS, intimin, Tir, secreted proteins (EspA, B and D) and other TTSS structural proteins, translocators and effectors. The LEE operons are directly activated by the LEE-encoded regulator (Ler), which is the first gene in the *LEE1* operon (Kaper *et al.*, 2004). Regulation of LEE gene expression is complex and is under coordinated activities of three regulatory elements: Ler, GrlR and GrlA (Bustamante *et al.*, 2001). Ler is a central activator necessary for the transcription of most of the LEE genes that is further positively and negatively modulated by GrlA and GrlR. The negative effect of GrlR on LEE expression depends on the GrlA function (Goldberg *et al.*, 2001). Since recent studies have shown that LEE expression is under the control of various regulatory elements outside the locus, its regulatory mechanisms are complicated and require more research for clarification (Grant *et al.*, 2003; Kaper *et al.*, 2004).

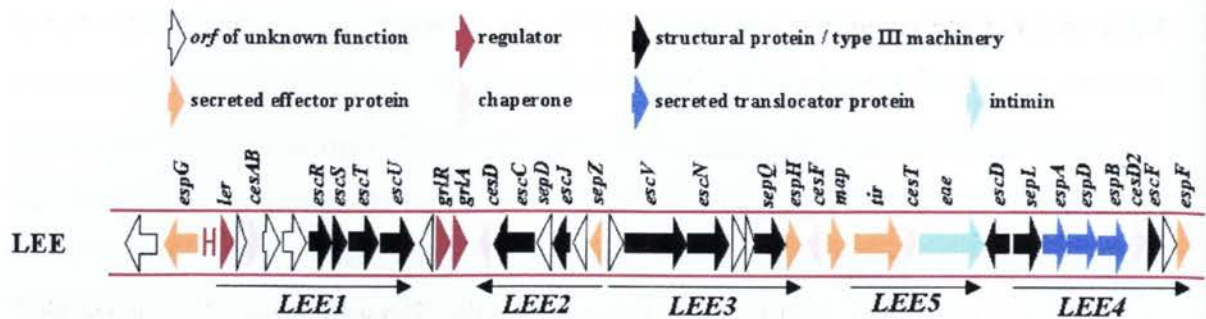


Figure 1.2-5 Genetic organization of LEE genes in EHEC.

The image was generated using coliBase (<http://colibase.bham.ac.uk/>). Reproduced from Garmendia *et al.*, 2005 with the permission of *Infection and Immunity*, American Society for Microbiology.

1.2.4 Type Three Secretion System

In order to sabotage the cellular functions and establish the disease, *E. coli* O157:H7 uses highly sophisticated macromolecular syringes on its surface called type III secretion systems (TTSS). The TTSS apparatus, remarkable similar to a flagellar hook basal body complex, is composed of three distinct parts: a hollow extracellular needle, a cylindrical base body (which crosses two membranes and stabilizes the whole structure upon the cell envelope and bulb) and bulb (like the flagellar cytosolic complex composed of an ATPase and other proteins (Garmendia *et al.*, 2005). EHEC uses this structure to penetrate and inject its toxins into host cells (Tampakaki *et al.*, 2004; Pizarro-Cerdá & Cossart, 2006). The basal body is composed of the secretin EscC and EscR, EscS, EscT, EscU and EscV which form the inner membrane proteins (Gauthier *et al.*, 2003). The lipoprotein EscJ bridges the inner and outer membrane ring structures and EscF forms the needle complex of the TTSS (Wilson *et al.*, 2001). EspA, EspB and EspD together constitute a needle like structure; EspA subunits polymerize to the tip to form the EspA filament and EspB and EspD make up the translocon pore (Delahay *et al.*, 1999). SepD and SepL are cytoplasmic components of this system, and it has been suggested that these proteins may play a role in “switching” the type of proteins secreted through the TTSS, from translocator proteins to effector proteins (O’Connell *et al.*, 2004). Cytoplasmic

ATPases from the bacterial F_0F_1 ATPase provides energy to the system and promotes protein secretion (Garmendia *et al.*, 2005).

1.2.5 TTSS Effectors

Once the molecular syringe has been formed, the effector proteins are translocated into the host cell cytosol where they modulate the host cell signaling through molecular mimicry and hijack the host cell signaling events (Garmendia *et al.*, 2005). A model of translocation of LEE encoded and other proteins into the host cell cytosol is presented in Figure 1.2-6. In addition, phosphorylation/dephosphorylation of critical residues in effectors often triggers the host cell signaling cascade (Garmendia *et al.*, 2005). These result in cytoskeleton rearrangements (Tir, EspH, EspF and EspG), disruption of the epithelial barrier and tight junctions (EspF and Map), altered ion secretion, cytotoxicity (EspF and Cif), neutrophil recruitment, actin polymerization, disruption of the cell cycle, and transcriptional changes that will ultimately cause watery diarrhea (Dahan *et al.*, 2004; Garmendia *et al.*, 2005). Following the delivery of the effector proteins, EspA filaments and the needle-like complex are removed from the bacterial surface to allow intimate attachment of the bacteria and host. Once the bacteria are intimately attached, the LEE-encoded genes are down-regulated (Garmendia *et al.*, 2005).

1.2.6 Non LEE Encoded Effectors

In addition to the above described effector proteins, new effector proteins encoded on prophages or other PAIs have been found to be translocated into the host cell. TccP (Tir-cytoskeleton coupling protein), located within the prophage CP-933U, is an effector protein that triggers actin polymerization and is essential for recruitment of α actinin and A/E lesion formation (Campellone *et al.*, 2004). Once translocated through the TTSS, TccP binds indirectly with Tir and then activates the neuronal Wiskott-Aldrich syndrome protein (N-WASP) and is able to then stimulate Nck-independent actin polymerization leading to pedestal formation (Carlier *et al.*, 2003).

1.2.7 EHEC Adherence Factors

To date, a number of adhesion factors, both fimbrial and non-fimbrial, have been suggested to play a role in EHEC host cell adhesion (Torres *et al.*, 2005a).

1.2.7.1 Fimbrial Adhesins

Since intimin mutants are still able to colonize host cell epithelial cells, it has been suggested that bacteria produce other yet unidentified adhesins (Torres *et al.*, 2003). To date, several fimbriae implicated in the binding of EHEC to host intestinal cells have been identified. Torres *et al.* (2004) have characterized 2 loci-encoding long polar fimbriae which have been proposed to mediate bacterial adherence to tissue culture cells (Torres *et al.*, 2004). EHEC mutants in *lpf* showed reduced adherence to epithelial cells and formed fewer microcolonies than the wild type (Torres *et al.*, 2004). The expression of *lpf* was observed in response to environmental signals, such as temperature (37°C), pH (pH 6.5), and maximal expression occurred only during the late exponential phase of growth (Torres *et al.*, 2002; Torres *et al.*, 2004). In addition, a cluster of *sfp* genes present only on the large plasmid of sorbitol-fermenting EHEC (pSFO157) has been suggested to mediate mannose-resistant hemagglutination and be sufficient in the expression of a novel type of Sfp fimbria (Brunder *et al.*, 2001). Other reports have implicated curli (Kim & Kim, 2004), a type I pilus homolog (F9) (Low *et al.*, 2006), and a type IV pilus (TFP) in LEE-negative non-O157: H7 STEC (Srimanote *et al.*, 2002) as mediators of adherence. Recent data demonstrating a role for a fimbrial factor in the adherence of EHEC were published by Xicohtencatl-Cortes *et al.* (2007). They propose that EHEC produces adhesive type IV pili, termed HCP (hemorrhagic coli pilus), that form bridges between bacteria adhering to human and bovine host cells. Since the expression of these putative adhesins was observed only under specific environmental conditions, additional experiments are required to elucidate the detailed mechanisms of colonization of the gastrointestinal tract of human and bovine organisms (Xicohtencatl-Cortes *et al.*, 2007).

1.2.7.2 Nonfimbrial Adhesins

Intimin is a key adhesion factor demonstrated to play a role in EHEC adherence to intestinal epithelial cells (McKee *et al.*, 1995; Sinclair & O'Brien, 2002). McKee *et al.* (1995) has shown that mutants defective in the *E. coli* O157:H7 *eae* (*Escherichia coli* attaching and effacing) gene were unable to adhere to epithelial cells, however when intimin was reintroduced, the phenotype was fully restored. They also provided evidence that *eae* introduced in either pEB310 or pEB311 was not sufficient to confer adherence on K-12 strains, resulting in a similar phenotype of impaired microcolony formation in HEp-2 cells. Their data together with those of Dytoc *et al.* (1993) and Jerse *et al.* (1991) and suggest that additional factors not directly involved in adherence of EHEC may aid in the localization of intimin by the bacteria.

In addition to intimin, several nonfimbrial surface proteins have been proposed as novel adhesion factors including Iha (adherence conferring molecule), OmpA (Outer membrane protein A), Saa (autoagglutinating adhesion), ToxB, Efa-1 (EHEC factor for adherence) (Nicholls *et al.*, 2000; Tarr *et al.*, 2000; Paton *et al.*, 2001; Stevens *et al.*, 2002).

As described above, intimin is an outer membrane protein adhesin, which recognizes and binds to its translocated intimin receptor (Tir) to mediate intimate attachment. Intimin, a 94-97 kDa protein, is encoded by the *eae* gene within the LEE (Elhanafi *et al.*, 2004). It is exported to the periplasm via the general secretory pathway and inserted into the outer membrane where it can interact with its own injected receptor or bind to host cell integrins and nucleolin (Sinclair & O'Brien, 2002; Garmendia *et al.*, 2005). Intimin has two functional regions, a highly conserved N-terminal region and a highly variable C-terminal region which defines the types of intimin (Touzé *et al.*, 2004). Intimin is classified as follows; types α , β , γ , δ , and ϵ (Sinclair and O'Brien 2004). Intimin types ϵ and γ are present in EHEC strains, whereas type α is mainly associated with EPEC (Caprioli *et al.*, 2005). Intimin β is found in EPEC and EHEC and is associated with rabbit Peyers's patch lymphoid follicles (Caprioli *et al.*, 2005). EHEC serotype O157:H7 produce γ intimin (Caprioli *et al.*, 2005).

Intimin is composed of two monomeric subunits, each monomer consisting of a transmembrane region and an extracellular rod formed by three immunoglobulin-like domains (D0 to D2) and one lectin-like domain (D3) (Adams *et al.*, 2005). The N-terminal region of intimin is inserted into the bacterial outer membrane, forms a β -barrel-like structure and mediates dimerization, whereas the C-terminal region of intimin extends from the bacterium, and interacts with receptors in the host cell plasma membrane (Adams *et al.*, 2005). Each type of intimin binds to Tir to promote tight adherence of the organism to the host-cell plasma membrane (Sinclair & O'Brien, 2002). γ intimin also binds to nucleolin (host cell receptor) (Sinclair & O'Brien, 2002). This interaction has been shown to promote initial adherence to the host cells (Campellone & Leong, 2003). Once Tir has been translocated into the host cell, it moves to the plasma membrane where it forms a hairpin loop structure. The cytosolic domains of Tir interact with cytoskeleton proteins which lead to formation of actin pedestal structures (Garmendia *et al.*, 2005).

Besides intimin, a number of other adhesins have been suggested to play a role in EHEC host cell adhesion. *E. coli* factor for adherence (Efa) has recently been proposed to contribute to adherence of O157:H7 to Chinese hamster ovary cells. Mutants defective in this non-fimbrial factor showed a significant decrease in adherence to bovine intestinal epithelium and human tissue cultures relative to the parent strain (Stevens *et al.*, 2002; Badea *et al.*, 2003). Since serotype EHEC O157:H7 strains (EDL933 and O157Sakai) have a truncated form of *efa-1*, it has been proposed that this gene has a role in bacterial adhesion and intestinal colonization (Nicholls *et al.*, 2000). Interestingly, *efa-1* has a significant homology to the *toxB* gene which has been associated with adherence to epithelial cells (Nicholls *et al.*, 2000). Stevens *et al.* (2004) have shown that mutants defective in the *E. coli* O157:H7 *toxB* gene resulted in lower expression and secretion of type III secreted proteins and decreased microcolony formation on epithelial cells. They also provided evidence that a mutation in the truncated *efa-1* resulted in a similar phenotype of impaired microcolony formation on HeLa cells. Their data together with those of Tatsuno *et al.* (2001) suggest that both genes may function to influence the expression of TTSS at a posttranscriptional level (Tatsuno *et al.*, 2001).

The non-fimbrial Iha (IrgA homologue adhesion) has also been reported to play a role in EHEC host cell adhesion. Iha is a 67 kDa protein found in EHEC strains and is an outer membrane protein which confers adherence to non-adherent laboratory strains (Tarr *et al.*, 2000). Tarr *et al.*, (2000) proposed that Iha mediates adherence to HeLa cells only when it is expressed in non-fimbriated *E. coli* strain. Probably K-12 strains that lack intimin are able to use Iha for adherence and colonization.

Outer membrane protein A (OmpA) has also been shown to act as an *E. coli* O157:H7 adhesin, playing a role in bacterial adherence to HeLa and Caco-2 cells (Torres & Kaper, 2003). Various other adhesins including long polar fimbriae (Torres *et al.*, 2004), curli (Uhlich *et al.*, 2001) and F9 fimbriae (Dziva *et al.*, 2004; Low *et al.*, 2006) have been implicated as mediators of adherence with no apparent pathogenic role. However, the expression of these described putative adhesins is under tight regulation and thus far intimin is the only factor clearly demonstrated to play a role in EHEC adherence (Torres & Kaper, 2003).

1.2.8 pO157:H7 Plasmid

E. coli O157:H7 carry a large plasmid (pO157), about 93-104 kB in size, which has been suggested to play a role in the pathogenesis of EHEC (Ostroff *et al.*, 1990; Paros *et al.*, 1993). pO157 is a dynamic structure containing mobile genetic elements such as transposons, prophages and parts of other plasmids, and as a consequence, the putative virulence factors harboured by this plasmid, appear to be unevenly distributed in EHEC O157 strains (Caprioli *et al.*, 2005). The plasmid typically carries several virulence genes including *hlyA*, *katP*, *espP*, *toxB*, *tagA*, *etcC-O*, and *hlyA-D* (Tatsuno *et al.*, 2001). *katP* and *espP* genes encode a katalase-peroxydase and a serine protease, respectively. Their role is still unclear, however studies have shown that antibodies to the *espP* product are present in sera from children with EHEC infection (Caprioli *et al.*, 2005). Little is known about EspP, although it has been suggested it may mediate the cleavage and the degradation of the human coagulation factor V, which could result in mucosal hemorrhage, common in HC (Law, 2000). ToxB may confer the ability to allow

adherence to host-cells since strains mutated in *toxB*, have been demonstrated to reduce adhesion to cultured epithelial cells (Stevens *et al.*, 2004). Additionally, a type II secretion system has been associated with this plasmid, yet the secreted targets pertaining to this system have not been identified (Tatsuno *et al.*, 2001; Caprioli *et al.*, 2005). StcE, a protease, which also is present on the virulence plasmid, has been proposed to contribute to the intimate adherence of *E. coli* O157:H7 to host cells (Grys *et al.*, 2005). The *stcE* gene, upregulated by Ler, is able to destroy the layer of mucins and glycoproteins, and thereby aids the bacterium to adhere to the host cells by subverting the host mucosal defenses (Grys *et al.*, 2005).

1.2.9 Regulation of the EHEC Virulence Genes

In order to colonize the host, activate virulence genes, and initiate the disease process, EHEC must sense its surrounding and respond accordingly to any environmental changes (Mekalanos, 1992). Thus, the ability to respond rapidly is essential for the success of the pathogen. Appropriate expression of TTSS genes is achieved through the integration of several signal transduction systems, which allow pathogens to respond to changing microenvironments during infection (Spear *et al.*, 2006). The complex regulatory cascades reflect their ability to adapt to different niches both inside and outside of the host.

LEE-encoded virulence factors are regulated by the LEE-encoded regulator (Ler) which are further positively and negatively modulated by GrlA and GrlR respectively (Deng *et al.*, 2004). It has also been reported that regulatory mechanisms of activation of LEE1 require perC (plasmid encoded regulator) in EPEC and pch A, B and C (renamed perC homologue pchA, pchB and pchC) in *E. coli* O157:H7 (Spear *et al.*, 2006). Although LEE1 expression in EPEC is tied to perC, however this is not the case in EHEC which possesses a number of perC chromosomal homologues that act to increase LEE1 expression (Spear *et al.*, 2006). LEE genes may be regulated at the post-transcriptional level (Roe *et al.*, 2003; Stevens *et al.*, 2004; Deng *et al.*, 2005). Zang *et al.* (2004) proposed that LEE expression may be repressed by a cryptic second type III

secretion island (Zhang *et al.*, 2004). There is likely a cross talk between regulators from other O-island and TTS-related genes such as flagella (Zhang *et al.*, 2004). Cell to cell signaling between bacteria (quorum sensing) is activated via the LuxS, QseA and SdiA systems (Sperandio *et al.*, 1999; Sperandio *et al.*, 2001; Sperandio *et al.*, 2002); however the mechanism for SdiA binding of signaling HSL to repress EspD and intimin remains to be elucidated. Additionally recent evidence has shown that EHEC may hijack signals produced by other microbial flora and the host that lead EHEC to regulate its virulence genes in a coordinated manner (Clarke & Sperandio, 2005). According to the model proposed by Hughes & Sperandio (2008) EHEC sense autoinducer (AI-3), adrenaline and noradrenaline (NA) signals to activate motility genes (flhDC), Stx genes and LEE genes (components of a type III secretion system).

Overall, TTSS expression in EHEC is under tight regulatory control, however with considerable variation between strains that may contribute to their epidemiology in both humans and cattle (Spear *et al.*, 2006).

1.3 Modulation of Host Cell Function

Multiple functions including disruption of epithelial barrier, altered ion secretion, disruption of tight junctions, neutrophil recruitment, actin polymerization, disruption of cell cycle and transcriptional changes explain the physiological outcome: watery persistent diarrhea (Garmendia *et al.*, 2005).

1.4 Disruption of the Barrier Function

EHEC infection alters the barrier function through disruption of the epithelial barrier and increased monolayer permeability through alteration of tight junctions. EHEC activates several pathways contributing to the disruption of barrier function (Garmendia *et al.*, 2005). The pathway is the redistribution and activation of ezrin by phosphorylation on threonine and tyrosine (leads to disruption of tight junctions). A second pathway is the redistribution of tight junction proteins: occludin is dephosphorylated by ser/threonine

phosphatases and moves from tight junctions to the cytosol (Berryman *et al.*, 1993). Another pathway is phosphorylation of myosin light chain (MLC): MLC associates with the cytoskeleton and is phosphorylated by MLC kinase – ATP hydrolysis and movement of filaments past each other to cause an increase in paracellular permeability (Manjarrez-Hernandez *et al.*, 1996). A fourth pathway is the disruption of adherent junctions: phosphorylation of protein kinase C (PKC) which associates with cadherins – dissociation of cadherin/ β catenin complex which forms adherent junctions. Several effectors are involved in induction of these effects including EspF, EspFu/TccP and Map (Malladi *et al.*, 2004).

1.4.1 Factors Contributing to Diarrhea

In addition to the disruption of the barrier function, several factors contribute to EHEC induced secretory diarrhea. Changes in the host cell electrolyte transport and decrease in membrane potential and increase in short circuit current are observed in EHEC infections. Additionally, changes in chloride secretion and bicarb-dependent chloride transport are observed (Hecht & Koutsouris, 1999). EHEC induces tyrosine phosphorylation (Tyr-P) of phospholipase C γ 1 (PLC- γ 1), cleaves phosphatidyl-inositol 4,5-bisphosphate (PI45P2) producing 1,4,5-triphosphate (IP3) and (diacylglycerol) DAG which activates PKC for secretion of ions and fluids (Crane *et al.*, 1999). Recent research revealed that several ion transport and ion channel proteins are upregulated during TTSS-EHEC infection (Hardwidge *et al.*, 2004).

1.4.2 Host Cell Cytoskeletal Rearrangements

Initial stages of infection are characterized by filopodia of the host tissue at the site of infection that extend and sway for about 20 min and retract into the host cell (Map and Cdc42 dependent) (Kenny *et al.*, 2002). These are followed by massive rearrangement of the host actin microfilaments triggering A/E lesions and formation of pedestal like structures of host tissue under adherent bacterium – 10 μ m outward from host cell. EHEC

can move across the surface of infected cells at speeds of up to 0.1 $\mu\text{m}/\text{sec}$ (Figure 1.4-1) (Sanger *et al.*, 1996). Y474 of EHEC Tir is phosphorylated and the Y474 containing region recruits and activates N-WASP activating Arp2/3 and triggering actin polymerization and pedestal formation (Cantarelli *et al.*, 2002).

Watery diarrhea may be generated by disruption of the epithelial barrier, cytoskeleton rearrangements, the inflammatory response to infection, and active chloride secretion resulting from signal transduction, whereas bloody diarrhea may be induced by verotoxin damage to small blood vessels in the large intestine (Law, 2000; Gyles, 2004 personal communication). In addition to causing cytotoxicity, Stx have also been implicated in inducing programmed cell death or apoptosis (Ceponis *et al.*, 2003). HEp-2 cells exposed to the toxins for 24-48 hours have been shown to show classic morphologic features associated with apoptosis such as cell shrinkage, membrane blebbing, and nuclear chromatin condensation (Ceponis *et al.*, 2005).

