

***ESCHERICHIA COLI* O157:H7 LINEAGE PERSISTENCE AND  
COLONIZATION OF CATTLE *IN VITRO***

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*To my beloved Sadie,  
for all of the love and support.*

## ABSTRACT

*Escherichia coli* O157:H7 is an important human pathogen that resides primarily in cattle and feedlot environments. *E. coli* O157:H7 can be divided into phylogenetic groups termed lineages; lineage I strains are responsible for most human illnesses. An understanding of the etiology of these lineages within cattle and the feedlot environment could allow for more effective surveillance and mitigation strategies. There were no lineage associated differences in growth or survival of *E. coli* O157:H7 in bovine feces at 4°C, 12°C or 25°C. Lineage I strains more readily colonized cattle jejunum tissue and a bovine colonic cell line than lineage II and intermediate type strains. Enhanced colonization of cattle by lineage I strains may increase the persistence of these strains in feedlots via re-infection and increased shedding. This outcome could increase the transmission of lineage I strains to the food supply and increase the potential for these strains to cause human illness.

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## LIST OF ABBREVIATIONS

A/E – attaching and effacing

AIC – Akaike Information Criterion

$a_w$  – water activity

bp – base pairs

ca - circa

CFU – colony forming unit

$C_t$  – threshold cycle

CT-KASMAC – sorbitol MacConkey agar supplemented with cefixime, potassium tellurite, kanamycin and ampicillin

CT-SMAC – sorbitol MacConkey agar supplemented with cefixime and potassium tellurite

CT-SMACnal – sorbitol MacConkey agar supplemented with cefixime, potassium tellurite and nalidixic acid

DM – dry matter

*eae* – *E. coli* attaching and effacing (gene encoding intimin)

EHEC – enterohemorrhagic *E. coli*

FBS – fetal bovine serum

HUS – hemolytic uremic syndrome

IVCC – *in vitro* cell culture

IVOC – *in vitro* organ culture

Kan<sup>R</sup>/Amp<sup>R</sup> – kanamycin and ampicillin resistant

LB – Luria-Bertani broth

LEE – locus of enterocyte effacement

LSPA-6 – lineage-specific polymorphism assay-6

Nal<sup>R</sup> – nalidixic acid resistant

NSF – non-sorbitol fermenting

OBGS – octamer-based genome scanning

ORF – open reading frame

PBS – phosphate buffered saline

PCR – polymerase chain reaction

RAJ – recto-anal junction

RAMS – recto-anal mucosal swabs

SMAC – sorbitol MacConkey agar

STEC – Shiga toxin producing *E. coli*

Stx1 – Shiga toxin 1

Stx2 – Shiga toxin 2

Tir – translocated intimin receptor

TSB – tryptic soy broth

VAP – variable absent or present

VFA – volatile fatty acid

VTEC – verotoxin producing *E. coli*

w/v – weight by volume

## INTRODUCTION

*Escherichia coli* O157:H7 is a pathogenic bacterium of the gastrointestinal tract of humans and other animals. First isolated and implicated as the source of human disease in the early 1980's (Riley et al. 1983; Wells et al. 1983), this pathogen has now emerged as one of the leading causes of gastrointestinal disease in the North America, the United Kingdom and Japan (Chase-Topping et al. 2008). *E. coli* O157:H7 causes a number of symptoms in humans ranging from watery diarrhea to hemorrhagic colitis and potentially fatal hemolytic uremic syndrome (Karmali 1989). Ruminants, especially cattle, are the primary reservoirs of the bacterium; however they are generally considered to be asymptomatic carriers. Regardless, the elimination of *E. coli* O157:H7 at the farm site/feedlot and its passage to the food supply is expected to have the greatest positive impact on food safety (Jordan et al. 1999).

Recent research has identified two predominant phylogenetic lineages of *E. coli* O157:H7, lineages I and II (Kim et al. 1999; Kim et al. 2001). Further, there seems to be a bias in the distribution of these lineages among hosts. Initial studies found that *E. coli* O157:H7 strains associated with clinical human illnesses predominantly belonged to the lineage I branch while strains isolated from feedlots belonged predominantly to the lineage II branch (Kim et al. 1999; Kim et al. 2001). This was further confirmed with a more diverse set of strains and found that lineage I strains comprised 73.8% and 49.5% of human and bovine isolates respectively (Ziebell et al. 2008). Intermediate lineage type strains of *E. coli* O157:H7 have been identified, however the role that these strains play in human illness and the cattle reservoir is not well understood (Sharma et al. 2009).