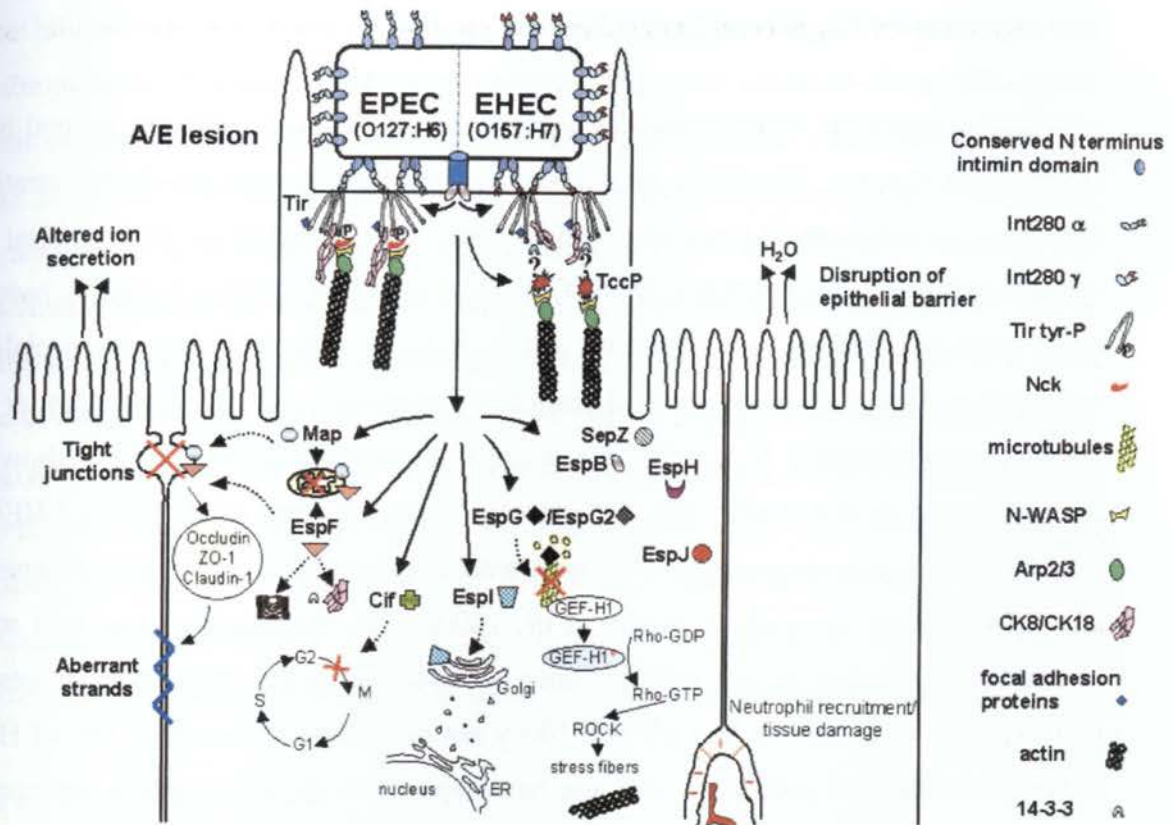


Figure 1.4-1 EPEC and EHEC translocate effector proteins into the host cell cytosol.

These effectors trigger cytoskeleton rearrangements (Tir, EspH, EspF, and EspG), disruption of the epithelial barrier (EspF and Map), cytotoxicity (EspF and Cif), and host cell responses that ultimately generate watery diarrhea. Different from EPEC Tir, EHEC Tir is not tyrosine phosphorylated and does not interact with Nck in order to trigger actin polymerization at the site of bacterial adhesion; instead, EHEC translocates an effector protein, TccP/EspFu, which has an Nck-like activity and is essential to promote the reorganization of the actin cytoskeleton underneath adherent EHEC bacteria. ER, endoplasmic reticulum. Reproduced from Garmendia *et al.*, 2005 with the permission of *Infection and Immunity*, American Society for Microbiology.

1.5 Impact of Ingestion Stress on Virulence

During ingestion, enteropathogens are exposed to, and must overcome several host-environment stresses. The ability of pathogens to withstand physiological concentrations of bile salt as well as short chain fatty acids is likely to be important for their survival and consequently for their persistence in the GI tract of their host. During infection, bacteria must adapt to changing environments and it is likely that they express specialized colonization factors in a hierarchical manner (Cummings *et al.*, 1987; Begley *et al.*, 2005). Enteropathogenic *E. coli* (EPEC), which is a related pathogen, has been shown to have enhanced virulence after bile salt and acid stress (de Jesus *et al.*, 2005). EHEC is also well suited to overcoming acid stress in order to infect and cause disease (Elhanafi *et al.*, 2004). Recent research by House *et al.*, (2006), has demonstrated that acid stress increases the adhesion to host cells but does not affect verotoxin production, suggesting that exposure to extremely low pH will likely not contribute to increased risk of HUS. Other gastrointestinal pathogens have also been reported to show changes in virulence in the presence of bile. Ramos-Morales and coworkers (2003) have shown that synthesis of enterobacterial common antigen (ECA) contributes to *Salmonella* virulence by protecting the pathogen from BS. *Shigella* grown in BS have been shown to enhance Ipa protein secretion and increase invasion of epithelial cells (Pope *et al.*, 1995). *Vibrio parahaemolyticus* has been shown to have increased expression of TDH virulence factor (Osawa *et al.*, 2003). Contrary to the positive effect of bile on *V. parahaemolyticus*, Schuhmacher *et al.* (1999) suggested that *Vibrio cholerae* in the presence of bile, resulted in decreased virulence gene expression.

1.6 Bacterial Response to Bile Salts

1.6.1 Bile Composition and Function

After overcoming the acid barrier in the stomach, enteric bacteria including EHEC are exposed in the intestinal tract to bile salts. Bile is a detergent-like digestion fluid that disrupts the lipid bilayer structure of cellular membranes, induces protein mis-folding and cause oxidative damage to DNA (Begley *et al.*, 2005). In humans, the major components

of the bile include bile acids, cholesterol, phospholipids (primarily phosphatidylcholine) and the pigment biliverdin (Carey *et al.*, 1994). Bile acids represent about 50% of the organic components of bile and are conjugated as N-acyl amidates with glycine or taurine before secretion. The carboxyl group of the bile acid and the amino group of the amino acid are linked by an amide bond (peptide linkage) (Hofmann, 1999). Amidation has significant effects on the bile acid solubility since the unconjugated bile acids are hardly soluble at physiological pH. Conjugation allows bile acids to be soluble over a wide range of ionic strengths, calcium concentrations and pH values (Hofmann, 1999). Taurine and glycine conjugates are called bile salts since they are totally ionized at physiological pH while the unconjugated bile salts are not (Begley *et al.*, 2005). The major BS present in the intestine are deoxycholate (NaDOC), chenodeoxycholate (NaCDC), ursodeoxycholate (NaUDC) and glycolate (NaGIC) (Gabig *et al.*, 2002). The approximate concentrations entering the small intestine are presented in Table 1-1:

Table 1-1 Bile Salts in Duodenum

<i>Bile salt</i>	<i>Duodenum percent of bile salts</i>
NaGIC	31.1 ^a
NaUDC	0.8
NaCDC	35.7
NaDOC	14.4

A litre of bile, which is a 'biological detergent', passing through the duodenum each day represents a serious challenge to intestinal pathogens and thus forms an important part of the body's defense system (Hofmann, 1999).

^a from ratio of glycine: taurine amidates and percent cholate (Bernstein *et al.*, 1999).

1.6.2 Antimicrobial Actions of Bile

The main antimicrobial action of bile has been demonstrated to be through damage to cell membranes by altering membrane integrity/permeability (Pazzi *et al.*, 1997). There are several important factors that determine the exact outcome of bile on cell membranes (Begley *et al.*, 2005). The concentration of bile is a major important factor. High levels of BS have been shown to dissolve membrane lipids and dissociate membrane proteins, causing disruption of membrane integrity (Coleman *et al.*, 1980). BS at low concentrations can also affect the physical chemical properties of cell surfaces. The type and structure of bile also may determine the outcome of bile action on the cell membrane (Begley *et al.*, 2005). For example, bovine bile which passively diffuses much more slowly across the lipid bilayer than porcine bile is also less inhibitory (Legrand-Defretin *et al.*, 1991). Although the composition of porcine bile is more similar to that of human bile, bovine bile is commonly chosen to assess the *in vitro* bile tolerance of bacteria (Begley *et al.*, 2005). The membrane architecture and composition are also key factors; charge, hydrophobicity and lipid fluidity have been shown to have significant consequences. Chou and Cheng, (2000) observed that freezing significantly damages the cell membrane lipopolysaccharides making them more susceptible to BS. Besides membrane damage, bile has been shown to induce DNA damage, alter conformation of proteins (misfolding or denaturation) and cause oxidative stress. Research by Bernstein *et al.*, (1999) has shown that BS induce the expression of several stress response genes: *micF*, *osmY* and *dinD*.

1.6.3 Bile Tolerance

Bile tolerance is poorly understood, particularly in Gram negative bacteria, however it is believed that they are more resistant to bile than Gram positive bacteria (Begley *et al.*, 2005). Prouty *et al.* (2004) showed that *Salmonella* is capable of colonizing the gall bladder where bile is in extremely high concentrations (Prouty *et al.*, 2004). *E. coli* is also considered to be very bile resistant since it has been isolated from gallbladder and

bile of animals and humans (Brook, 1989; Cable *et al.*, 1997; Carpenter, 1998; Flores *et al.*, 2003). Another study by Gänzle *et al.* (1999) reported that *E. coli* may inhabit the small intestine of a gastrointestinal model in the presence of high concentrations of porcine bile extract whereas *Listeria* was rapidly killed. *Helicobacter* and *Campylobacter* are also bile resistant and have been isolated from bile ducts, liver and gallbladder (Fox *et al.*, 1995). In contrast, *H. pylori* has been shown to be sensitive to bile which may explain why it has never been observed in faecal samples or cholecystectomy specimens (Begley *et al.*, 2005).

1.6.4 Genetics of Bacterial Bile Tolerance

The pathogenic strains of *E. coli* are able to survive the BS stress by developing adaptive strategies to the stress, including: drug efflux pumps (AcrAB pump), porin proteins (OmpF, OmpC), transport proteins, murein lipoproteins, peptidoglycan associated lipoproteins (Gunn, 2000). Research by Bernstein *et al.* (1999) has shown that BS induces the expression of several stress response genes: *micF*, *osmY* and *din*. Another study by Wang (2002) suggested that the outer membrane protein OmpA plays a major structural role in *E. coli* membrane stability. *E. coli* K1 *ompA* mutants are significantly more sensitive to environmental stresses (cholate, acidic environment and high osmolarity) resulting in reduced stress resistance. OmpF and OmpC, two major outer membrane proteins, are responsible for the passive diffusion of small molecules in the periplasm. Their expression is conversely regulated; because the OmpF porins form channels larger than those of OmpC. It has been reported that OmpC can exclude larger molecules more efficiently than OmpF porins (Bernstein *et al.*, 1999).

In addition, several other factors have been demonstrated to contribute to bacterial resistance to environmental stress. Among these are colanic acid (CA) exopolysaccharides (EPS) which are cell surface structures and that provide the first line of defence for bacterial cells (Mao *et al.*, 2006). According to these studies, CA EPS of *E. coli* O157:H7 provides a protective barrier to the pathogen while they are exposed to the various stress conditions in the gastrointestinal tract. The resistance of *Vibrio*

cholerae and other *Vibrio* species to BS has been attributed to the lipopolysaccharide (LPS) and outer membrane porins (Provenzano *et al.*, 2000).

1.6.5 Bile Sensing and Regulation of Bile Stress Response

The mechanisms used by bacteria to sense and respond to bile are poorly understood. Bile is thought to be detected by a two-component system (TCSs) (Parkinson and Kofoed, 1992). TCSs are composed of a membrane-associated histidine kinase and a cytoplasmic response regulator. The histidine kinase detects the presence of bile, it transduces the signal to the response regulator which instructs the cell to respond to the change via changes in the expression of specific virulence factors (Parkinson and Kofoed, 1992). Bile may also be sensed by responding to the disruption of membrane integrity. Bacterial membrane damage sensors such as *E. coli* Cpx and RcsCb, which sense alterations in the cell surface, may function as sensors and signal transducers (De Wulf *et al.*, 2002; DiGiuseppe and Silhavy, 2003). In addition, the accumulation of proteins damaged or denatured by bile may activate a general stress response. Transcription of stress genes with significant roles in bile resistance has been shown to be controlled by two well characterized transcriptional regulators: Sigma B (σ^B) and RpoS (σ^S) (Cheville *et al.*, 1996; Ferreira *et al.*, 2001).

1.6.6 Bile and Pathogenesis

Continuous monitoring of environmental changes is essential for survival for enteric bacteria when making the transition to the human host. It is not surprising that enteric pathogens use bile as an environmental cue to establish the organism's intestinal location and co-ordinately modulate the expression of virulence factors. It is likely that some gene products expressed in bile tolerance assist in survival and colonization of the intestinal tract and thus in their own right, function as virulence factors (Begley *et al.*, 2005). Recent work has uncovered the pathogen responses to bile that alter the expression of virulence factors in some of the GI pathogens including *Salmonella*,

Shigella, *Vibrio*, *Campylobacter* and enteropathogenic *E. coli* (Pope *et al.*, 1995; Prouty & Gunn, 2000; Osawa *et al.*, 2002; Prouty *et al.*, 2004; de Jesus *et al.*, 2005; Torres *et al.*, 2007).

Pathogens respond to bile in different fashions in order to facilitate their own pathogenic mechanisms (Begley *et al.*, 2005). Prouty *et al.* (2000, 2004) demonstrated that *Salmonella* sense the bile as environmental signal which consequently down-regulates invasion in the intestinal lumen where there are high concentrations of bile. However, upon transit of the mucus layer, where bile concentrations decrease, the expression of the invasion machinery is initiated resulting in an increase in SP-1 genes involved in epithelial cell entry. Pope *et al.* (1995) provide evidence that *Shigella* (*S. flexneri* and *S. dysenteriae*) respond to bile in a contradictory fashion; Ipa protein secretion is up-regulated, and invasion of epithelial cells is increased due to enhanced adherence. A study by Osawa *et al.* (2006) on *Vibrio parahemolyticus* showed that bile acids enhance production of the virulence factor thermostable direct hemolysin (TDH). In addition to these findings, another study by Pace *et al.* (1997) also demonstrated that bile acids favor growth of *V. parahaemolyticus* and enhance adherence to epithelial cells. Contrary to the positive effect of bile to *V. parahaemolyticus*, two independent studies demonstrated that growth of *V. cholerae* in bile results in decreased virulence expression (Gupta and Chowdhury, 1997; Schuhmacher and Klose, 1999). Growth in the presence of bile resulted in a decrease in cholera toxin (CT) production and toxin-coregulated pilus (TCP) but an increase in motility. Another study reported that bile inactivates the transcription factor ToxT which induces numerous virulence promoters including those for CT and TCP (Schuhmacher and Klose, 1999). The model proposed by these authors suggests that once bacteria swim into the mucosal gel where bile concentrations are low, motility is decreased, ToxT activity is restored and virulence gene expression required for colonization and disease is induced (Schuhmacher & Klose, 1999).

The role of bile in EPEC pathogenesis has also been identified. De Jesus *et al.* (2005) identified profound changes in host adhesion of EPEC after exposure to BS stress. BS stress of EPEC significantly increases the adhesion of surviving EPEC to epithelial cells compared to unshocked EPEC. The authors also observed a slight increase in flagella expression and bundle-forming pilus (BFP) after BS (de Jesus *et al.*, 2005). To

our knowledge there are no studies on the impact of BS on changes in *E. coli* O157:O7 virulence factor expression.

1.7 Short Chain Fatty Acid Stress Response

1.7.1 Short Chain Fatty Acid Composition and Function

Transiting to the large intestine, EHEC must also resist the stress of exposure to short chain fatty acids (SCFA) which are key end products of colonic fermentation (Arnold *et al.*, 2001; Polen *et al.*, 2003). Although the pH of the intestine is almost neutral, there are high concentrations of SCFA present in the large intestine, mainly acetate, propionate and butyrate (Cummings *et al.*, 1987). The organic acids are preferentially absorbed across the epithelial membranes in the human ileum and colon. Butyrate is of particular importance because it is the major source of energy for colonic epithelial cells (Borthakur *et al.*, 2006). Propionate is largely taken up by the liver whereas acetate enters the peripheral circulation to be metabolized by peripheral tissues (Puchowicz *et al.*, 1999). The concentration of SCFAs is estimated to be low, between 20 and 40 mM total SCFAs in the ileum, and ranges from 100-300 mM in the colon, depending on species and diet (Cummings *et al.*, 1987; Macfarlane *et al.*, 1992). Because the protonated form of SCFA equilibrates across the cytoplasmic membrane, SCFA can lower internal pH even when the external pH is neutral (Roe *et al.*, 1998). The pH of the colon and ileum are similar at around 6.7 (Lawhon *et al.*, 2002).

1.7.2 Short Chain Fatty Acid Tolerance and Pathogenesis

Exposure to SCFAs is considered a stress condition and likely impacts the survival of microorganisms, evidenced by the fact that they are widely used as food preservatives to prevent the growth of bacteria (Abdul-Raouf *et al.*, 1993). A study by Lasko *et al.* (2000) investigated the response of several probiotics and *E. coli* K12 after exposure to varying acetate challenges. The authors pointed out that capacity of non-pathogenic *E. coli* to tolerate acetate was dependent on the carbon source. *E. coli* exhibits higher acetate tolerance in the glycerol grown cultures compared to glucose supplemented media. Moreover, *E. coli* grown in the presence of glycerol is almost tolerant to acetate as are the acetic acid bacteria (AAB) which are best known to be tolerant to acetate (Lasko *et al.*, 2000). Another study by Polen *et al.* (2003) investigated gene expression patterns *E. coli* O157:H7 due to the presence of acetate. The adaptive response of nonpathogenic *E. coli* exposed to SCFA (acetate and propionate) has been shown to include an increased expression of chemotaxis and flagellum genes, both of which can play a role in colonization of the gastrointestinal tract (Polen *et al.*, 2003). Lawhon *et al.* (2002) demonstrated that SCFAs alter the expression of *Salmonella typhimurium* invasion genes. Acetate activated *S. typhimurium* invasion whereas propionate and butyrate (primarily found in the colon) had the opposite effect. The authors suggested that the composition of SCFAs in the small intestine provides a cue to activate *Salmonella* invasion while that in the large intestine inhibits invasion (Lawhon *et al.*, 2002). Consistent with these findings, Gantois *et al.* (2006) observed that most genes significantly downregulated after exposure with butyrate are genes of the pathogenicity island 1. Another study by Kirkpatrick *et al.* (2001), using a proteomic approach, has revealed that *E. coli* grown with acetate increased the expression of several amino acid and peptide transporters. The authors suggested that these transporters might enhance the uptake of alternative energy source if the cells perceive acetate uptake as a sign of decreasing availability of carbohydrates. Accentuated expression of components of the RpoS regulon (stress response system) was observed after short term exposure with acetate (Kirkpatrick *et al.*, 2001).

1.8 Stress and EHEC Virulence

While significant advances have been made in EHEC pathogenesis, we still do not fully understand the impact of environmental stress on the virulence properties of EHEC including adhesion to host endothelial cells and production of Shiga toxins. Environmental stresses have been clearly demonstrated to enhance the virulence of many enteropathogens. Recent research by de Jesus *et al.* (2005) has identified profound changes in host adhesion of a related pathogenic EPEC after exposure to major components of bile. BS stress of EPEC also resulted in a significantly increased adhesion of surviving bacteria to epithelial cells. In addition, our laboratory has also shown that acid shock enhances the adhesion of *E. coli* O157:H7 and its virulence (House *et al.*, 2006). The role of bile on EHEC adhesion to host endothelial cells and toxin production has yet to be described.

The effect of SCFA on *E. coli* O157:H7 virulence is not well understood. Arnold *et al.* (2001) is one of the first reports that investigated the impact of SCFAs on *E. coli*. This group only reported gene expression changes of *E. coli* O157:H7 which was grown with inhibitory concentrations of 100 mM acetate for 30 min. Their results provided evidence that acetate increases expression of a member of RpoS regulon, as well as various transporters and metabolic enzymes. Another study by Tao *et al.* (1999) has shown that *E. coli* grown on rich media have elevated expression of the RpoS regulon as well as OmpC and TpiA, the authors pointing out a role of glucose in generating high acetate levels. A second study by Oh *et al.* (2002) investigated the transcript profile of *E. coli* grown also in acetate as the sole carbon source and compared it with that one of the culture grown in glucose. When grown in the poor carbon source, genes involved in cell replication, transcription and translation were down-regulated whereas metabolic genes were up-regulated. Other studies also revealed increased expression of general stress response genes after short exposure of *E. coli* to high concentrations of acetate. Louise *et al.* (1995) have established the role of butyrate on Shiga toxin binding, internalization, intracellular transport and activation in eukaryotic cells. They suggest that butyrate may increase the sensitivity and intracellular traffic in HUVEC as well as Shiga toxin binding to the cell surface (Louise *et al.*, 1995). Interestingly, recent research by Borthakur *et al.*

(2006) showed that enteropathogenic *E. coli* which is a related EHEC pathogen, modulates the butyrate uptake in Caco2 cells. EPEC infection inhibits butyrate uptake in Caco2 cells whereas infection with nonpathogenic *E. coli* had no effect. Other complementary studies have also demonstrated that butyrate is implicated in suppressing mucosal inflammation (Inan *et al.*, 2000; Segain *et al.*, 2000). Taken together, these results suggest that decreased butyrate uptake caused by EPEC infection may compromise the colonic epithelial integrity, resulting in inflammation of the epithelium (Borthakur *et al.*, 2006).

Recent studies by Carey *et al.* (2008) used several probiotic strains to evaluate the effect of SCFAs on Stx2 gene expression in *E. coli* O157:H7. All strains tested down regulated *stx2A* expression to different degrees, the authors suggesting that may be attributed to the type and amount of organic acid produced by each probiotic strain. Takahashi *et al.* (2004) also suggested that due to butyric acid production, *Clostridium butyricum* might be able to inhibit EHEC growth. However further research is needed to fully understand the impact of SCFA stress on EHEC toxin production since these published reports have only examined the impact of probiotic strains. The role of major SCFA including acetate, propionate and butyrate as well as that the mixture mimicking the large intestine, on *E. coli* O157:O7 needs to be explored.

1.9 Purpose of Investigation and Hypothesis

1.9.1 Research Rationale

It is becoming increasingly clear that pathogens respond to BS and SCFA in a variety of fashions to facilitate their specific pathogenic mechanisms. Bile represses *Salmonella* invasion but stimulates invasion of *Shigella* (Begley *et al.*, 2005). It is evident that that BS and SCFA can trigger changes in virulence properties of gastrointestinal pathogens. However, there is little known about how these stresses modulate the virulence potential of *E. coli* O157:H7. Further research is required to fully delineate the effect of BS and SCFA stress on EHEC virulence gene expression. This study will contribute to the developing picture of a complex relationship between *E. coli* O157:H7

and its environment within the host during infection. Moreover, it will be essential for describing novel approaches to prevent and heal EHEC infections.

1.9.2 Hypothesis

Exposure to physiological concentrations of BS and SCFA will significantly increase EHEC virulence including host cell adhesion and toxin production

1.9.3 The Main Objectives of this Thesis

- 1) Assess impact of exposure of *E. coli* H157:O7 to physiological relevant concentrations of BS on virulence including survival, host adhesion and toxin production
- 2) Assess impact of exposure of *E. coli* H157:O7 to physiologically relevant concentrations of SCFA on virulence, including host adhesion and toxin production
- 3) Clarify the role of known adhesins in the adhesion of BS and SCFA treated *E. coli* O157:H7.

2 Materials and Methods

2.1 Bacterial Cultivation

Table 2.1 below details the bacterial strains utilized in this thesis project.

Briefly, the Enterohaemorrhagic *E. coli* O157:H7 strains analyzed in this study were, 86-24(wt), 86-24*eae*Δ10, 86-24 *eae*Δ(pEB310), 86-24(Δ*iha*), ORN172(pSK+) and ORN172(pIHA). Bacteria were maintained as glycerol stocks and stored at -80°C, were routinely streaked onto Luria-Bertani (LB) agar plates (tryptone, 1% (w/v); yeast, (0.5% (w/v); sodium chloride, 1% (w/v); agar 1.5% (w/v) with or without addition of appropriate antibiotics and grown at 37°C. The mutants were checked to make sure they retained their characteristics. Strains were stored at 4 °C and subcultured every three weeks (Riordan *et al.*, 2000). The cultures inoculated from the LB plate were grown overnight under standardized conditions (shaking incubator at 37°C for 16 to 18 h at 200 rpm). For strain 86-24 *eae*Δ(pEB310), ampicillin (Sigma) was added to all plates and liquid media to a final concentration of 100 ug/mL of. 200 ug /ml Ampicillin (Sigma) was added for ORN172(pSK+) and ORN172(pIHA) strains and 20ug/ml nalidixic acid (Sigma) for 86-24(Δ*iha*).

Table 2-1 Bacterial Strains Used in this Study

Strains	Relevant characteristics	LEE	VT1	VT2	Source/Reference
86-24	Clinical Isolate O157:H7	+	-	+	(Gansheroff <i>et al.</i> , 1999)
86-24 <i>eae</i> Δ10	86-24 with deletion of <i>eaeA</i> gene encoding intimin protein required for attachment	+	-	+	(McKee <i>et al.</i> , 1995)
86-24 <i>eae</i> Δ(pEB310)	86-24 carrying plasmid pEB310, wild-type activity being restored	+	-	+	(McKee <i>et al.</i> , 1995)
86-24(Δ <i>iha</i>)	86-24 with deletion of <i>iha</i> gene encoding <i>iha</i> adherence-conferring protein	+	-	+	(Tarr <i>et al.</i> , 2000)
ORN172(pSK+)	Nonadherent laboratory <i>E. coli</i>	-	-	-	(Tarr <i>et al.</i> , 2000)
ORN172(pIHA)	Nonadherent laboratory <i>E. coli</i> carrying plasmid pIHA for adherence phenotype	-	-	-	(Tarr <i>et al.</i> , 2000)

2.2 Epithelial Cell Culture

Human epithelial cell lines, HEp-2 (laryngeal carcinoma cells), and CaCo-2 (colonic adenocarcinoma cells) were obtained from the American Type Culture Collection (ATCC) and were used in various adhesion assays. Vero cells (African Green Monkey Kidney cells) were kindly provided by Dr. Lingwood (Hospital of Sick Children, Toronto) and were used in Vero Cell Cytotoxicity Assays (see Table 2.2 below). All tissue cell culture conditions consisted of growing the cell lines in minimal essential media 1x Eagle's (modified) (Wisent; with Earle's salts and L-glutamine) supplemented with gentamycin (20, 000 ug/mL) and 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Wisent) for HEp-2 and Vero cell lines and 20% (v/v) for CaCo-2 cells (MEM with additives). All cell lines were grown to confluence at 37 °C and 5% CO₂ (v/v) in cell culture vented flasks. Vero and HEp-2 cells were maintained by subculturing the cells into fresh pre-warmed media every 3-4 days. Maintenance of the CaCo-2 cell line required fresh media after the 4th day of incubation and on the 6-7th day when the cells were transferred to fresh prewarmed media. One day prior to adhesion assay, cells were seeded into 24 well or 6 well plates and grown to 80-90% confluence.

Table 2-2 Cell Lines Used in This Study

<i>Cell Line (ATTC#)</i>	<i>Morphology</i>	<i>Species</i>	<i>Tissue/Tumor</i>	<i>Source</i>
HEp-2- (CCL23)	Epithelial	Human	Larynx, carcinoma	ATCC
CaCo-2 -(HTB37)	Epithelial	Human	Colon, adenocarcinoma	ATCC
Vero - (CCL 81)	Fibroblast	African Green Monkey	Kidney	Hospital for Sick Kids (Lingwood Lab)

2.3 Bile Salt Treatment of *E. coli* O157:H7

One day prior to the experimental day, bacteria (Table 2.1) were grown in LB broth supplemented with appropriate antibiotics when necessary. A 1:8 dilution of overnight culture was made into prewarmed Dulbecco's MEM (DMEM) pH 7.4 (Wisent; 4.5g/L

glucose, L-glutamine, sodium pyruvate) and grown statically for 2-2.5 hours at 37°C, 5% CO₂ to mid-log growth phase. Expression of LEE-encoded virulence factors is known to be influenced by bacterial growth phase or environmental factors such as culture medium, temperature and pH (Rosenshine *et al.*, 1996; Abe *et al.*, 2002; Lio and Syu, 2004). Bacteria were grown in prewarmed DMEM pH 7.4 as it has been reported that the secretion of LEE-encoded virulence factors are enhanced when EHEC strains are grown in DMEM and result in maximal adhesion to CaCo-2 cells (Abe *et al.*, 2002; Lio and Syu, 2004). It also has been reported that environmental factors similar to those in the intestine such as 5% CO₂ enhance secretion of pathogenic proteins and VT1 production in EHEC (Yoh *et al.*, 2003). Cells were grown to an OD₆₀₀ of 0.4-0.6 (logarithmic phase) as it has been shown that early-logarithmic-phase of EPEC enhances LEE –protein secretion and increases A/E activity to HeLa epithelial cells (Rosenshine *et al.*, 1996; Kenny *et al.*, 1997; Vanmaele and Armstrong, 1997). 4 mL of the induced bacteria culture was aliquoted to 15 mL Falcon tubes (one for each treatment type) and centrifuged at 3,500 rpm (2,000 x g) for 10 minutes at 18°C. The resulting supernatant was removed and BS were added to the DMEM pH 7.4 at the final concentration of 2.5 mM glycocholate (Sigma), 2.5 mM chenocholate (Sigma), 2.5 mM deoxycholate (Sigma), 2.5mM ursocholate (Sigma) and 0.15% BS mix (Sigma) (Pope *et al.*, 1995; Gunn, 2000). The control treatment (untreated) was resuspended in sterile filtered DMEM pH 7.4. Bacteria were incubated statically at 37°C, 5% CO₂ for 90 minutes and then centrifuged at 3500 rpm (2,000 x g) for 10 minutes. Supernatants were removed from all tubes and bacterial pellets were washed with 2 washes of phosphate buffered saline (PBS) (Wisent without calcium and magnesium) to ensure that all traces of BS were removed. After the second wash, the supernatant was removed and all treatments were resuspended in 4 ml of sterile filtered DMEM pH 7.4. An OD₆₀₀ reading was taken again to ensure that the culture was in the logarithmic phase of growth (0.4-0.6). Table 2.3 summarizes the various experimental BS treatments used in this study.

Table 2-3 Bile Salt Experimental Treatments

Experimental Treatment	Description
UU	Untreated
Glyco	Exposed for 90 minutes with Glycocholate (2.5 mM)
Cheno	Exposed for 90 minutes with Chenodeoxycholate (2.5 mM)
Deoxy	Exposed for 90 minutes with Deoxycholate (2.5 mM)
Urso	Exposed for 90 minutes with Ursodeoxycholate (2.5 mM)
BS	Exposed for 90 minutes with BS Mix (0.15%)
Note:	BS solutions were originally made as 10x in dH ₂ O and diluted in DMEM pH 7.4 during the stress treatments.

Following BS exposure, the samples were used in viability, adhesion and cytotoxicity experiments.

2.4 Short Chain Fatty Acid Stress of *E. coli* O157:H7

Prior to addition of the short chain fatty acids (SCFA), strains were grown in 10 ml LB with appropriate antibiotics when necessary, in a shaking incubator at 37°C, 200 rpm for approximately 4-5 h. 2 ml aliquots of culture were transferred to 14 Falcon tubes (one for each treatment), and centrifuged at 3500 rpm (2,000 x g) for 10 minutes at 18°C. The bacterial pellet from each tube was resuspended in LB broth containing SCFAs at following final concentrations: 110 mM sodium acetate (EMD), 70 mM sodium propionate (Alfa Aesar), 20 mM sodium butyrate (Alfa Aesar) and ~ 90 mM SCFAs mixture. SCFAs mixture is composed of 60.5 mM acetate (69%), 24.5 mM propionate (28%) and 3 mM butyrate (3%) (Lawhon *et al.*, 2002). Following overnight growth, LB broth cultures were diluted 1:8 in DMEM pH 7.4 with appropriate SCFAs (as the same concentrations as above) and grown to mid-log growth phase. The control treatment (unshocked) was resuspended in sterile filtered DMEM pH 7.4. All bacteria were incubated statically at 37°C, 5% CO₂ for 2-2.5 hours at, 5% CO₂ and then centrifuged at 3500 rpm for 10 minutes. The resulting pellets were washed two times with PBS to remove any traces of SCFAs. After the second wash, the supernatant was removed and all treatments were resuspended in DMEM pH 7.4. OD₆₀₀ readings were taken again to ensure that the cultures were in the logarithmic phase of growth (0.4-0.6) and samples were ready for use in viability, adhesion and cytotoxicity experiments. Table 2.4

summarizes the various experimental short chain fatty acid stress treatments used in this study.

Table 2-4 Short Chain Fatty Acid Stress Experimental Treatments

<i>Experimental Treatment</i>	<i>Description</i>
UU	Unshocked
Acetate	Shocked for 16-18 h with Sodium Acetate (110 mM)
Propionate	Shocked for 16-18 h with Sodium Propionate (70 mM)
Butyrate	Shocked for 16-18 h with Sodium Butyrate (20mM)
Mix	Shocked for 16-18 h Short Chain Fatty Acid Mixture (~ 90mM)
Note:	SCFA solutions were originally made as 10x in dH ₂ O and diluted in LB broth/DMEM pH7.4 during stress treatments. The colon environment was represented by a mixture containing ~ 90mM composed of 69% acetate, 28% propionate and 3% butyrate equal to 60.5 mM acetate, 24.5 mM propionate and 3 mM butyrate)

2.5 Percent Survival of Treated *E. coli* O157:H7

Bacteria exposed to either BS or SCFA were obtained using the appropriate Stress Treatments, as described above. A dilution series for each experimental treatment was made in PBS and the bacteria were plated out on LB agar plates, with or without appropriate antibiotic. All plates were incubated at 37°C for 16-18 hours. Colony counts for plates that contained 30-300 colony forming units (CFU) were completed and used in subsequent calculations to determine the percentage of surviving organisms after BS and SCFA exposure (as assessed by culturability).

2.6 Bacteria- Host Cell Adhesion Assay: Colony Plate Count Method

Adhesion of BS and SCFA treated EHEC strains to either HEP-2 or CaCo-2 cells was assessed by a colony plate count assay as described previously by De Jesus *et al.* (2005). This method allows quantification of live organisms after stress. Using a haemocytometer (Hausser Scientific), epithelial cells grown in a 25 cm² vented flask (BD Falcon) were enumerated and seeded in a 24-well flat bottom plates (BD Falcon) to

approximately 80-90% confluence on the day of the experiment ($\approx 2 \times 10^5$ (24-well)). MEM with additives was added to each well to bring it to 0.5 mL (24-well) and plates were then incubated overnight at 37°C, 5% CO₂. Bacterial cultures of strains grown on LB with the appropriate antibiotics were either BS or SCFA treated using the previously described methods. Using a haemocytometer, epithelial cell counts (cells/mL) from several wells were determined and averaged. This cells/mL value was used in conjunction with the bacterial OD₆₀₀ value to determine the approximate amount of bacterial solution to be added to each well to achieve a multiplicity of infection (MOI) of 100:1. Results indicated that actual MOIs varied from 22- 444. According to preliminary results obtained in the lab the MOI value is critical since there is a correlation between the number of bacteria loaded and adherence to epithelial cells. Results show that the more bacteria are loaded, the less they adhere to epithelial cells. To confirm the MOI, serial dilutions of all bacterial treatments were made in PBS (Wisent without calcium and magnesium) and plated out on LB agar. Only plates with 30-300 colonies were used in subsequent calculations. Prior to cell infection, the media from each well (24-well plates) was removed and cells were washed three times with PBS, pH 7.4. 10^5 cells were infected with 10^7 bacteria so the MOI of 100:1 was achieved and fresh DMEM without antibiotics was added to each well to bring the volume up to either 0.5 mL (24 well plate) or 3 mL (6 well plate). The infected monolayers were incubated at 37°C, 5% CO₂ for 4 hours of infection. After a total of 4 h incubation, the media and non-adherent bacteria were removed, and the wells washed five times with PBS pH 7.4. Adherent bacteria and remaining epithelial cells were detached by adding 250 uL trypsin-EDTA (0.25% trypsin; 1 mM EDTA·4Na). After approximately two minutes incubation at 37°C, 5% CO₂, 50 uL of FBS was used to stop the trypsin reaction, and the cells from each well were added to 1.5 mL microcentrifuge tubes. The wells were rinsed with 200 uL of cold sterile distilled deionized H₂O (ddH₂O) and this was added to the tubes. Complete detachment of cells from wells was verified microscopically. Epithelial cells were lysed with 500 ul cold sterile ddH₂O and then were added to each tube (total 1 mL per well, for 24 well plates). The number of viable bacteria bound to adherent cells, were quantified by serial dilution in PBS performed for each treatment (with triplicates). The appropriate dilutions (10^{-5} , 10^{-6} , 10^{-7}) were plated on LB agar containing antibiotics when necessary, and incubated

between 16-18 hours at 37°C. The following day, the colonies were counted and plates that had between 30-300 colonies were used for further calculations and statistical analysis.

2.7 Impact of Erythromycin-Mediated Inhibition of Protein Synthesis on Bacteria-Host Adhesion

A modified version of a previously described method by de Jesus *et al.* (2005) was used to assess the involvement of newly synthesized adhesins after stress. *E. coli* 86-24 cultures were grown on LB followed by either BS or SCFA treatment. Following the stress treatment, 4 mL of neutralized bacterial suspension, in DMEM pH 7.4 for each treatment, was divided into two tubes of 2 mL aliquots; 2 mL of each treatment will be subjected to erythromycin treatment and the other without. All tubes were centrifuged at 3,300 rpm (2,000 x g) for 10 minutes at 18°C and resuspended in 2 mL DMEM pH 7.4 with or without 0.06 mg/mL erythromycin (Sigma), depending on the treatment. All tubes were left at room temperature for 30 minutes prior to cell infection. The effect of erythromycin on BS stressed bacterial adhesion to HEP-2 cells was determined by a 24 well colony plate count assay as previously described in Section 2.6.

2.8 Bile Salt/Short Chain Fatty Acids Treated *E. coli* O157:H7 86-24 Growth during Infection Time

This experiment was performed to assess the impact of BS and SCFA exposure on the growth of *E. coli* 86-24 throughout the infection time in a four hour bacterial-host cell adhesion assay. Cultures of *E. coli* 86-24 were grown in LB, then either BS or SCFA stressed using the previously described BS or SCFA treatment in Section 2.3, Section 2.4. After stress, the treatments were normalized using prior viability data to ensure that the initial inoculum was approximately the same. To validate the normalization, a dilution series was prepared in PBS for each normalized treatment and plated on LB agar and incubated at 37°C for 16-18 hours. 100 uL of normalized bacteria from each treatment was added to an empty (cell-free) well from a 24-well plate and MEM (without additives) was added to each well to bring the volume to 0.5 mL, and incubated at 37°C, 5% CO₂.

After 4 h incubation time, the media and bacteria were removed and added to 1.5 mL microcentrifuge tubes. To determine the impact of BS and SCFA stress on bacterial growth, serial dilutions using PBS were performed for each treatment. The appropriate dilutions (10^{-5} , 10^{-6} , 10^{-7}) were plated on LB agar (triplicates) and incubated between 16-18 hours at 37°C. Following incubation, colonies were counted and plates that had between 30-300 colonies were used in subsequent calculations and statistical analysis.

2.9 Qualitative Microscopic HEp-2 cell Adhesion Assay after Exposure to Bile Salts/Short Chain Fatty Acids

One day prior to experiment, epithelial cells grown in a 25 cm² vented culture flask (BD Falcon) were enumerated with the aid of a haemocytometer (Hausser Scientific) and then added to 6-well flat bottom plates with coverslips (BD Falcon) so that the cell monolayer would be approximately 80-90% confluent on the experimental day; 8×10^5 cells/well in a 6-well plate. DMEM with additives was then added to each well to bring the volume to 3 mL. The plates were then incubated overnight at 37°C, 5% CO₂. LB culture of *E. coli* 86-24 was subjected to either BS or SCFA stress using previously described BS or SCFA Treatment (Section 2.3 and 2.4). Epithelial cell counts (cells/mL) from 2-3 wells and the approximate amount of bacterial solution to be added to each well were determined as previously described in Bacteria- Host Cell Adhesion Assay: Colony Plate Count Method (Section 2.6). DMEM (without additives) was added to each well to bring the volume up to 3 mL and the well plates were incubated at 37°C, 5% CO₂ for four hours. After 4-hour infection time, media and non adherent cells were removed and wells with coverslips were washed five times with PBS. The cells were fixed by adding 2 ml of 100% methanol (Sigma) and incubated at room temperature for 5 minutes. After incubation, the cells were stained by adding 2 ml of Giemsa stain (Sigma) and incubated at room temperature for 45 minutes. Cells were washed few times with dH₂O. For microscopic preparation, the coverslip from each well was carefully removed with forceps and rinsed with acetic acid solution (1%), placed on a glass slide (25 mm x 75 mm, VWR). Coverslips were then sealed on the glass and were ready for examination

under the light microscope. A Leica DM 5000B Microscope equipped with camera was used. Images were taken at 630x magnification using immersion oil.

2.10 Vero Cell Cytotoxicity Assays

Shiga toxin produced during stress of *E. coli* O157:H7 86-24 was assessed by exposing Vero cells in microtiter cultures to dilutions of toxin. A modified version of The Quantitative Microtiter cytotoxicity assay as previously described by Karmali *et al.*, (1985) and Khine *et al.*, (2004) was used to assess Shiga toxin produced during stress. Briefly, one day before the experiment, Vero cells grown to confluency (80-90%, 75 cm² flasks) were trypsinized with 2 mL of trypsin-EDTA and incubated for approximately 2 minutes at 37°C, 5% CO₂. Following trypsinization, cells were counted and suspended to the desired concentration in growth medium. Seven 96-well microtiter plates (BD Falcon), one for each experimental treatment type (BS or SCFA) and one for the control were seeded with 200 μ L of the vero cell solution using a multichannel pipettor. Monolayers were established by incubating the plates overnight at 37°C, 5% CO₂. Negative controls were wells containing cells not exposed to toxin that were included on each plate. LB culture of 86-24 was subjected to either BS or SCFA stress as previously described in Section 2.3 and 2.4. After stress, the treatments were normalized using prior viability data to ensure that the initial inoculum was approximately the same. To validate the normalization, a dilution series was prepared in PBS for each normalized treatment and plated on LB agar and incubated at 37°C for 16-18 hours. For verotoxin production, 1 mL of each bacterial treatment was added to 14 mL of Penassay broth (antibiotic medium no. 3; Difco Laboratories) and incubated overnight at 37°C and 200 rpm. Following overnight incubation, 2 mL of each treatment were used to obtain OD₆₀₀ readings and/or serial dilutions were performed in PBS. The appropriate serial dilution were plated on LB agar and incubated at 37°C for 16-18 hours. After all treatments, cells were centrifuged at 3,500 rpm (2,000 x g) for 10 minutes at 18°C, 10 mL of the supernatant was filter sterilized using 0.2 μ m membrane filters and 10 cc Leur-Lok syringes (BD Falcon). The supernatant filtrates were stored at -20°C for no longer than a week until use. The remaining supernatant was aspirated and bacterial pellet was

resuspended in 1.5 mL of 0.1 mg/mL colymycin (Parker Davis) and incubated statically at 37°C for 30 minutes. The treatments were then centrifuged at 3300 rpm for 10 minutes at 18°C and the periplasmic extracts were filter sterilized using 0.2 µm membrane filters and 3 cc leur-lok syringes (BD Falcon). The resulting periplasmic extracts were stored at -20°C for no longer than a week until use. On the day of the experiment, frozen experimental extracts and filtrates were thawed and dilutions of the samples 1/1, 1/5, 1/25, 1/125, 1/625 and 1/15625 were made in MEM (no additives) and 50 µL of each dilution for each experimental treatment (both periplasmic extracts and supernatant filtrates) was added, with replicates, to the wells. For the control wells, 50 µL of MEM (no additives) were added. All plates were incubated for 2-3 days at 37°C, 5% CO₂. After incubation, the detached cells, medium and toxin in the wells were removed by vigorous shaking. Remaining live cells in each well were fixed with 65 µL of a 2% solution of formalin (0.05% of a 40% formaldehyde (v/v), 95% PBS (v/v)). The plates were incubated at room temperature for approximately 1-2 minutes and fixative was removed from the wells. The wells of the plates were stained with 65 µL of Crystal Violet Tissue Culture stain (0.26% of 0.5% Crystal Violet Stock Solution (v/v), 0.05% absolute ethanol (v/v), 0.05% of 40% formaldehyde (v/v), 0.64% of PBS (v/v)) and incubated at room temperature for a minimum of 20 minutes. The excess crystal violet stain was removed and the wells were rinsed four times with ddH₂O and the plates were left to air dry. To quantify the results, 100 µL of 10% acetic acid solution (Fisher Scientific) was added to solubilize the Crystal Violet and the absorbance was determined at 570 nm in an ELISA plate reader.

2.11 Statistical Analysis

The mean and standard deviations of all quantitative adhesion and cytotoxicity assay results were calculated. A two-tailed Student *t*-test was used to determine if the results for *E. coli* O157:H7 after exposure to BS or SCFA were significantly different from the control unstressed cells. *P*-values less than or equal to 0.05 were considered statistically significant.

3 Results

3.1 Bile Salt Treatment Enhances *E. coli* O157:H7 Survival

The viability of *E. coli* O157:H7 was predicted to be affected after BS exposure. To determine whether *E. coli* O157:H7 strains were capable of survival after BS treatment, the viability of wild-type strain 86-24 was assessed before and after BS as described in the Methods Section 2.6 using a plate count method. The viability of BS stressed EHEC 86-24 wt was increased 1.2 - 1.5 fold relative to that of unshocked (Fig 3.1). BS treatment resulted in significantly increased viability of log phase *E. coli* O157:H7 strains relative to that of untreated. Similar trends were obtained with other *E. coli* O157:H7 strains such as 8624 *eae* Δ 10 and 8624 *eae* Δ (pEB310) which showed significantly increased survival levels compared to untreated bacteria (data not shown).

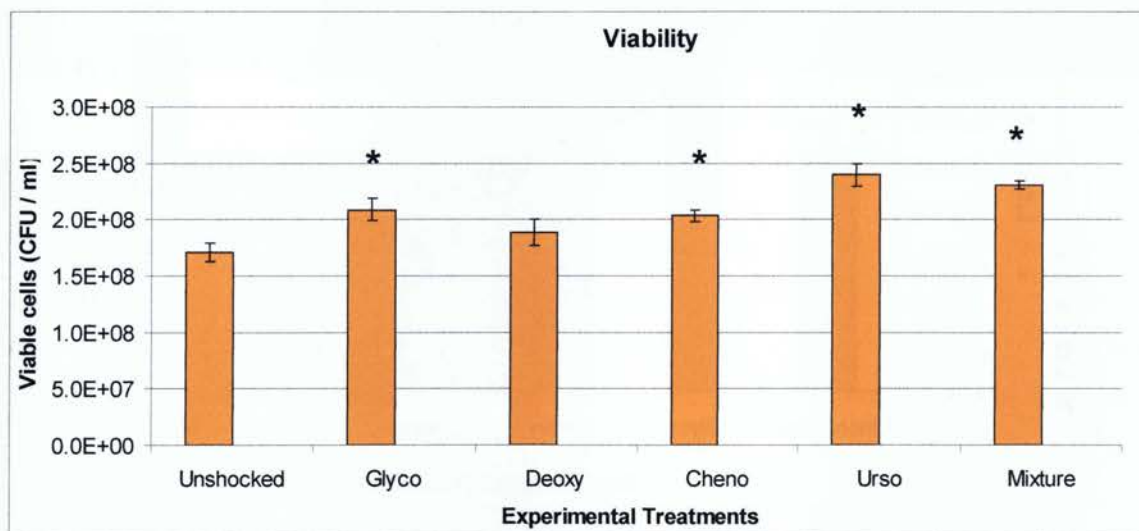


Figure 3.1-1 BS Treatment Enhances EHEC O157:H7 Survival

Effect of BS exposure on *E. coli* O157:H7 86-24 viability was assessed by plate count assay (n=3). Values are means \pm standard deviations and are representative of a minimum 4 experiments. BS Stress Treatment: 90 min, 37°C, 5% CO₂ Cheno/Deoxy/Urso/Glyco: 2.5mM; BS Mixture: 0.15% w/v. [Untreated: Control (Untreated for 90 minutes); Glyco: Glycocholate treated for 90 minutes; Deoxy: Deoxycholate treated for 90 minutes; Cheno: Chenodeoxycholate treated for 90 minutes;;); Urso: Ursodeoxycholate treated for 90 minutes; Mixture: BS Mixture treated for 90 minutes]. *Significantly different from corresponding control (P < 0.05).

3.2 Bile Salt Treatment Enhances *E. coli* O157:H7 Adhesion to Human Epithelial Cells

To judge the level of adherence of *E. coli* O157:H7 strains to human epithelial cells, adhesion assays before and after BS stress were conducted. Bacterial adhesion was determined by plate count assay (Fig 3.2-1) and light microscopy was used as a complimentary method (Figure 3.2-2). Adhesion of BS mixture and glycholate treated EHEC 86-24 to epithelial cells was significantly higher than that of the untreated control EHEC 86-24. Adhesion enhancement factors ranged from 1.5 - 2.1 for HEP-2 cells to 4.8 for Caco-2 cells (Fig 3.2-1, Figure 3.3-1 and Table 1 Supplementary Data).

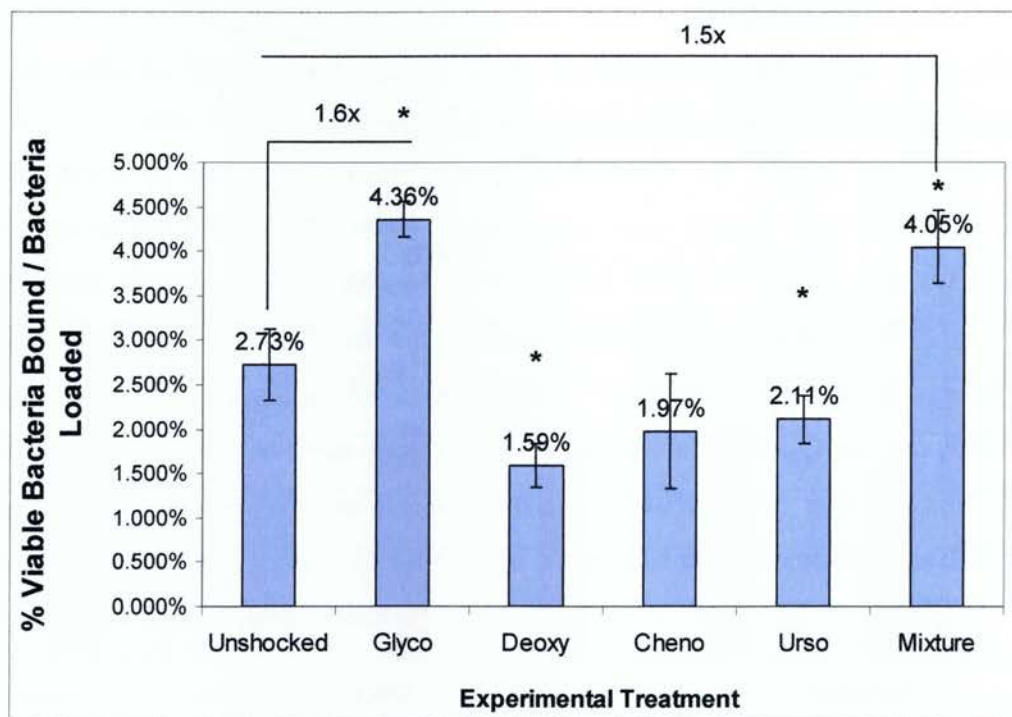


Figure 3.2-1 BS Treated *E. coli* O157:H7 86-24 Show Enhanced Adhesion to HEP-2 Cells

Effect of BS treatment of *E. coli* O157:H7 86-24 on adhesion to HEP-2 cells (MOI range 45-57) was assessed by plate count assay (n= 4-6 for each experiment). The results of 2 independent experiments are shown and the values are means \pm standard deviations. BS Stress Treatment: 90 min, 37°C, 5% CO₂ Cheno/Deoxy/Urso/Glyco: 2.5mM; BS Mix: 0.15% w/v. [Untreated: Control (Untreated for 90 minutes); Glyco: Glycocholate treated for 90 minutes; Deoxy: Deoxycholate treated for 90 minutes; Cheno: Chenodeoxycholate treated for 90 minutes;; Urso: Ursodeoxycholate treated for 90 minutes; Mixture: BS Mixture treated for 90 minutes]. *Significantly different from corresponding control (P < 0.05);

In agreement with the previous results obtained, light microscopy images of infected Giemsa stained cells showed obvious increased adherence of BS mixture and glycocholate treated *E. coli* O157:H7 as compared with untreated strains (Figure 3.2.2). HEp-2 cell monolayers were incubated with BS treated EHEC 86-24 for 4 hours as described in Materials & Methods (Section 2.6). Adhesion patterns were typical of those reported for EHEC adherence to cultured epithelial cells (Torres and Kaper, 2003).

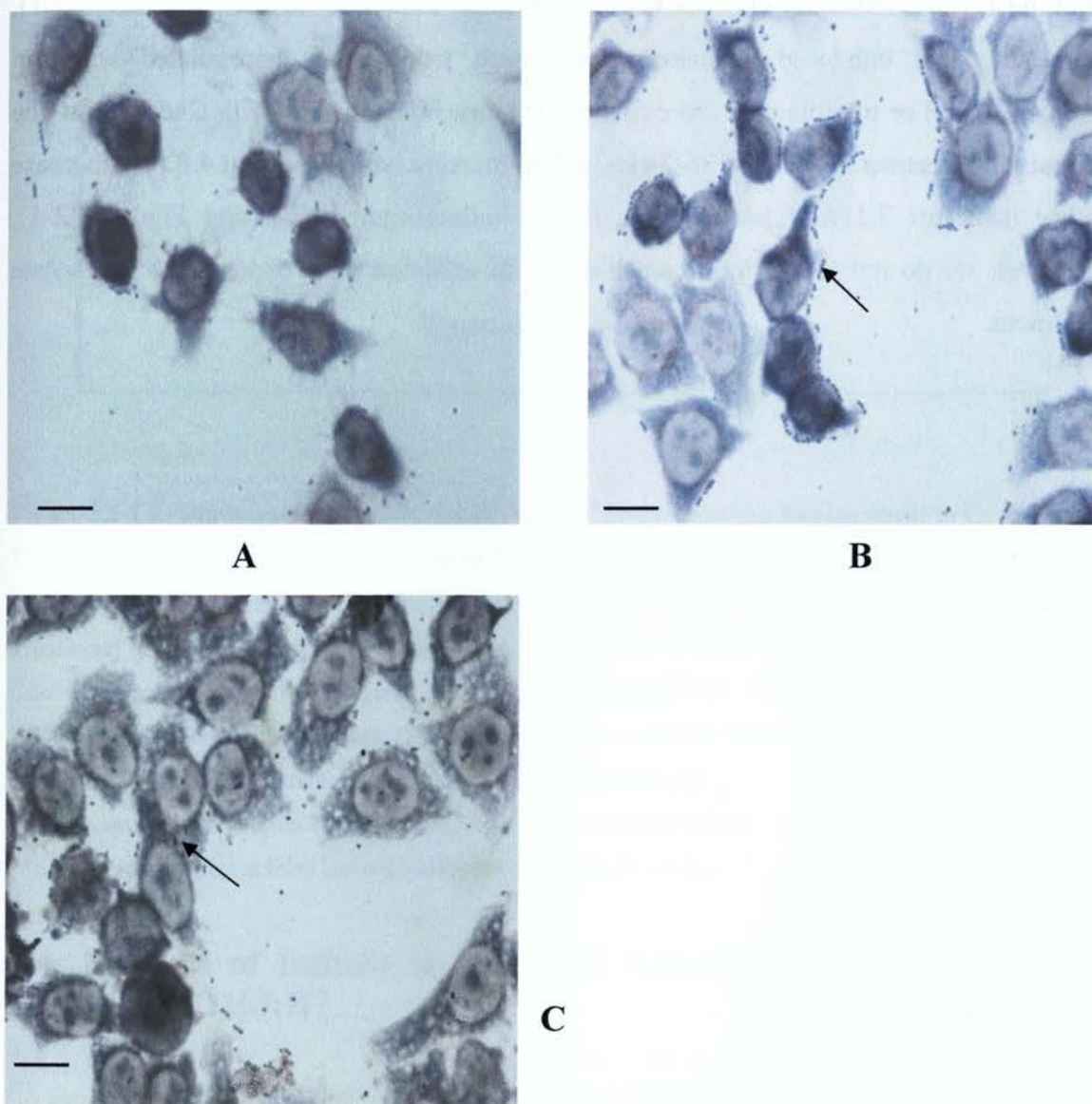


Figure 3.2-2 Qualitative Comparison of BS Treated *E. coli* O157:H7 86-24 Adhesion to HEp-2 Cells
 Effect of BS Treatment of *E. coli* O157:H7 86-24 on adhesion to HEp-2 cells using light microscopy. A: Untreated control; B: BS Mixture Treatment; C: Glycocholate Treatment; BS Treatment: 90 min, 37°C, 5% CO₂ Glyco: 2.5mM; BS Mixture: 0.15% w/v. The black arrows indicate either BS or Glycocholate treated *E. coli* cells attached to HEp-2 cells. Scale bar on A,B, and C represents 7 microns

3.3 Erythromycin Treatment Abrogates EHEC O157:H7 Adhesion Enhancement

To investigate further whether the adhesion enhancement is a result of increased expression of adhesins, erythromycin, below the MIC, was used to inhibit protein synthesis (de Jesus *et al.*, 2005). Pretreatment of BS treated bacteria with erythromycin eliminated the adhesion enhancement seen after exposure to BS (Figure 3.3-1), suggesting that enhanced adhesion could result from either upregulated adhesion expression and or modifications to existing adhesins. Interestingly, with Caco2 cells, the BS mixture treatment of EHEC 86-24 caused an increase which is about 4.8 fold increase rather than just 2.1 fold increase for HEp-2 cells (Figure 3.3-1 and Figure 3.2-1). However, we do not see a significant increase in adhesion after exposure to glycholate treatment.

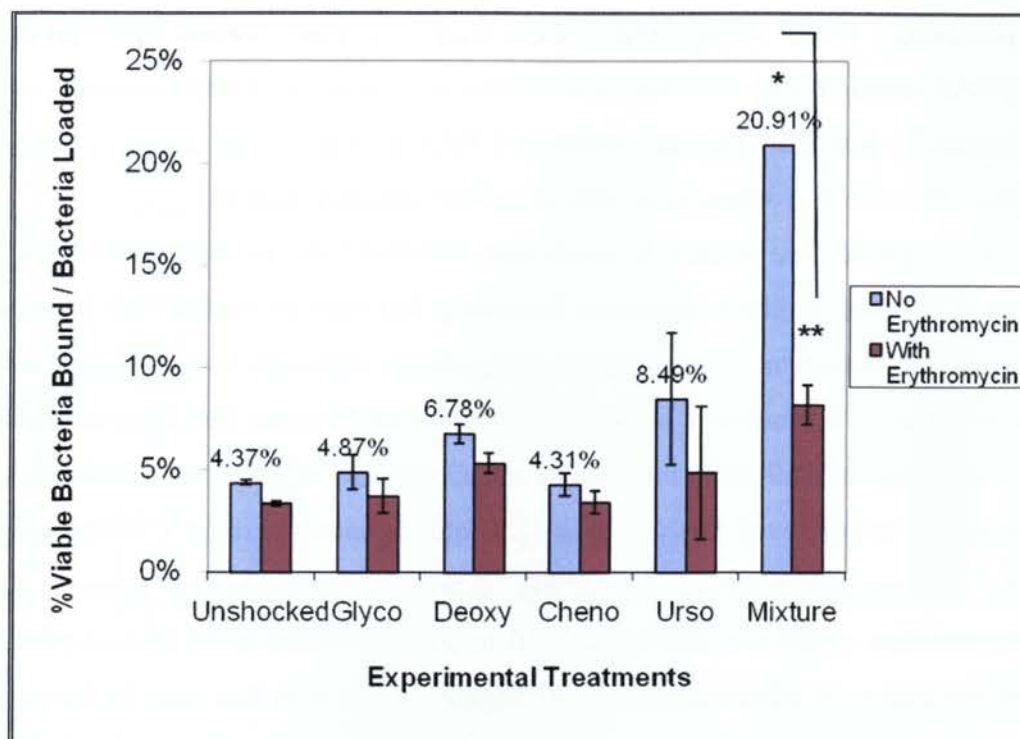


Figure 3.3-1 Erythromycin Abrogates *E. coli* O157:H7 86-24 Adhesion Enhancement to Caco-2 Cells.

The effect of erythromycin on BS treatment of *E. coli* O157:H7 86-24 adhesion to Caco-2 cells (MOI range 114-150) was assessed by plate count assay ($n=6$), and the values are means \pm standard deviations. BS Treatment: 90 min, 37°C, 5% CO₂ Cheno/Deoxy/Urso/Glyco: 2.5mM; BS Mix: 0.15% w/v. [Untreated: Control (Untreated for 90 minutes); Cheno: Chenodeoxycholate treated for 90 minutes; Deoxy: Deoxycholate treated for 90 minutes; Glyco: Glycocholate treated for 90 minutes); Urso: Ursodeoxycholate treated for 90 minutes; Mixture: BS Mixture treated for 90 minutes].

*Significantly different from corresponding unshocked control ($P < 0.05$)

**Significantly different from corresponding BS treatment ($P < 0.05$)

3.4 No Role of Intimin in Enhanced Adhesion of Bile Salt Treated EHEC O157:H7

To explore the contribution of intimin, a known adhesin in increased adhesion after BS stress, intimin mutants (86-24*eae* Δ 10 and 86-24*eae* Δ (pEB310)) were used to investigate adherence to human intestinal cells with and without BS exposure. To achieve this aim, first we ran a control experiment to compare adhesion capability of *E. coli* O157:H7 wild-type 86-24 and intimin mutants obtained with the tissue culture

adherence assays. It was verified that intimin negative mutant adhered significantly less than parental strain (8 fold decrease) and intimin recomplemented mutant strain adhered only marginally less than parental strain (1.4 fold decrease). This result confirms the importance of intimin in adhesion to cells in culture (data not shown).

Although the *eae* mutant is much less adherent than wild-type, adhesion after exposure to BS was similarly increased, indicating that intimin is likely not involved in the adhesion enhancement (Figure 3.4-1). Interestingly, although both untreated and BS treated 86-24*eae*Δ10 were only weakly adherent (about 10 times less than untreated wt 86-24) there was a significantly increase in adhesion of BS mixture treated 86-24*eae*Δ10 relative to that of untreated 86-24*eae*Δ10 (2.3 fold adhesion increase), which suggests that the BS mixture may be triggering expression of adhesins (Figure 3.4-1). Complementation of the *eae* gene in pEB310 in 86-24 (resulted in 86-24*eae*Δ(pEB310)) showed restoration of adherence to a level slightly higher than that seen in the parental strain (1.5 fold adhesion increase after BS mixture treatment) (Figure 3.4-2). Taken together, the adherence data suggest that intimin is not the adhesin responsible for the increase in adhesion observed after BS treatment. Other colonization factors may contribute to increased adhesion to human intestinal cells.

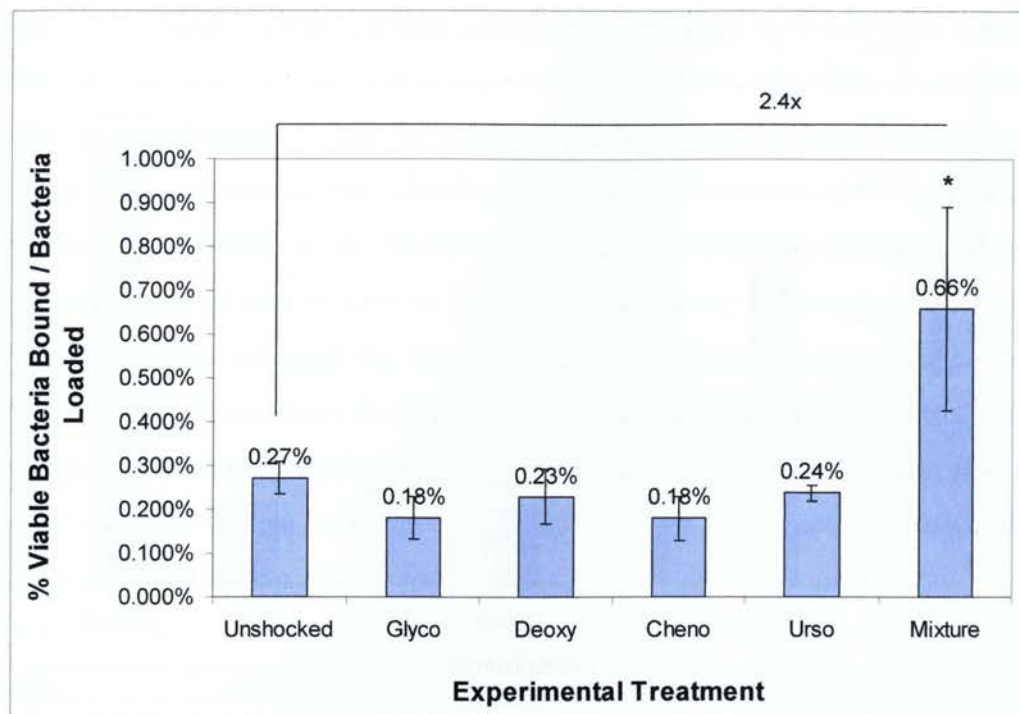


Figure 3.4-1 BS Treated *E. coli* O157:H7 86-24 *eae*Δ10 Show Enhanced Adhesion to HEp-2 Cells

Effect of BS shock of *E. coli* O157:H7 86-24 *eae*Δ10 on adhesion to HEp2 cells (MOI range 57-105) was assessed by plate count assay (n=3). Data shown are representative of 2 independent experiments and the values are means \pm standard deviations. BS Treatment: 90 min, 37°C, 5% CO₂ Cheno/Deoxy/Urso/Glyco: 2.5 mM; BS Mixture: 0.15% w/v. [Untreated: Control (Untreated for 90 minutes); Glyco: Glycocholate treated for 90 minutes; Deoxy: Deoxycholate treated for 90 minutes; Cheno: Chenodeoxycholate treated for 90 minutes; ; Urso: Ursodeoxycholate treated for 90 minutes; Mixture: BS Mixture treated for 90 minutes].

*Significantly different from corresponding control ($P < 0.05$);

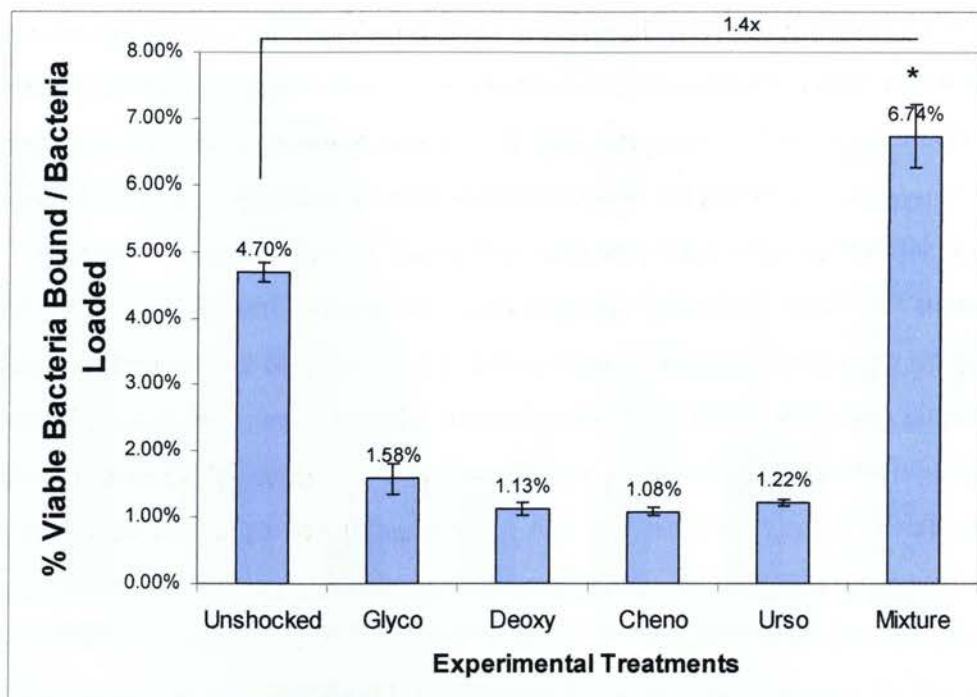


Figure 3.4-2 BS Treated *E. coli* O157:H7 8624 *eae*Δ(pEB310) Show Enhanced Adhesion to HEp-2 Cells.

Effect of BS shock of *E. coli* O157:H7 86-24 *eae*Δ10 (pEB310) on adhesion to HEp-2 cells (MOI range 67-92) was assessed by plate count assay (n=3). Data shown are representative of 2 independent experiments and the values are means \pm standard deviations. BS Treatment: 90 min, 37°C, 5% CO₂ Cheno/Deoxy/Urso/Glyco: 2.5 mM; BS Mixture: 0.15% w/v. [Untreated: Control (Untreated for 90 minutes); Glyco: Glycocholate treated for 90 minutes; Deoxy: Deoxycholate treated for 90 minutes; Cheno: Chenodeoxycholate treated for 90 minutes; Urso: Ursodeoxycholate treated for 90 minutes; Mixture: BS Mixture treated for 90 minutes].

*Significantly different from corresponding control ($P < 0.05$);

3.5 No Role of Iha in Enhanced Adhesion of Bile Salt Treated EHEC O157:H7

To investigate whether Iha (bacterial adherence-conferring protein) is a prerequisite for increased adhesion in BS treated EHEC, *iha* mutants and nonpathogenic *E. coli* complemented with *iha* were used in quantitative adherence assays. Firstly, 86-24Δ*iha* mutant adhered at about 70% of the wt strain 86-24 level (1.69% and 2.5%

respectively) (Figure 3.5-1). These results are consistent with previous reports that indicate that *iha* plays a role in adhesion of EHEC to host cells (Tarr *et al.*, 2000). To confirm the involvement of *iha*, adhesion of wt 86-24 and 86-24 Δ *iha* were compared before and after exposure to BS. Similar adhesion enhancements were noted for strains 86-24 Δ *iha* (1.7 fold adhesion increase) and wt 86-24 (2.1 fold adhesion increase) relative to their untreated 86-24 Δ *iha* and wt 86-24 control strains (Figure 3.5-1 and Table 1 Supplementary Data). Although the *iha* mutant is significantly less adherent (2.88%) than wt 86-24 (5.29%), there was a similarly increased adhesion after exposure to BS mixture relative to that of untreated controls (Figure 3.5-1). These results suggest that *E. coli* O157:H7 86-24 does not utilize *iha* for the increased adherence observed after BS exposure, and other factors must be involved.

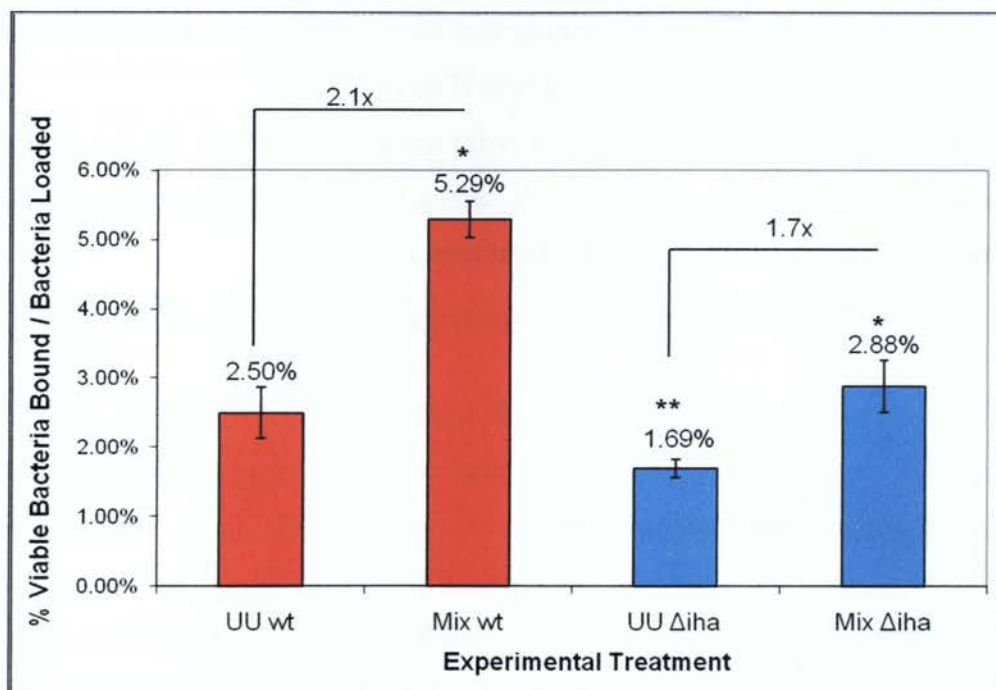


Figure 3.5-1 BS Treated *E. coli* O157:H7 86-24 Δ *iha* Show Enhanced Adhesion to HEp-2 Cells

Effect of BS shock of *E. coli* O157:H7 8624 Δ *iha* on adhesion to HEp-2 cells (MOI range 99-101) was assessed by plate count assay (n=3). Results shown are representative of 2 independent experiments. Values are means \pm standard deviations. BS Treatment: 90 min, 37°C, 5% CO₂ BS Mix: 0.15% w/v. [Untreated: Control (Untreated for 90 minutes); Mixture: BS Mixture treated for 90 minutes].

*Significantly different from corresponding control (P < 0.05)

**Significantly different from corresponding untreated (P < 0.05);

Since Iha has been described to contribute to enhanced adherence of non-adherent laboratory *E. coli* strains, we tested BS treated ORN172(pIHA) and ORN172(pSK+) to determine if Iha is responsible for the enhanced adhesion after BS exposure. *E. coli* ORN172(pIHA) is a nonvirulent laboratory strain which expresses the *iha* in trans, while ORN172(pSK+) carries the plasmid alone and does not carry *iha* in its chromosome (Tarr *et al.*, 2000). Untreated *E. coli* ORN 172(pIha) adhered 4x more than ORN172 (pSK+) demonstrating that Iha plays a major role in adhesion when transgenically expressed in a non-pathogenic *E. coli* background (Figure 3.5-2). There is no significant increase in adhesion of mixture treated ORN 172(pIHA) relative to the untreated, confirming that Iha does not likely play a role in BS induced adhesion of *E. coli* O157:H7 (Figure 3.5-2). There is also no significant increase in the mixture treated in ORN172 (pSK+) relative to the untreated, suggesting that there is no upregulation of adhesins in the nonpathogenic ORN 172 after BS treatment (Figure 3.5-2).

Taken together both sets of results point toward no role for *iha* suggesting that other adhesins or virulence factors which are not present in laboratory strains are necessary for increased adhesion after BS treatment.

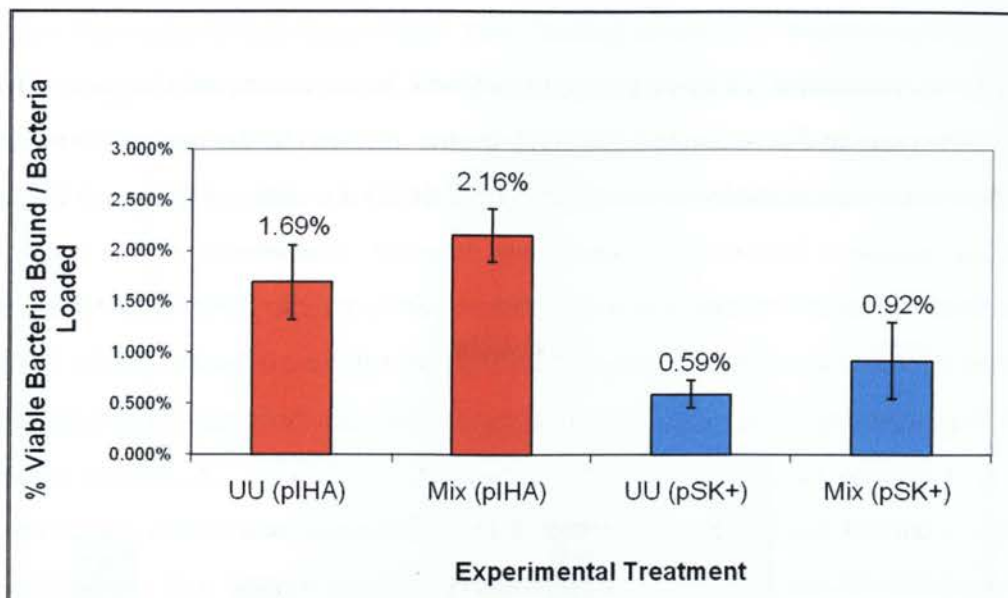


Figure 3.5-2 BS Treated *E.coli* ORN 172(pIHA) and (pSK+) Adhesion to HEp-2 Cells

Effect of BS shock of *E.coli* ORN172(pIHA) and ORN172(pSK+) on adhesion to HEp-2 cells was assessed by plate count assay (n=3). Values are means \pm standard deviations. BS Stress Treatment: 90 min, 37°C, 5% CO₂; BS Mixture: 0.15% w/v. [Untreated: Control (Untreated for 90 minutes); Mixture: BS Mixture treated for 90 minutes]. MOI range for pIHA 182-199 and MOI range for pSK+ 424-444

3.6 Short Chain Fatty Acids Decrease *E. coli* O157:H7 Viability

Several species of Gram-negative bacteria are known for their considerable tolerance to SCFA (Lasko *et al.*, 2000; Arnold *et al.*, 2001; Lawhon *et al.*, 2002; Polen *et al.*, 2003; Maurer *et al.*, 2005). Less toxic effect on some microorganisms has been attributed to the weak acid nature of most of the organic acids. However, SCFA are capable of exhibiting bacteriostatic and bactericidal properties. Most of these compounds can easily penetrate the lipid membrane of the bacterial cell where they dissociate into anions and protons and may stress bacteria by depleting cellular energy (Ricke, 2002). Our hypothesis was that survival of EHEC will be reduced after SCFA stress. To test this hypothesis, we employed a plate count method to assess whether *E. coli* O157:H7 86-24 was capable of survival after SCFA exposure. As predicted, in the presence of either individual treatments or a mixture of all SCFAs, *E. coli* O157:H7 86-24 resulted in significantly lower viabilities relative to that of the control by a factor of 1.3 - 1.5.

Butyrate and propionate treatments showed even significantly lower survival levels over the other two treatments; acetate and mixture. These observations may suggest a higher impact of butyrate and propionate treatments on the survival of the bacteria. The results of SCFA stress on the viability of *E. coli* O157:H7 86-24 are shown in Figure 3.6-1.

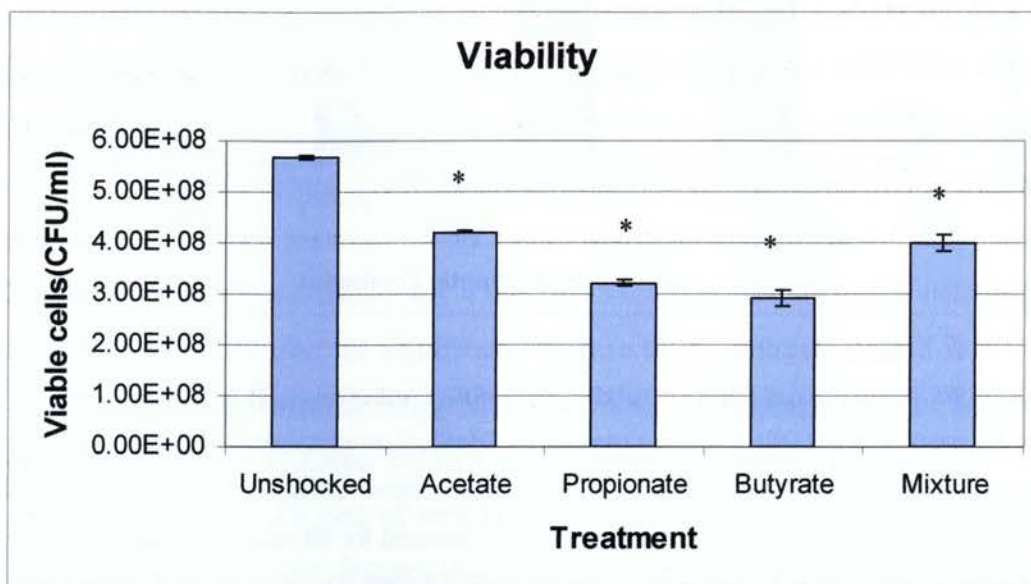


Figure 3.6-1 SCFA Treatment Decreases EHEC O157:H7 86-24 Survival

Effect of SCFA stress on *E. coli* O157:H7 86-24 viability was assessed by a plate count assay (n=3). This is the result of 2 independent experiments. Values are means \pm standard deviations. Short Chain Fatty Acid Treatment: 16-18 hr, 37°C, 5% CO₂, Sodium Acetate (110 mM), Sodium Propionate (70 mM), Sodium Butyrate (20 mM) and a Mixture: combined acetate-propionate-butyrate. [Unshocked: Control (Unshocked for 16-18 hr); Acetate: Sodium Acetate shocked for 16-18 hr; Propionate: Sodium Propionate shocked for 16-18 hr; Butyrate: Sodium Butyrate shocked for 16-18 hr; Mixture: Mixture of the above shocked for 16-18 hr].

*Significantly different from corresponding control (P < 0.05)

3.7 Short Chain Fatty Acids Stress Enhances *E. coli* O157:H7 Adhesion to Human Epithelial Cells

In order to establish infection, *E. coli* O157:H7 must overcome the short chain fatty acid stress environment of the large intestine. Since there are no previous studies that have addressed the role of SCFA on EHEC adherence capability, the present study tried to clarify whether SCFA stress has any impact on EHEC adhesion to epithelial cells.

To judge the level of adherence of *E. coli* O157:H7 strains to human epithelial cells, adhesion assays before and after SCFA stress were conducted. Quantification of bacterial adherence to human intestinal cells was determined by plate count assay as previously described by de Jesus *et al.* (2005). The effect of different SCFAs was examined, individually and in combination (acetate, propionate, butyrate and a mixture of above). EHEC 86-24 (O157:H7) strains were assessed with either human colonic Caco-2 or non-intestinal HEp-2 epithelial cells for 4 hours. No significantly different bacterial adherence on epithelial cells was produced with either acetate or propionate treatments relative to unshocked control.

However, adhesion of butyrate and SCFA mixture treated EHEC 86-24 to epithelial cells (either HEp-2 or Caco-2 cells) was significantly higher than that of untreated control EHEC 86-24. Adhesion enhancement factors ranged from 1.5 – 1.8 for butyrate and 1.6 - 3 for SCFA mixture (Fig 3.7-1; Figure 3.8-1 and Table 2 Supplementary Data). It is possible that changes in bacterial adherence to eukaryotic cells after treatment could depend on the concentration of SCFA used. Future research should examine the role of concentration of SCFA in the adhesion of treated bacteria.

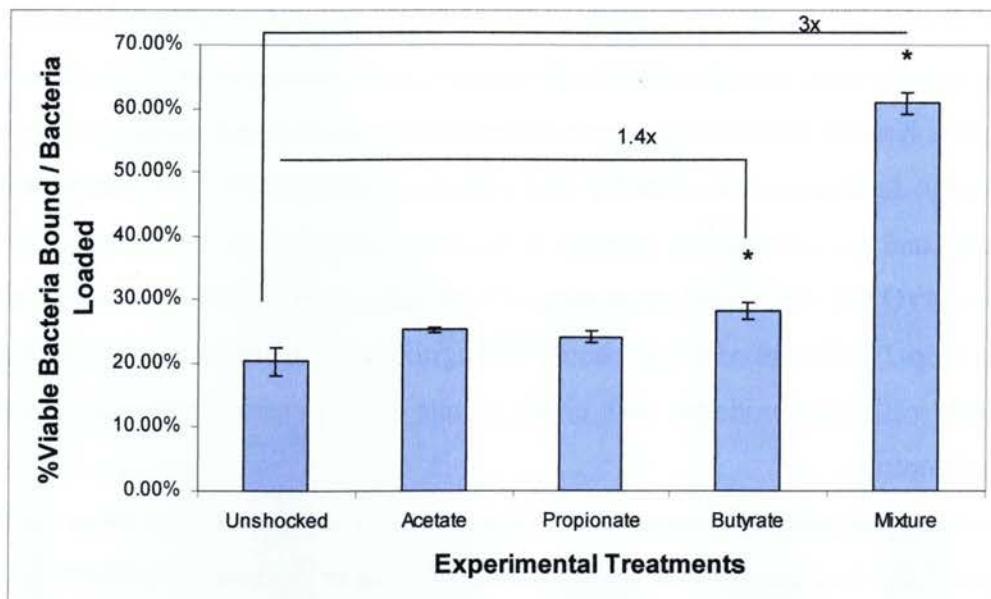


Figure 3.7-1 SCFA Shocked *E. coli* O157:H7 86-24 Show Enhanced Adhesion to HEp-2 Cells

Effect of SCFA stress on *E. coli* O157:H7 8624 in adhesion to HEp-2 cells (MOI range 34-60) was assessed by plate count assay (n= 3). Results representative of 2 independent experiments are shown and the values are means \pm standard deviations. Short Chain Fatty Acid Stress Treatment: 16 hr, 37°C, Acetate: 110 mM; Propionate: 70 mM; Butyrate: 20 mM; Mixture of combined acetate-propionate-butyrate: ~ 90 mM

*Significantly different from corresponding control ($P < 0.05$);

3.8 Erythromycin Treatment Abrogates Short Chain Fatty Acids Host Adhesion Enhancement

To determine whether the adhesion enhancement observed after SCFA stress was a result of increased expression of adhesins, erythromycin, below the MIC, was used to inhibit protein synthesis (de Jesus *et al.*, 2005). Following stress, all treatments were incubated at room temperature for 30 minutes with or without 0.06 mg/mL erythromycin. The adhesion capability of *E. coli* O157:H7 86-24 after SCFA stress exposure to HEp2 cells was examined in the presence of erythromycin. Pretreatment of bacteria with erythromycin significantly decreased the adhesive capability of *E. coli* O157:H7 to Caco-2 cells (Figure 3.8-1).

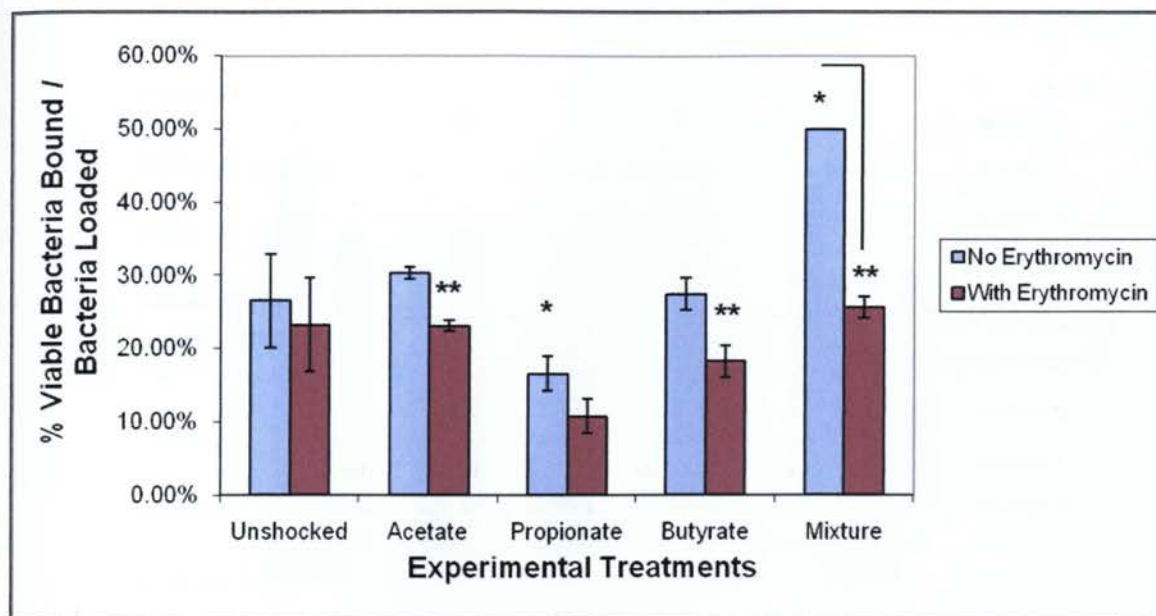


Figure 3.8-1 Erythromycin Treatment Reduces SCFA Host Adhesion Enhancement to Caco-2 Cells

The effect of erythromycin pretreatment on SCFA stressed *E. coli* O157:H7 in adhesion to HEP-2 cells (MOI range 22-60) was assessed by plate count assay (n= 6). Values are means \pm standard deviations.

* Significantly different from unshocked control ($p \leq 0.05$).

** Significantly different from corresponding SCFA control without erythromycin ($p \leq 0.05$);

3.9 The Effect of Bile Salts and Short Chain Fatty Acids Stress on *E. coli* O157:H7 86-24 Growth during Infection Time

The enhanced adhesion of BS and SCFA stressed *E. coli* O157:H7 to tissue culture cells may be due to increased growth that may arise after BS or SCFA stress. To test this possibility, control adhesion assays were employed; *E. coli* O157:H7 86-24 was exposed to either BS or SCFA and then incubated, but without epithelial cells, simulating the conditions of the adhesion assays previously described. After 4 h incubation at 37°C with 5% CO₂ bacteria were plated out and the next day counted (Figure 3.9-1 and Figure 3.9-2). Three of the treatments (glycholate, deoxycholate and ursocholate) showed significantly higher final viability compared to the control. These results may point out the possibility that the enhanced adhesion of BS stressed *E. coli* O157:H7 86-24 might be due to increased growth after stress.

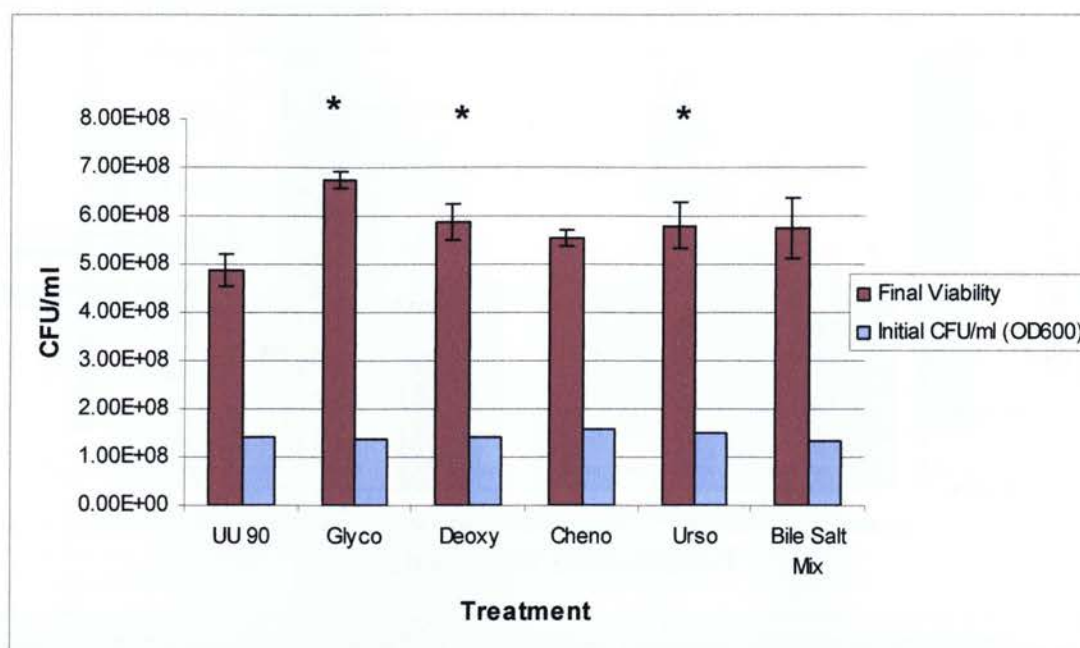


Figure 3.9-1 The Effect of BS Treatment on *E. coli* O157:H7 86-24 Viability during 4 h Incubation

The effect of BS Treatment on *E. coli* O157:H7 86-24 viability was assessed by plate count assay (n= 3 for each experiment) Initial viability: initial amount of bacteria loaded. Final viability: Culturability after 4 h incubation at 37°C and 5% CO₂. The results are representative of 2 independent experiments and the values are means ± standard deviations. BS Stress Treatment: 90 min, 37°C, 5% CO₂

Cheno/Deoxy/Urso/Glyco: 2.5 mM; BS Mix: 0.15% w/v. [Unshocked: Control (Unshocked for 90 minutes); Cheno: Chenodeoxycholate shocked for 90 minutes; Deoxy: Deoxycholate shocked for 90 minutes; Glyco: Glycocholate shocked for 90 minutes); Urso: Ursodeoxycholate shocked for 90 minutes; Mixture: BS Mixture shocked for 90 minutes].

*Significantly different from corresponding control (P < 0.05)

The growth of SCFA stressed *E. coli* O157:H7 86-24 during 4 hr incubation time showed a significant decrease in viability for all treatments in comparison to the control. These results are consistent with previous viability results of EHEC 86-24 after exposure to SCFAs and indicate that increased adhesion of SCFA stressed *E. coli* O157:H7 86-24 is not mediated through increased growth during infection time.

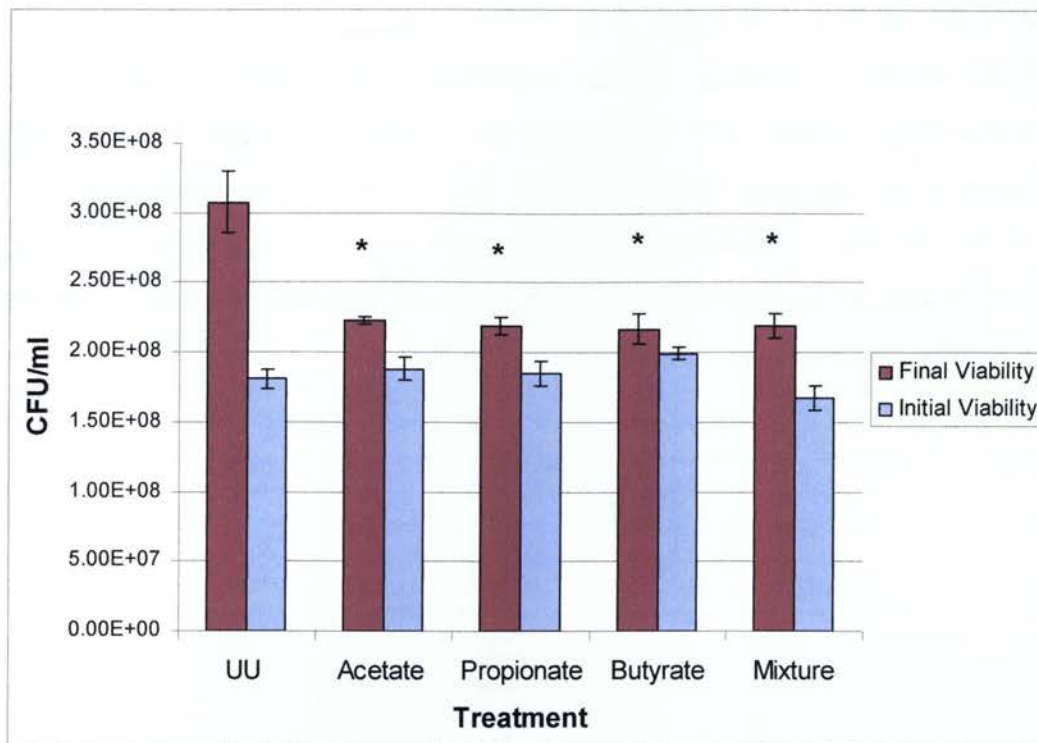


Figure 3.9-2 The Effect of SCFA Stress on *E. coli* O157:H7 86-24 Viability during 4 h Incubation

The effect of BS Treatment on *E. coli* O157:H7 86-24 viability assessed by plate count assay (n= 3 for each experiment). Initial viability: initial amount of bacteria loaded. Final viability: Culturability after 4 h incubation at 37 °C and 5% CO₂. The results are representative of 2 independent experiments and the values are means ± standard deviations. Short Chain Fatty Acid Stress Treatment: 16 hr, 37°C, Acetate: 110 mM; Propionate: 70mM; Butyrate: 20 mM; Mixture of combined acetate-propionate-butyrate: ~ 90 mM

* Significantly different from corresponding untreated (P < 0.05)

3.10 No Change in Secreted and Periplasmic VT Production after BS Treatment

The effect of BS stress on both the verotoxin secreted, as well as that retained in the periplasmic space of 86-24 was examined using the vero cell cytotoxicity assay. The bacteria were subjected to either BS or SCFA stress, then normalized based on prior viability experiments and grown overnight in rich media to induce verotoxin production. Various dilutions of the supernatant filtrates and periplasmic extracts were incubated with semi-confluent monolayers of vero cells for 2-3 days at 37°C with 5% CO₂. The remaining live cells were fixed with 2% formalin and stained with crystal violet. The

crystal violet staining of verocells is an indirect measure of levels of toxin produced. Levels of secreted verotoxin (in the supernatant) and verotoxin retained in the periplasmic space formed with BS-stressed 86-24 were determined by measuring the absorbance of crystal violet with an ELISA plate reader (Figures 3.10-1 and 3.10-2). None of the BS stress treatments appeared to show any significant change in verotoxin production either in the periplasmic extracts or in the supernatant filtrates compared to the untreated controls.

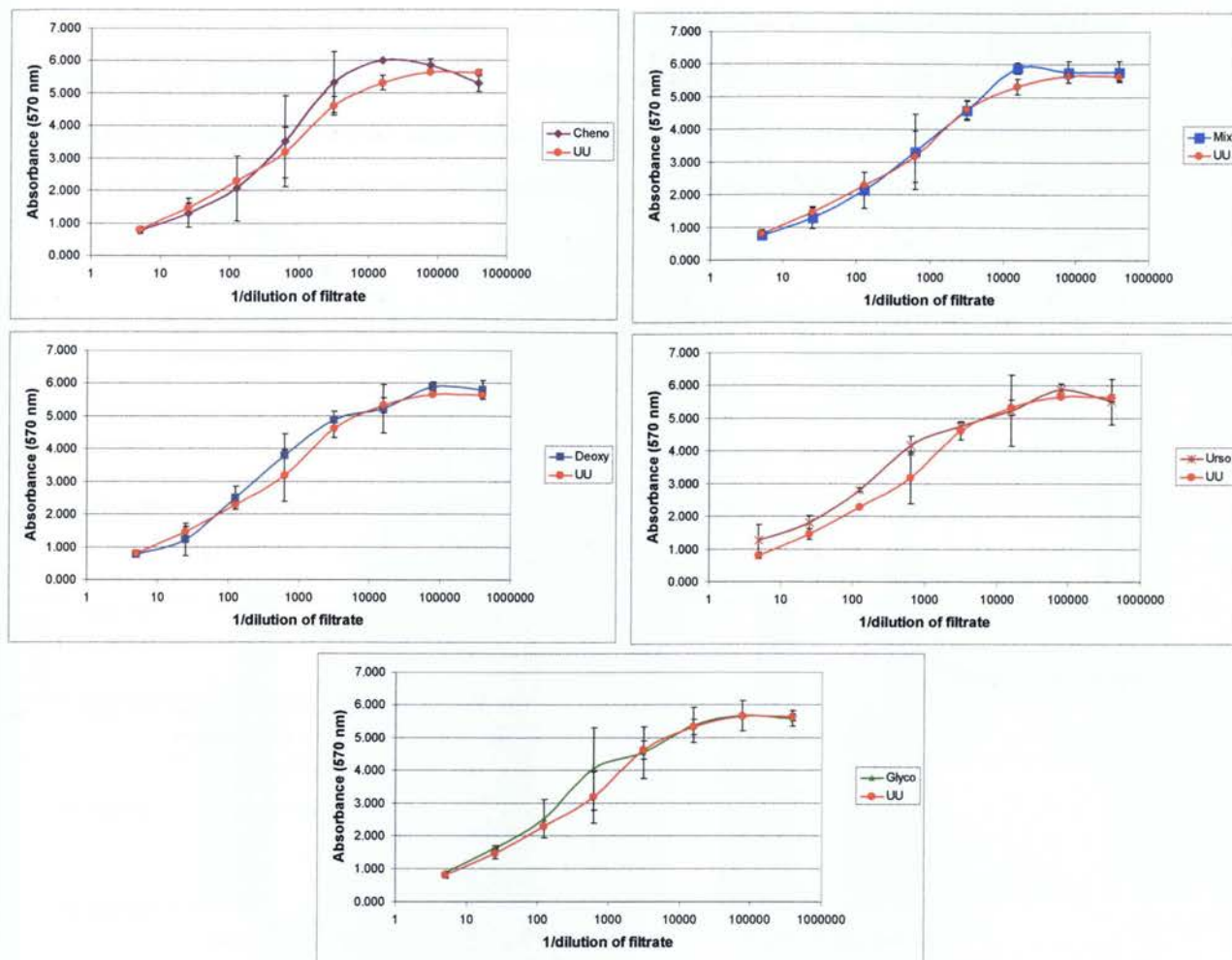


Figure 3.10-1 Secreted Verotoxin Production of *E. coli* O157:H7 86-24 after Exposure to BS

The effect of BS treatment on the verotoxin secreted in the supernatant by *E. coli* O157:H7 86-24 was measured by the vero cell cytotoxicity assay. Results are expressed as the mean absorbance \pm standard deviations (corresponding to the remaining live vero cells) versus the 1/dilution of verotoxin in the supernatant and are representative of two independent experiments. Experimental treatments: Unshocked: Control (Unshocked for 90 minutes); Cheno: Chenodeoxycholate shocked for 90 minutes; Deoxy: Deoxycholate shocked for 90 minutes; Glyco: Glycocholate shocked for 90 minutes; Urso: Ursodeoxycholate shocked for 90 minutes; Mixture: BS Mixture shocked for 90 minutes].

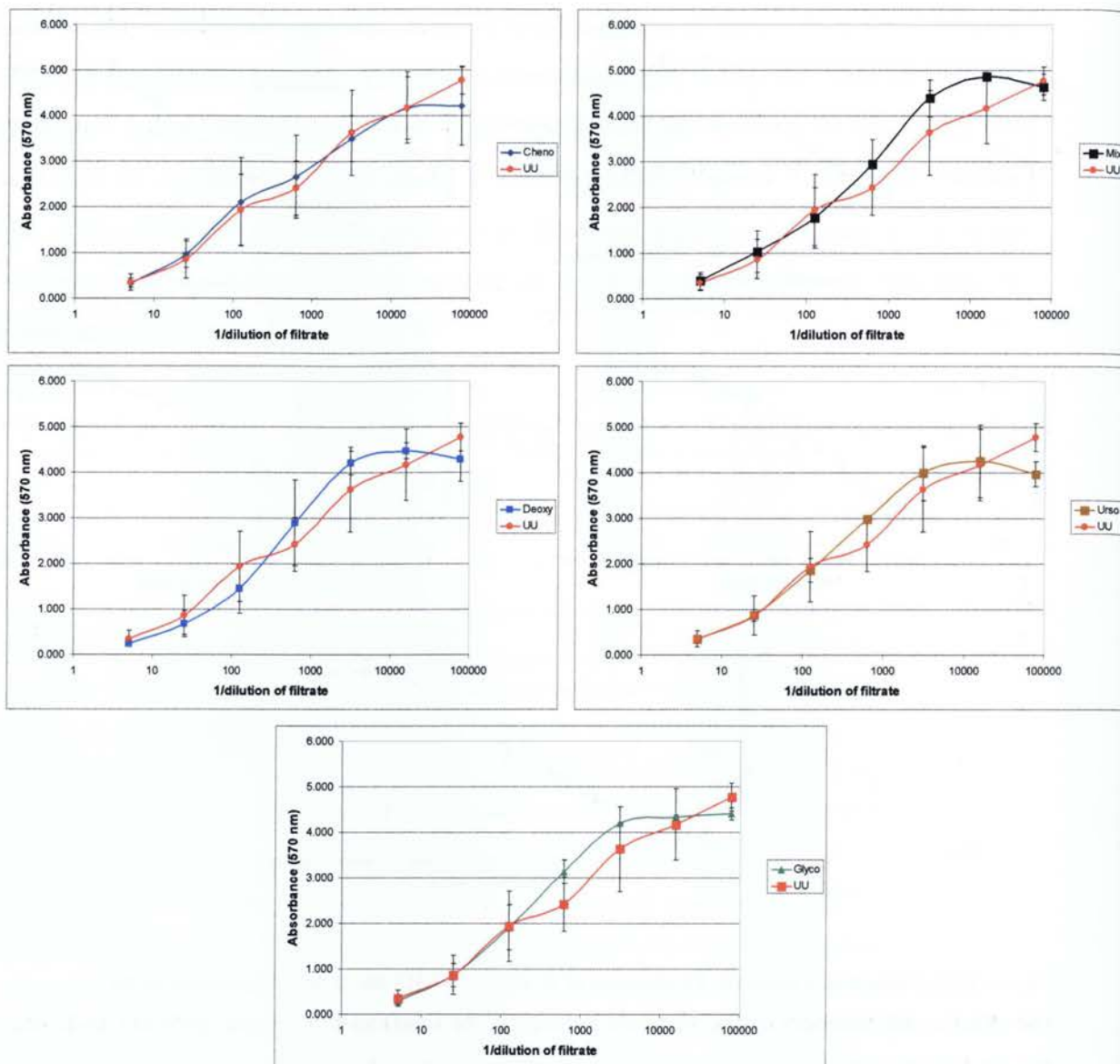


Figure 3.10-2 Periplasmic Verotoxin Production of *E. coli* O157:H7 86-24 after Exposure to BS

The effect of BS treatment on the verotoxin produced in the periplasmic space by *E. coli* O157:H7 86-24 was measured by the vero cell cytotoxicity assay. Results are expressed as the mean absorbance \pm standard deviations (corresponding to the remaining live vero cells) versus the 1/dilution of verotoxin in the supernatant and are representative of two independent experiments. Experimental treatments: Unshocked: Control (Unshocked for 90 minutes); Cheno: Chenodeoxycholate shocked for 90 minutes; Deoxy: Deoxycholate shocked for 90 minutes; Glyco: Glycocholate shocked for 90 minutes; Urso: Ursodeoxycholate shocked for 90 minutes; Mixture: BS Mixture shocked for 90 minutes].

To confirm that these experimental results were not the product of significantly different initial bacteria inoculums, the initial and final CFU/mL for each treatment prior to and after overnight growth, were measured (Figure 3.10-3). Results demonstrate that initial and final bacteria concentrations were the same across all treatments.

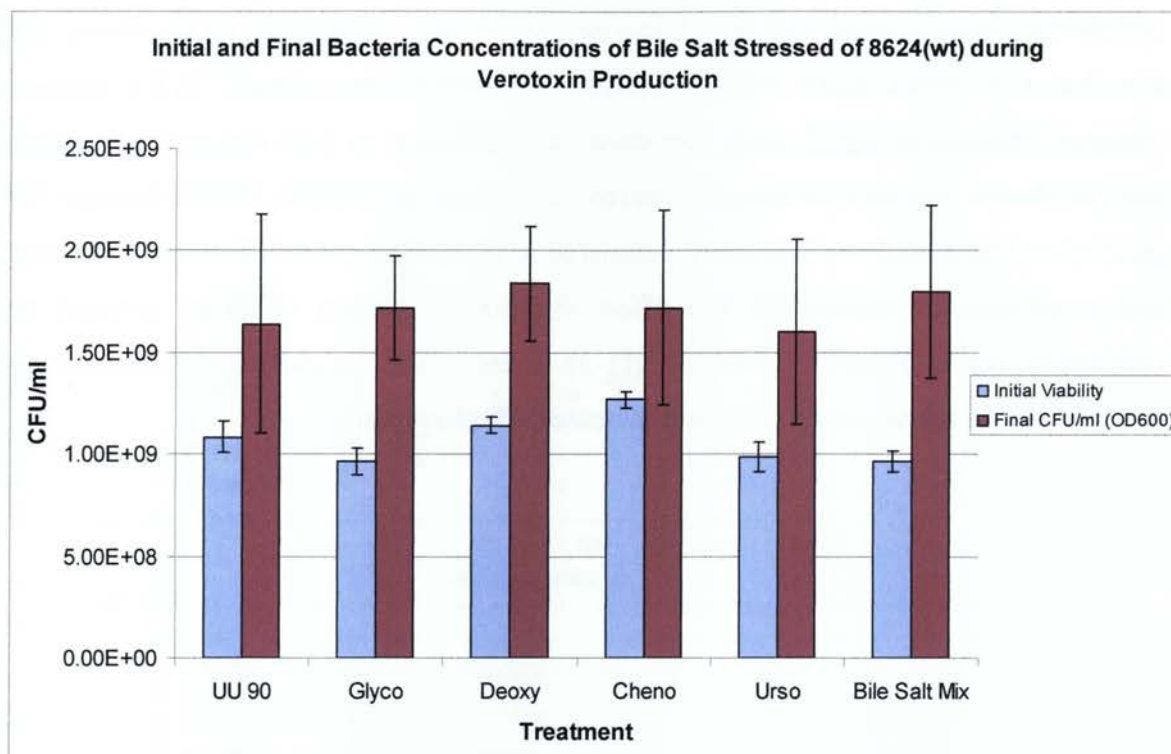


Figure 3.10-3 Initial and Final Bacteria Concentrations of BS Treated *E. coli* O157:H7 86-24 during Verotoxin Production

The initial and final bacteria concentrations of BS treated *E. coli* O157:H7 86-24 were measured either by plate count or OD₆₀₀ to confirm that the initial inoculums and growth during verotoxin production were the same across all treatments. After BS exposure, a plate count assay (n=3) was completed to determine the initial CFU/ml for each treatment. Following overnight growth, the OD₆₀₀ of bacterial cells after each treatment was taken and converted to a final CFU/mL using a standard curve previously completed. OD₆₀₀ results are from two independent experiments and the results are the means \pm standard deviations. For both final and initial bacteria concentrations, $P < 0.05$.

3.11 VT Levels Increased after SCFA Mixture

A similar experiment to investigate the verotoxin secreted into the supernatant and that retained in the periplasmic space were conducted for bacteria exposed to SCFA. Levels of secreted verotoxin (in the supernatant) and verotoxin retained in the periplasmic space formed with SCFA-stressed 86-24 were determined by measuring the absorbance of crystal violet with an ELISA plate reader. Interestingly, SCFA mixture treatment resulted in significantly increased Stx production in both supernatant filtrates and periplasmic extracts compared to untreated controls. In contrast, acetate induced Stx production was significantly dropped compared to unshocked control. However, butyrate and propionate treatments did not affect the Stx production of either secreted or periplasmic levels (Figures 3.11-1 and 3.11-2). These results suggest that SCFAs mixture that mimics the human colon condition increases VT production.

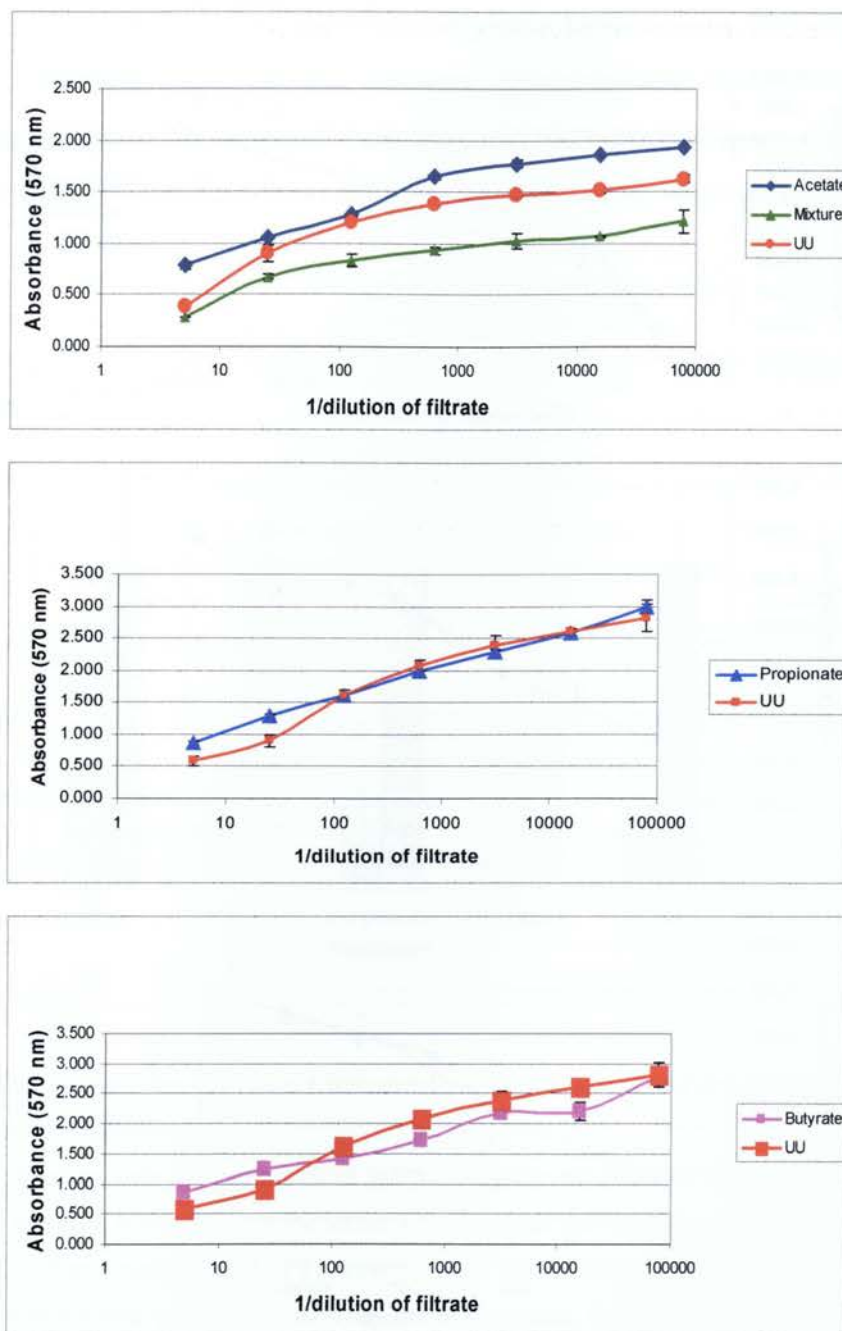


Figure 3.11-1 Secreted Verotoxin Production of SCFA Stressed *E. coli* O157:H7 86-24

The effect of SCFA stress on the verotoxin secreted in the supernatant by *E. coli* O157:H7 86-24 was measured by the vero cell cytotoxicity assay. Results are expressed as the mean absorbance \pm standard deviations (corresponding to the remaining live vero cells) vs. the 1/dilution of verotoxin in the supernatant and are representative of two independent experiments. Experimental treatments: Short Chain Fatty Acid Stress Treatment: 16 hr, 37°C, Acetate: 110 mM; Propionate: 70mM; Butyrate: 20 mM; Mixture of combined acetate-propionate-butyrate: ~ 90 mM

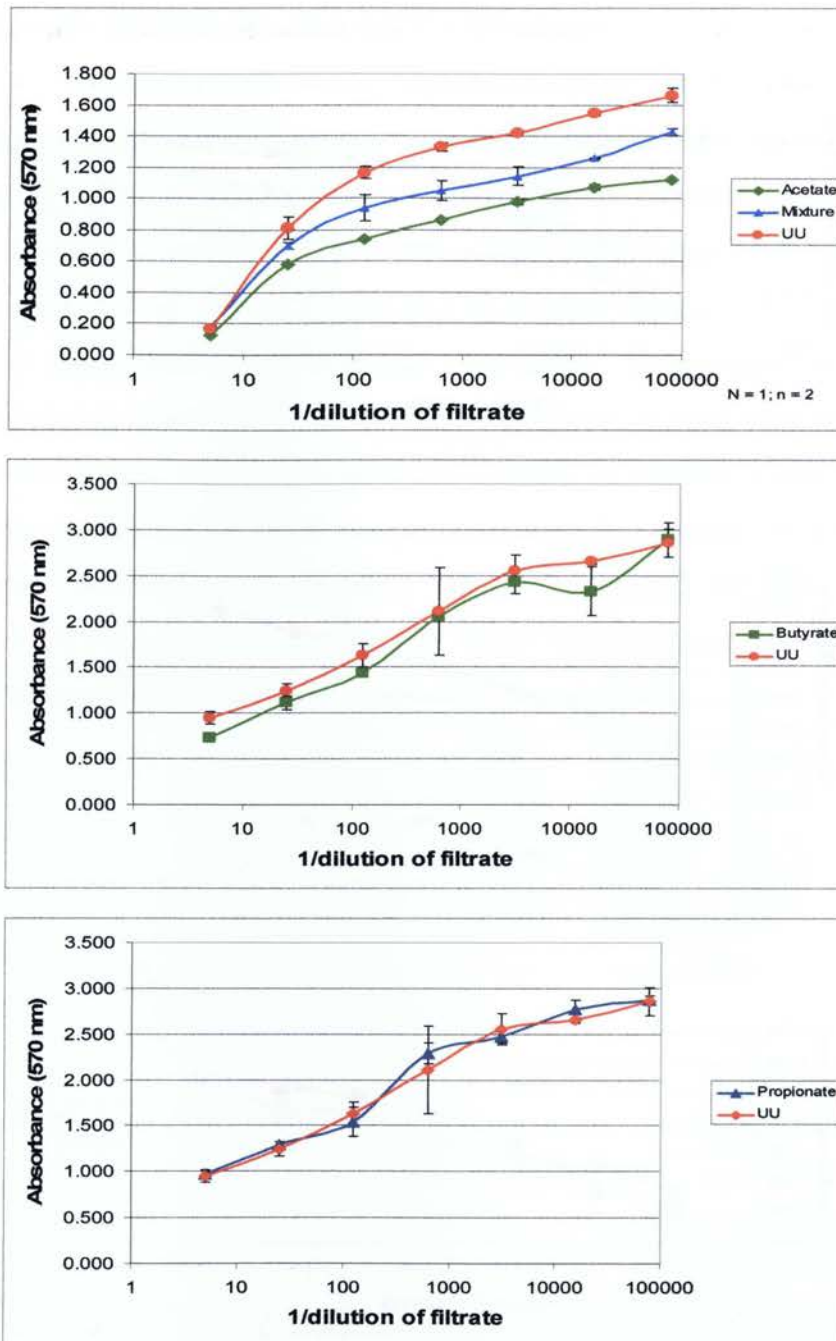


Figure 3.11-2 Secreted Verotoxin Production of SCFA Stressed *E. coli* O157:H7 86-24

The effect of SCFA stress on the verotoxin produced in the periplasmic space by *E. coli* O157:H7 86-24 was measured by the vero cell cytotoxicity assay. Results are expressed as the mean absorbance \pm standard deviations (corresponding to the remaining live vero cells) versus. the 1/dilution of verotoxin in the supernatant and are representative of two independent experiments. Experimental treatments: Short Chain Fatty Acid Stress Treatment: 16 hr, 37°C, Acetate: 110 mM; Propionate: 70mM; Butyrate: 20 mM; Mixture of combined acetate-propionate-butyrate: ~ 90 mM

Again, to confirm that these experimental results were not the product of significantly different initial bacteria inocula, the initial and final CFU/mL for each treatment prior and after overnight growth were measured (Figure 3.11-3). Results demonstrate that initial and final bacteria concentrations were the same across all treatments.

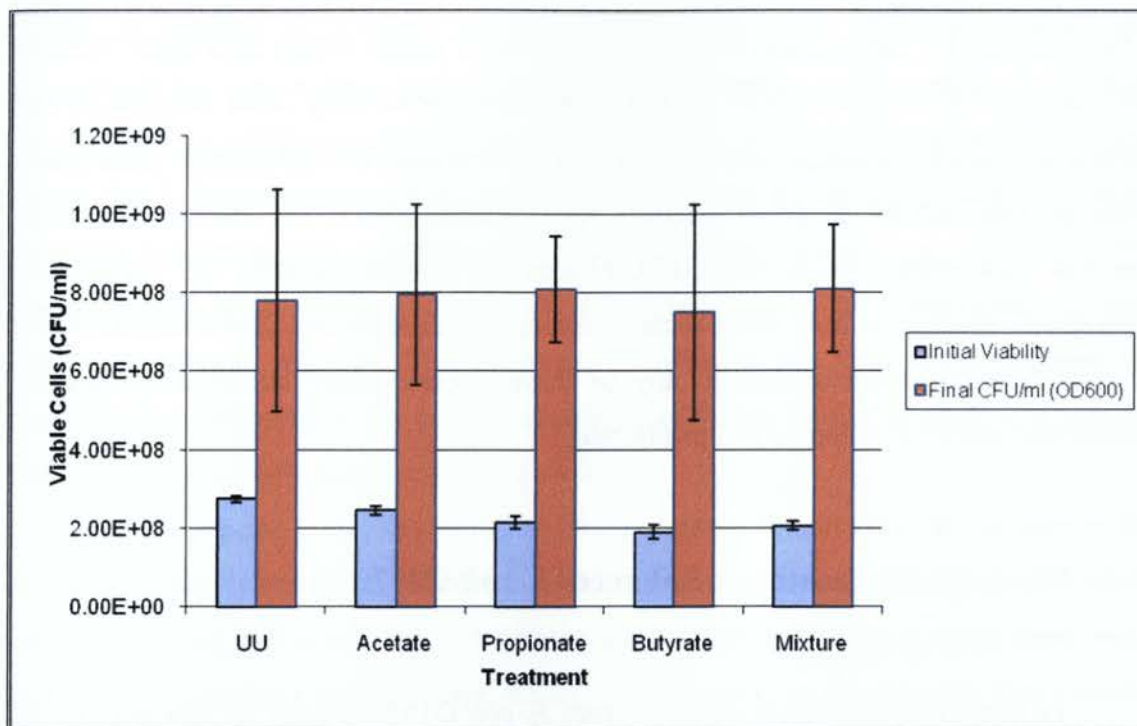


Figure 3.11-3 Initial and Final Bacteria Concentrations of SCFA Stressed *E. coli* O157:H7 86-24 during Verotoxin Production

The initial and final bacteria concentrations of SCFA treated *E. coli* O157:H7 86-24 were measured either by plate count or OD₆₀₀ to confirm that the initial inoculums and growth during verotoxin production were the same across all treatments. After SCFA stress, a plate count assay (n=3) was completed to determine the initial CFU/ml for each treatment. Following overnight growth, the OD₆₀₀ of each treatment was taken and converted to a final CFU/mL using a standard curve previously completed. OD₆₀₀ results are from two independent experiments and the results are the means \pm standard deviations. For both final and initial bacteria concentrations, $P < 0.05$.

4 Discussion

4.1 Effect of Intestinal Stress on *E. coli* O157:H7 Survival: Summary

The ability to adapt to environmental stresses, such those imposed by human hosts, is critical for survival and proliferation of EHEC strains within their hosts (Merrell and Camilli, 2002). When these bacteria enter their hosts they must respond to the extreme and rapidly changing conditions such as gastric acid, BS and organic acids which challenge their ability to grow (Merrell and Camilli, 2002). The effects of human intestinal tract stresses on *E. coli* O157:H7 survival and pathogenicity are not entirely understood. Studies on how these bacteria respond to these stress conditions will allow us to understand how exposure to any of these stressors may influence subsequent survival and ability to colonize and proliferate.

4.1.1 Bile Salt Mix Treatment Enhances *E. coli* O157:H7 Survival

To survive in the human intestinal tract, *E. coli* O157:H7 must tolerate high levels of BSs in the upper small intestine at physiological concentrations of between 0.1 - 1.3% (Pumbwe *et al.*, 2007). BSs have detergent like properties which can permeabilize bacterial membranes and can lead to membrane collapse (Begley *et al.*, 2005). Several pathogens including, *E. coli* O157:H7, have adapted mechanisms to evade the antimicrobial activity of BSs. Various effects of BSs on bacteria have been observed after exposure which depend on factors including the type, length and severity of the stress, the growth phase of the organism, and the ability of different strains to survive the encountered stress. For example de Jesus and colleagues (2005) reported significant reductions in viability of BS stressed EPEC relative to that of untreated controls. Another study by Pumbwe *et al.* (2007) demonstrated that *B. fragilis* exposed to 0.15% BS mixture extracted from porcine bile had a longer lag phase, and slower growth rate compared with bacteria grown with media alone.

In contrast Pace *et al.* (1997) showed that pathogenic *V. parahaemolyticus* strains have the ability to survive to levels 0.5 - 1 log higher on the agar containing 0.15% oxgall bile compared with strains grown in the presence of no bile. Little difference was observed in survival of non-virulent strains of the bacterium with 0.15% bile. However, when pathogenic strains were grown on plates containing higher concentrations of bile (1.5%), it resulted in reduced survival. Interestingly, this effect was particularly pronounced for non-virulent strains suggesting that virulent strains appear to be more resistant to bile inhibitory activity (Pace *et al.*, 1997). Additionally, other studies have shown that *L. monocytogenes* is capable of colonizing the human gall bladder indicating an ability to tolerate high concentrations of bile (Allerberger and Guggenbichler, 1989; Briones *et al.*, 1992; Begley *et al.*, 2002). The role of BS in this context has not been fully explored in *E. coli* O157:H7 strains, and therefore in this study we investigated the contribution of BS stress to the survival capability of the bacteria. Therefore, it is possible that BS exposure might increase EHEC survival.

In the present study, the ability of mid-log phase *E. coli* O157:H7 to survive BS exposure at physiological concentrations was examined. BS treated cells were found to survive better on agar compared with untreated cells, after exposure to either three major individual components of BS (cheno, deoxy, glycocholate) or BS mixture. The viability of BS treated EHEC increased 1.2 - 1.4 fold relative to that of untreated EHEC ($p \leq 0.05$). These findings are not surprising as the most commonly-used selective media for detection of *E. coli* O157:H7 is sorbitol- MacConkey agar (SMAC medium) which contains BS (March and Ratnam, 1986).

Interestingly, studies of the impact of acid stress on *E. coli* O157:H7 revealed significantly lower survival of the cells compared to the untreated controls (House *et al.*, 2006). It is possible that BS present in the small intestine may favour growth of EHEC to facilitate recovery from the acid stress of gastric passage and proliferation prior to entry and colonization in the large intestine.

4.1.2 Short Chain Fatty Acids Treatment Reduces *E. coli* O157:H7 Survival

E. coli O157:H7 that survive and transit the small intestine enter the large intestine where they encounter stresses associated with SCFA. The SCFA concentrations in the human colon, the major site of EHEC colonization, are 110 mM, 70 mM and 20 mM for acetate, propionate and butyrate, respectively (Cummings *et al.*, 1987; Macfarlane *et al.*, 1992; Lawhon *et al.*, 2002). It is well documented that SCFA have antimicrobial activity, and they are widely used as preservatives in food products (Maurer *et al.*, 2005). Organic acids are able to exhibit antimicrobial effect by penetrating the bacterial membrane and dissociation into anions in the cytoplasm. Consequently this results in an increase of the internal osmotic pressure and a depletion of cellular energy to maintain a near neutral pH (Ricke, 2003). Various SCFAs including acetate, propionate, and butyrate are produced by the microbial flora of the large intestine such as *Lactobacillus* sp., *Bifidobacterium* sp. and *Bacteroides* sp. These resident flora can enhance host defence by producing SCFA that in turn create an unfavorable environment for enteric pathogens (Lu and Walker, 2001). On the other hand, enteric pathogens including *E. coli*, *Shigella* and *Salmonella* appear to only generate acetate. Acetate can pass through the bacterial membrane into the cytoplasm where it dissociates, generates a large accumulation of anions and lower internal pH (Maurer *et al.*, 2005). Growth inhibition occurs as a result of lower pH and the ability of anions to inhibit metabolism (Salmond *et al.*, 1984). This evidence was supported by recent studies which revealed that acetate concentrations increase as bacterial density increases (Arnold *et al.*, 2001; Kirkpatrick *et al.*, 2001). Other studies have also shown that SCFAs can have inhibitory effects on *Salmonella* growth and colonization of gastrointestinal tract. However, there are no previous studies that have addressed the role of SCFA on EHEC pathogenesis. We reasoned that exposure to SCFA may decrease EHEC survival. Because the gastrointestinal tract contains a mixture of acetate, propionate and butyrate, individual SCFAs as well as a mixture of SCFAs were investigated for their affects on EHEC. The survival of the cells with exposure to acetate, propionate, butyrate and the mixture was significantly lower than untreated cells by a factor of 1.3 - 1.5 . Here we show for the first

time that a mixture of SCFAs predicted to be similar to that present in the human large intestine significantly decreased viability relative to unstressed cells by a factor of 1.3.

4.2 Bile Salt Mix Treatment Enhances *E. coli* O157:H7 Adhesion to Epithelial Cells

The ability of many gastrointestinal bacteria including *E. coli* O157:H7 to adhere to host is essential for colonization and requires survival through a wide range of changing environments in the human digestive tract, including BS in the small intestine followed by SCFA in the large intestine. Only those pathogens that evade all these host defenses are able to cause infection. Many enteric pathogens have evolved several complex defense mechanisms, which are induced under different environmental conditions dependant on many factors such as growth phase, the type and the severity of the stress encountered (Lu & Walker, 2001). These important stress responses of *E. coli* O157:H7 enhance virulence and correlate to the low infectious dose of approximately 100 organisms (de Jesus *et al.*, 2005). BS have been shown to play a role in host cell adhesion for several gastrointestinal pathogens including *Shigella flexneri*, *Vibrio parahaemolyticus* and *Listeria monocytogenes* (Pope *et al.*, 1995; Pace *et al.*, 1997; Begley *et al.*, 2002). Enteropathogenic *E. coli* which is a related pathogen has also been reported to show enhanced host-cell adhesion following exposure to BS stress (de Jesus *et al.*, 2005). Host adhesion of typical EPEC was significantly enhanced by a factor of 1.3 to 2.6 after BS stress. The observed enhanced adhesion was dependent on the different culture conditions employed. Similarly, Torres *et al.* (2007) identified that BS increase expression of the afimbrial adhesin (LDA) in atypical EPEC that mediates binding to tissue-cultured cells in a diffuse adherence pattern of aEPEC. Another study by Maroncle *et al.* (2006) reported that *Klebsiella pneumoniae* host-cell attachment was significantly increased, mounting a 2-fold higher adhesion than bacteria cultured without bile. The results reported by Pumbwe and colleagues (2007) showed that BS also contribute to enhanced colonization of *B. fragilis* through increased bacterial co-aggregation, bacterial host adhesion, biofilm formation and antimicrobial resistance.

In addition, research by Hung *et al.* (2006) has shown that the gastrointestinal pathogen, *V. cholerae* had a significant increase in biofilm formation in response to bile acids.

In this study, various adhesion assays were performed with human epithelial cell lines derived from adenocarcinoma including HEp-2 (laryngeal carcinoma cells) and Caco-2 (colonic adenocarcinoma cells). However, the use of tissue cells that are cultured has the limitation of obtaining approximative results compared with *in vivo* models. Regardless of the epithelial cell lines used (either HEp-2 cells or Caco-2 cells) the *E. coli* O157:H7 86-24 strains exposed to BS for 90 min, resulted in significantly enhanced adhesion to host cells after glycocholate and BS mixture treatments compared to the untreated controls. Even though we eliminated the role of several predominant individual components of bile salts in enhanced adhesion, however we could not rule out the role of other additional components of the bile complex such as taurocholate, cholate, cholesterol and phospholipids. Even though the viabilities for these treatments were not significantly different relative to the control cells, *E. coli* O157:H7 strains subjected to BS treatments were able to better adhere to either HEp-2 cells or Caco-2 cells by adhesion enhancement factors that ranged from 1.6 to 3.2 depending on treatment. In agreement with these results, light microscopy photomicrographs of infected Giemsa stained cells demonstrated as well obvious increased adherence of BS mixture and glycocholate treated *E. coli* O157:H7 as compared with untreated strains.

E. coli O157:H7 strain 86-24 had the highest percentage of viable bacteria bound to Caco-2 cells (>4%) and the lowest adherence levels to HEp-2 cells (2.4 - 2.6%) . EHEC adherence capability after exposure to BS which was also more significantly increased to colonic Caco-2 cells (4x more) than to human non-intestinal HEp-2 cells (2x). Sherman *et al.* (1987) have also shown differences associated with host-cell adhesion capabilities. Adherence of clinical O157:H7 isolates to both HEp-2 and Intestinal 407 cells did not follow the same morphology of binding (Sherman *et al.*, 1987). These results may suggest that EHEC mediates adherence to various epithelial cell lines through recognition of different surface receptors.

Since the Caco-2 cell line is a colonic cell line which has characteristics of mature enterocytes and is more similar to the *in vivo* situation, these results may suggest that the adhesion enhancement seen after BS exposure may be more dramatic *in vivo* as well.

The importance of adhesion in the life style of EHEC is reflected by the high number of different structures bacteria use for adhesion (Wells *et al.*, 2008). Interestingly, Boerlin and colleagues (1999) suggested a strong association for Stx2, intimin and increased adherence on HEp-2 cells. Perhaps this synergism is one factor that may contribute to the differences among strain adhesion phenotypes and ultimately severe EHEC-induced diseases as not all O157:H7 strains produce VT2. In the present study, all of the EHEC O157:H7 8624 strains used in the adhesion assays were VT2 positive strains

Since treatment with BS enhanced the adhesion of O157:H7 86-24 cells to host cells we wanted to determine if this phenotype was the result of upregulation of the expression of adhesins, and or modifications to existing adhesins. We sought to clarify the role of protein synthesis in the enhanced adhesion phenotype by pretreatment of bacterial cells with erythromycin. Huesca *et al.* (1996) found that erythromycin, used at concentrations below the minimal inhibitory concentration (MIC), can inhibit bacterial protein synthesis. When BS treated *E. coli* O157:H7 86-24 were pre-treated with erythromycin, the adhesion enhancement to HEp-2 cells was abrogated. This result suggests that the enhanced adhesion of BS stressed *E. coli* O157:H7 is likely the result of enhanced expression of existing adhesins and or expression of new adhesin(s).

The ability of EHEC to adhere to host cells and subsequently colonize the large intestine is a key virulence trait and a requirement for the production of diarrhea (required for dissemination of the bacteria) (Torres *et al.*, 2005a). To date, intimin is the only well characterized adhesin that appears to be required for intimate adherence to host cells *in vivo* (Donnenberg *et al.*, 1993; McKee *et al.*, 1995; Torres *et al.*, 2005b). Several proteins have been implicated as novel EHEC adhesive factors, both fimbrial and non-fimbrial, including long polar fimbriae 2, outer membrane protein A (OmpA), Iha, and Efa-1 (EHEC factor for adherence) (Nicholls *et al.*, 2000; Tarr *et al.*, 2000; Paton *et al.*, 2001; Stevens *et al.*, 2002). In addition, EspA filaments also appear to be important adhesion factors (Garmendia *et al.*, 2005). Since BS stressed *E. coli* O157:H7 resulted in enhanced host cell adhesion after two of treatments (glychocolate and BS mixture) we

sought to clarify the role of known adhesins in the BS induced increase in EHEC adhesion to epithelial cells.

Intimin is the best characterized adhesin and has been clearly demonstrated to play a key role in bacterial adhesion (Donnenberg *et al.*, 1993; McKee *et al.*, 1995; Torres *et al.*, 2005b). Therefore, it is possible that intimin may play a role in increased adhesion after BS stress. To explore this hypothesis, an intimin mutant of 86-24 (8624 *eae* Δ 10) and its complemented wild-type 86-24 *eae* Δ (pEB310) were used to investigate the adhesive capacity to human intestinal cells in tissue culture. First it was confirmed that the intimin negative mutant adhered much less than the parental strain or complemented intimin mutant, supporting the notion that intimin serves as a major adhesin (data not shown). We did note an adhesion enhancement of *eae* mutant after exposure to the BS mixture. The enhancement was 2.3 fold relative to that of the untreated correlate ($P < 0.05$). Similar adhesion enhancement after treatment with the BS mixture was seen in 86-24 wt (2.1 fold increase) compared to the untreated control, which demonstrates that intimin is likely not the adhesin responsible for enhanced cell adhesion post-BS stress. Complementation of the intimin mutant with pEB310 restored adhesion to that of wt levels for both the unstressed and BS-stressed treatments. These results indicate that other colonization factors are involved in the increased adhesion to human intestinal cells after BS-treatment observed *in vitro*.

Since we concluded that intimin was not responsible for the increased bacterial adhesion observed after BS treatment, we choose to investigate another known bacterial adherence-conferring protein (Iha). This protein is another adhesin which has been described in *E. coli* O157:H7 to be involved in bacterial adhesion (Tarr *et al.*, 2000).

To confirm that Iha is an adhesin and to exclude the effect of other adhesins, adherence to epithelial cells was assessed using *iha* positive strains (e.g. 86-24), an *iha*-negative mutant of 86-24 and a nonpathogenic *E. coli* strain (ORN172) that naturally lacks *iha*, as well as this strain complemented with *iha* on a plasmid. Preliminary results showed that the 86-24(Δ *iha*) mutant adhered significantly less ($P < 0.04$) than the parental strain. This is consistent with previous reports, which indicated that Iha plays a role in adhesion of EHEC to host cells (Tarr *et al.*, 2000).

Additionally, an adhesion assay was performed for the ORN172(pSK+) (plasmid alone, no *iha*) and ORN172(pIha) and results showed that *E. coli* ORN172(pIha) adhered 4x more than ORN172(pSK+), confirming that *iha* plays a major role as an adhesion in a non-pathogenic *E. coli* background. The low levels of adherence in ORN172(pSK+) can likely be attributed to type I fimbriae (Tarr *et al.*, 2000). No significantly increased adherence was observed in either ORN172(pIha) or ORN172(pSK+) after BS mix treatment as compared with untreated strains, suggesting that Iha is not responsible for the increase in adherence observed after BS stress, in these strains. This suggests that other adhesins or virulence factors that are not present in laboratory strains might be necessary to cause an increased adhesion after BS stress.

In order to confirm these results, protein expression under the same conditions needs to be examined. Further research is needed to fully explore the roles that these virulence factors have in host adhesion of BS stressed *E. coli* O157:H7 and their possible effects on Stx production.

4.3 Short Chain Fatty Acid Stress Enhances Adhesion of *E. coli* O157:H7 to Epithelial Cells

In order to establish infection, *E. coli* O157:H7 must finally overcome the short chain fatty acid stress environment of the large intestine. SCFA represent approximately two-thirds of the colonic anion concentration (100-300 mM) mainly as acetate, propionate and butyrate (Cummings *et al.*, 1987). It has been shown that gastrointestinal pathogens including EHEC possess sophisticated protective mechanisms to survive these conditions (Bearson *et al.*, 1997; Kwon and Ricke, 1998; Durant *et al.*, 1999; Ricke, 2003). Maintenance of this competitive edge is a prerequisite for efficient colonization and establishing infection of the large intestine by food-borne pathogens.

Significant research has been devoted to understanding the complex regulatory systems that mediate *E. coli* response to the external environment. However, there is very little information on the potential role of SCFA on virulence of gastrointestinal pathogens. To date several studies have demonstrated that bacteria exhibit enhanced virulence after SCFA stress. For example, Durant *et al.* (2000) showed that SCFA exposure increases the ability of stationary phase *Salmonella typhimurium* to adhere to

HEp-2 cells. Recently another study by Rishi *et al.* (2005) reported that SCFA exposure of *Salmonella enterica* increased intracellular survival within macrophages, induced an acid tolerance response and enhanced expression of outer membrane matrix proteins (porins). According to Polen *et al.* (2003), *E. coli* growth in acetate and propionate showed enhanced flagellar motility. In contrast with previous studies, two other reports provided evidence for suppression of motility and chemotaxis at high pH (Polen *et al.*, 2003). It is clear from these contradictory reports that the role of SCFA on virulence of *E. coli* O157:H7 in the host gastrointestinal tract is still currently unknown.

Since there are no previous studies that have addressed the role of SCFA on EHEC adherence capability, the present study aimed to clarify whether SCFA stress has any impact on EHEC adhesion to epithelial cells. Interestingly, the adhesion of SCFA stressed EHEC to HEp-2 cells was significantly higher compared to untreated EHEC ($p < 0.05$). Adhesion-enhancement factors ranged from 1.2 - 1.4, depending on SCFA treatment. We reasoned that the SCFA-induced adhesion of *E. coli* O157:H7 to host cells might be conferred by increased synthesis of existing or new adhesins. To determine whether the adhesion enhancement is a result of increased expression of adhesins, erythromycin, below the MIC, was used to block protein synthesis (de Jesus *et al.*, 2005). Following SCFA-stress, all treatments were incubated with or without erythromycin and the adhesion of *E. coli* O157:H7 86-24 to HEp2 cells was examined. Pretreatment of SCFA stressed bacteria with erythromycin completely abolished the increased host adhesion capability of *E. coli* O157:H7 to Caco-2 cells compared to that of untreated sample. These results indicate that a specific set of proteins synthesized within 30 min, after cells are exposed to SCFAs mixture, are responsible for the increased adhesion to host cells. Further studies are required to identify the nature of the adhesive factors involved in SCFA stressed increased adhesion.

4.4 The Effect of Bile Salts and Short Chain Fatty Acids Treatments on *E. coli* O157:H7 Growth during Infection Time

The enhanced adhesion to host epithelial cells by BS and SCFA stressed *E. coli* O157:H7 might be due to increased growth during infection time that may arise after BS

or short chain fatty acid stress. To confirm this hypothesis, control adhesion assays were employed. *E. coli* O157:H7 86-24 was treated with either BS or SCFA and incubated without epithelial cells, simulating the adhesion assays previously described. Bacteria were then plated out and quantified. Three of the treatments showed significantly higher final viability compared to the control. These results indicate that the enhanced adhesion of BS stressed *E. coli* O157:H7 86-24 may be due in part to increased growth during infection time after stress. This is actually not a surprising observation since the viability of BS stressed EHEC with three of the treatments was increased relative to that of unshocked. On the other hand, unexpected results were obtained on EHEC growth during infection time after SCFA stress. Although there were no significant differences in initial amount of bacteria loaded, after 4 hr incubation time the survival of all stressed bacteria was significantly lower in comparison to control. Therefore it is likely that enhanced adhesion after SCFA exposure is not mediated through increased growth during incubation time. Further studies will be needed to identify the requirements necessary for that butyrate and mixture adhesion enhancement.

4.5 The Effect of Bile Salts and Short Chain Fatty Acids Treatment on *E. coli* O157:H7 86-24 Verotoxin Production:

Stx production by EHEC O157:H7 is the primary virulence factor associated with the development of the life threatening HUS and vascular complications of HC (Karmali *et al.*, 1983). Karmaly *et al.*, (1985) reported that verotoxin production is strain variable and classified Shiga-like toxin producing *E. coli* as low- to moderate- and high level toxin producers. The authors also suggested that Stx accumulated into the periplasmic space is released into the supernatant due to a leaky outer membrane (Karmali *et al.*, 1985). Recently a study by House *et al.*, (2006) examined the effect of acid shock of EHEC O157:H7 86-24 on verotoxin production and secretion. No significant difference was found in verotoxin secreted in the supernatant and that one retained in the periplasmic space, suggesting that acid shock does not augment the severity of EHEC induced renal disease.

Additional concern is that Shiga toxin has also been reported to enhance EHEC binding to epithelial cells which could lead to increased colonization, HC and its

complications. One study by Robinson *et al.* (2006) reported that Stx2 produced during infection increases the cell surface expression of nucleolin and helps EHEC in promoting intestinal colonization. That Stx2 provides an adherence advantage of EHEC O157:H7 in establishing infection was supported by the finding that wt EHEC 86-24 adhere better to epithelial cells than the *stx2* isogenic mutant (Robinson *et al.*, 2006). Epithelial cells that were exposed to enzymatically active Stx2 toxin throughout the infection period not only harmed the host but also enhanced the bacterial adherence level of *stx2* isogenic mutants. This increased adherence of isogenic mutants correlated with toxin exposure was even higher to HEp-2 cells than its wt 86-24 alone. These findings are in agreement with their previous report that demonstrated that intimin interacts not only with Tir as a receptor but also with the eukaryotic protein nucleolin localized on the cell surface (Sinclair & O'Brien, 2002). Similar results were obtained by Barnett Foster *et al.* (2000) that demonstrated an enhanced adhesion of EHEC to Stx-treated epithelial cells. This finding was correlated with increased levels of outer leaflet phosphatidyletanolamine, the host receptor previously reported to bind EHEC (Barnett Foster *et al.*, 2000).

This study investigated the impact of BS and SCFA treatment on both secreted and periplasmic production of verotoxin. It is important to assay the levels of both secreted and periplasmic production because it is well documented that antimicrobial treatment of EHEC infections can activate the Stx phage thereby releasing the toxin accumulated in the periplasmic space and increasing the risk of HUS (Bushen and Guerrant, 2003; Kaper *et al.*, 2004).

4.5.1 No Change in Secreted and Periplasmic VT Production after Bile Salt Treatment

Firstly, the impact of BS stress on Stx production (both secreted and periplasmic extracts) of *E. coli* O157:H7 86-24 was examined. The results of the Vero cell cytotoxicity assay showed no significant changes in toxin production either in the supernatant filtrates or periplasmic extract across all BS various treatments. Since no significant differences were found across all the various treatments, it is likely that BS stress does not augment the severity of EHEC induced renal disease.

4.5.2 Increased VT Levels after Short Chain Fatty Acids Mixture Stress

Having shown the impact of BS on Stx production, further investigation was conducted to determine whether the Stx levels were altered by SCFA stress. SCFA-mixture treatment resulted in a significant increase in Stx production in both supernatant filtrates and periplasmic extracts compared to untreated controls. In contrast, acetate induced Stx production was significantly lower compared to untreated controls. Butyrate and propionate treatments did not affect the Stx production, either secreted or periplasmic. These data suggest that exposure to an SFCA mixture almost similar to that of the large intestine increases Stx production and extrapolated to the *in vivo* situation, may augment the severity of EHEC induced renal disease by increasing levels of Stx produced. This is consistent with the proposed mechanism in which environmental signals in the colon serve as cue for bacterial infection (Gyles *et al.*, 2006). Research by Louise *et al.* (1995) showed that butyrate increases the number of Stx receptors on HUVEC cell surface. Although it is tempting to suggest that butyrate may increase Stx binding to Gb3, further study is needed. However, it is possible that *E. coli* O157:H7 exploits the SCFA microenvironment using it as a signal to seek appropriate opportunities for infection in the large intestine. Further research is required to examine whether other *E. coli* O157:H7 strains produce the same observed results observed here under these experimental conditions. A better understanding will be obtained by analyzing the molecular basis of these changes in gene expression induced by BS and SCFA treatments of *E. coli* O157:H7.

5 Summary and Conclusions

5.1 Summary of Results

This research examines the impact of exposure of EHEC O157:H7 to physiologically relevant concentrations of BS and SCFA on critical virulence properties including the adhesion to host endothelial cells and the production of Stxs. This study has revealed significant stress-induced changes in a number of these properties including survival, ability to adhere to human epithelial cells and verotoxin production. Exposure to BS significantly enhanced the survival of EHEC O157:H7. It is tempting to speculate that this increase in survival first could facilitate the recovery from the acid stress of the stomach and further promote colonization in the large intestine. There was also significantly increased adhesion to tissue culture cells after BS stress. Our investigations on EHEC adherence capability after BS treatment were extended for two different epithelial cell lines and we found that EHEC had the highest percent of viable bacteria bound to Caco-2 cells (4x fold increase) and the lowest adherence level (2x fold increase) to HEp-2 cells.

Another interesting finding was that the pretreatment of the bacteria with erythromycin completely abolished the adhesion enhancement of BS stressed EHEC O157:H7. This suggests that the enhanced adhesion required increased bacterial protein synthesis, presumably of existing or new adhesins. The adhesion increase after BS exposure was likely not mediated by either an intimin or Iha since the same adhesion increase was noted for both the intimin-negative mutant and the Iha negative mutant. On the other hand, a nonpathogenic *E. coli* strain demonstrated decreased adhesion after BS stress. Adhesion levels of both BS treated and untreated nonpathogenic ORN172 strains were much lower relative to *E. coli* O157:H7 strain. No variation of the host adhesion capability of non-virulent laboratory K12 strains to HEp-2 cells indicate that virulence factors not present in the laboratory strains are responsible for an increased level of adhesion to host cells in EHEC.

Next, we hypothesized that the enhanced adhesion might be due to increased growth after BS stress. Preliminary data demonstrated that glycocholate, deoxycholate

and ursocholate treatments showed significantly higher final viability compared to the control. It is likely possible that the glycocholate treatment may have enhanced the adhesion of *E. coli* O157:H7 to epithelial cells through elevated growth after stress. This would suggest that there are more bacteria adhering because there are more bacteria present. These findings are consistent with previous studies in the lab that have also demonstrated that BS treatments significantly enhanced several other virulence properties including motility, flagella expression and resistance to several antibiotics (Prayitno *et al.*, unpublished data). None of the experimental BS treatments resulted in a significant change in Stx production in either secreted or periplasmic extracts. These results suggest that BS does not upregulate STx production and so this virulence factor is not expressed at higher levels after BS stress. This could then be used to suggest that BS stress may not lead to increased risk of VT –mediated pathology including HUS. However, further research needs to clarify whether more VT is produced than more is translocated into the blood stream and that more reaches the kidneys etc and causes damage. Since VT has also been implicated in increased adhesion of the bacteria, it is unlikely that changes in VT levels could explain the increased adhesion observed for BS-stressed EHEC. It is tempting to speculate that EHEC sense the BS as a signal of being in the small intestine and does not influence the Stx production in order to save resources and promote colonization in more appropriate environments such as the large intestine.

Since BS stress may be inducing novel adhesin expression, microarray analysis is currently underway (Barnett Foster, D.E., personal communication). Any potential adhesins upregulated will be confirmed with real time RT-PCR and BS adhesion assays.

Having shown the impact of BS on adherence capability and verotoxin production levels, further investigation was conducted to determine whether SCFA stress had any effect on the adhesion and cytotoxicity. SCFA stress, such as that present in the large intestine, significantly induces changes in bacterial survival, ability to adhere to human epithelial cells and verotoxin production. Interestingly, although the survival after exposure to SCFAs was significantly lower than that of untreated control, it seems that bacteria that survived the stress showed enhanced adhesion capabilities to host epithelial cells. Pretreatment of SCFA stressed *E. coli* O157:H7 strains with subinhibitory concentrations of erythromycin did not show increased adhesion to host epithelial cells.

These data again support the idea that protein synthesis was required for the increased adhesion phenotype and strongly suggest that the adhesion enhancement resulted from increased expression of existing or new adhesins.

Treatment with the SCFA-mixture resulted in significantly increased Stx production in both supernatant filtrates and periplasmic extracts compared to untreated controls. This finding lends support of the concept that EHEC is using local microenvironmental cues to regulate virulence factor expression. The mix of SFCAs approximates that of the local microenvironment of the large intestine and the data suggest that EHEC is responding to this cue by increasing the production of VT in a location where this virulence factor can be translocated across the intestinal epithelial for circulation to body organs including kidneys. Since the bacterial survival is lower after SCFA exposure, the data might also suggest that increased Stx production may assist in enhancing bacterial adhesion. Further work is needed to determine whether Stx mediated adherence contributes to and consolidates the SCFA induced changes. Again, these findings are consistent with previous studies in the lab that have also proven that SCFA treatments significantly enhanced other several virulence properties including flagella expression (Prayitno *et al.*, unpublished data).

5.2 Future Work

Since the results of this study highlighted changes in virulence factors, the molecular basis of the response of EHEC to localized stress in the small and large intestine should be examined. We hypothesize that the changes in expression of selected virulence factors is a direct response to the localized environmental stress encountered during transition to the small and large intestine. Whole-genome expression profiling (microarray analysis) and real-time PCR are currently underway to examine changes in the expression of genes up- and down-regulated post BS- and SCFA- treatment. This research should begin by focusing on genes encoding adhesin candidates since the most recent data from this study indicate significant changes in host adhesion after specific SCFA stress. Based on these results, the role of these candidate genes in host cell

infection should be assessed by constructing mutants defective in the individual genes and then should be tested in phenotypic virulence assays.

This study has highlighted changes in several virulence factors including adhesion to host tissues; the role(s) of known bacterial adhesins after BS and SCFA stress needs to be clarified. BS and SCFA adhesion assays with *espA*, *iha* and intimin mutants should be completed. Furthermore, Western blot analysis should be employed to assess expression levels of intimin, Iha and EspA in BS and SCFA-treated organisms. Additionally, the secretion phenotypes of BS and SCFA stressed *E. coli* O157:H7 should also be examined to further explore the possible posttranscriptional regulation of these virulence factors after either BS or SCFA treatments. The role of other potential adhesins, such as OmpA, EspA, long polar fimbriae 2, and Efa-1 should also be examined after either BS or SCFA stress. Adhesion assays with parental and mutant strains for each adhesin should be conducted. Microscopic investigation would also be useful to determine if there are differences between stressed and unstressed strains. This research will enhance our understanding of stress-induced virulence changes in this serious, potentially life-threatening gastrointestinal pathogen and explain the genetic basis of these changes.

5.3 Significance and Conclusions

It is obvious that most bacterial pathogens including *E. coli* O157:H7 must overcome hostile conditions, such those encountered in the intestinal tract, in order to successfully reach their target sites on the surface of enterocytes (Merrell and Camilli, 2002; Foster, 2004). The results of this study show that the environmental conditions of the human host, such as BS of the small intestine and SCFA of the large intestine, directly influence essential virulence factors involved in the intestinal colonization process of *E. coli* O157:H7 including bacterial attachment to epithelial cells as well as verotoxin production. Interestingly, this work suggests for the first time that *E. coli* O157:H7 uses BS and SCFA as a cue to establish location and influence virulence gene expression. The production of verotoxin is the major virulence factor in EHEC infection since it can ultimately lead to several life threatening diseases such as hemolytic uremic syndrome and thrombotic thrombocytopenic purpura. SCFA stress did significantly increased verotoxin production. This finding is significant and supports the idea that

exposure to SCFA stress will lead to increased cases of HUS due to increased verotoxin production. Adhesion to host cells is an important virulence factor of EHEC infection. Although not associated with the severe life threatening disease, adherence to host epithelial cells is still a requirement for the development of diarrhea (Tzipori *et al.*, 1987; Campellone and Leong, 2003). Since BS treatment has enhanced survival and adhesion capability to the host cells, the severity of EHEC-induced diarrhea may be increased and may occur faster in infected individuals. Taken together, these results indicate that *E. coli* is able to survive in hostile conditions, reproducing stresses encountered in the intestinal tract, a prerequisite for successful propagation of the microorganisms. However, the higher levels of adhesion to intestinal cells observed with SCFA stress could be attributed to an advantage due to the increased verotoxin production. The results of this research indicate that ingestion stresses such as BS and SCFA, which are part of the host's natural chemical assault on foreign organisms, may actually enhance the virulence properties of this pathogen including adhesion to human epithelial cells, Stx production, motility and flagella expression, resistance to several antibiotics contributing to, rather than, preventing infection. Furthermore, they suggest that this pathogen may use these ingestion stresses to cue the expression of numerous virulence factors for successful infection of local microenvironments.

This work makes a valuable contribution to understanding how these stresses modulate the virulence potential of this pathogen. In addition, we have contributed essential data for delineating the pathogenesis of disease caused by EHEC infection. Continued work in this field will surely contribute to identifying new genes and pathways involved in BS and SCFA stress-induced virulence changes. These data, combined with other results, may provide exciting possible alternatives for the treatment or prevention of EHEC infections. It may allow the design of a new class of antimicrobials that can block BS and SCFA signaling in bacterial pathogens. These potential antimicrobials will not only be useful against EHEC but possibly also against other pathogens which harbor similar response to stress. We can conclude that our hypothesis was correct. Finally, our results support the hypothesis that environmental stresses such as BS or SCFA encountered during transition to the small and large intestine increase EHEC virulence including host cell adhesion and toxin production.

6 Supplementary Data

Supplemental Table 1

Summary of Adhesion of *E. coli* strains to HEp-2/Caco-2 cells before and after BS Exposure as Determined by Plate-count assay

Table 6-1 Supplemental Data 1

Strain	Treatment	HEp-2		Caco-2	
		%	Fold increase	%	Fold increase
86-24	UU	2.5 - 2.73%		4.37%	
86-24	Gly	4.36%	1.5x	4.87%	1.1x
86-24	Mix	4.05 - 5.29%	1.5 - 2.1x	20.91%	4.7x
86-24 <i>eae</i> Δ10	UU	0.3 - 0.34%			
86-24 <i>eae</i> Δ10	Mix	0.70%	2.3x		
86-24 <i>eae</i> Δ(pEB310)	UU	4.70%			
86-24 <i>eae</i> Δ(pEB310)	Mix	6.74%	1.4x		
86-24Δ <i>iha</i>	UU	1.69%			
86-24Δ <i>iha</i>	Mix	2.88%	1.7x		
ORN172(pIHA)	UU	1.69%			
ORN172(pIHA)	Mix	2.16%			
ORN172(pSK+)	UU	0.59%			
ORN172(pSK+)	Mix	0.92%			

Note: Adhesion enhancement factors after BS exposure ranged from 1.1 -4.7x depending on the strain used and treatments.

Supplemental Table 2

Summary of Adhesion of *E. coli* strains to HEp-2/Caco-2 cells before and after SCFA Exposure as Determined by Plate-count assay

Table 6-2 Supplemental Data 2

Strain	Treatment	HEp-2		Caco-2	
		%	Fold increase	%	Fold increase
86-24	UU	15 - 20%		25.00%	
86-24	But	28 - 30%	1.5 - 1.8x	28.00%	1.1x
86-24	Mix	25 - 60%	1.6 - 3x	50.00%	2x

Note: Adhesion enhancement factors after SCFA exposure ranged from 1.1 -3x depending on the strain used and treatments

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