It has been recently established that cattle colonized by and shedding *E. coli* O157:H7 make up a heterogeneous population (Naylor et al. 2003; Low et al. 2005). Among this heterogeneous population are individuals which have been termed supershedders. Various studies have used different criteria for defining these animals but supershedders, which make up approximately 10% of cattle population, are likely responsible for greater than 90% of the total *E. coli* O157:H7 shed by cattle (Chase-Topping et al. 2008). It is possible that these supershedders are the result of increased colonization of the cattle, colonization of a different location within the intestinal tract of cattle or some combination thereof. Due to the often high concentrations of *E. coli* O157:H7 within the environment, an understanding of factors which alter its survival could have implications when implementing effective detection and mitigation strategies. As such, it is critical to understand what factors may influence the colonization of cattle as well as factors that could alter survival of *E. coli* O157:H7 in the environment.

This thesis encompasses three chapters which are aimed at furthering our understanding of how *E. coli* O157:H7 persists within the feedlot environment. The ultimate goal of this work is to contribute to the knowledge necessary to reduce or eliminate the passage of this important human pathogen from the feedlot to the food supply. Chapter 1 is a critical overview of the available literature regarding *E. coli* O157:H7 as it pertains to human infections, cattle and the feedlot environment. The second chapter is a research study which explores the possible role of lineage type alongside temperature and fecal matrix in the long term survival of *E. coli* O157:H7 in cattle feces. The final chapter contains a number of linked experiments attempting to

define *E. coli* O157:H7 colonization of cattle *in vitro* in terms of lineage type, strain origin and cytotoxin production.

## CHAPTER ONE

### Literature Review

#### 1.1. *Escherichia coli* O157:H7 and human illness

*Escherichia coli* are a large group of typically non-pathogenic bacteria that normally reside within the gastrointestinal tract of humans and other animals. A subset of bacteria within this group are the Shiga toxin (or verotoxin) producing *E. coli* (STEC or VTEC) that produce toxins similar to a toxin produced by *Shigella dysenteriae* I (O'Brien et al. 1982). These are known as the Shiga-like toxins, Stx1 and Stx2, and STEC strains may produce one or both of these toxins (Nataro and Kaper 1998). An extremely virulent group of STEC strains are known to cause hemorrhagic colitis and hemolytic uremic syndrome (HUS) and are termed enterohemorrhagic *E. coli* (EHEC)(Levine 1987). *E. coli* serotype O157:H7 is the most prevalent EHEC strain; however, there are other non-O157:H7 EHEC serotypes such as O111:H-, O117:H4 and O121:H19 (Karmali 1989; Johnson et al. 1996).

*Escherichia coli* O157:H7 was first linked with enteric disease in the early 1980's following outbreaks of human illness traced back to contaminated meat (Riley et al. 1983; Wells et al. 1983). Symptoms of these first reported infections with *E. coli* O157:H7 were abdominal cramping, watery diarrhea followed by bloody diarrhea (hemorrhagic colitis) (Riley et al. 1983; Wells et al. 1983). Soon after, a group of potentially fatal symptoms (acute renal failure, microangiopathic hemolytic anemia and thrombocytopenia) known collectively as hemolytic uremic syndrome (HUS) was added to the symptoms of *E. coli* O157:H7 infection (Karmali 1989). Children, the elderly and immuno-compromised individuals are at the highest risk for severe complications of *E.*

*coli* O157:H7 infection and severe cases of illness may require blood transfusions and kidney dialysis (Fremaux et al. 2008). Presently, *E. coli* O157:H7 is known to cause outbreaks which include thousands of illnesses and several deaths every year in North America (Centre for Infectious Disease Prevention and Control 2005; Centers for Disease Control and Prevention 2007; Chase-Topping et al. 2008). A review of *E. coli* strains that caused illness and were serotyped prior to the initial description of *E. coli* O157:H7 in 1983 suggests that the O157:H7 serotype is indeed an emerging pathogen (Nataro and Kaper 1998). Further, the economic cost of this disease in terms of medical costs, time missed from work and premature death is estimated to be in the hundreds of millions of dollars annually (Frenzen et al. 2005; United States Department of Agriculture 2006). Canada, the United States, Japan and Scotland have been reported to have the highest incidence rates of *E. coli* O157:H7 infection in the world over the last 20 years (Chase-Topping et al. 2008).

## **1.2. *E. coli* O157:H7 virulence factors and human infection**

*E. coli* O157:H7 cells cause attaching and effacing (A/E) lesions on the intestinal epithelium by effacing the microvilli and rearranging the cytoskeleton to create actin pedestals (Vallance and Finlay 2000). There are a number of essential virulence factors which are involved in the effective colonization of humans. The locus of enterocyte effacement (LEE) pathogenicity island contains 41 open reading frames (ORFs), and many of the products of the LEE are critical for colonization (Moxley 2004). The LEE is organized into five operons designated as LEE1 through LEE5 (Moxley 2004). Among those genes encoded on the LEE pathogenicity island are *ler* (LEE-encoded regulator),

*sep* and *esc* (encode a type III secretion system), the *espABD* genes (encode protein products that are secreted by the type III secretion system), *eae* and *tir* (Nataro and Kaper 1998). Intimin is encoded by *eae* while *tir* encodes Tir (translocated intimin receptor). Tir, the receptor for intimin, is translocated into the host cell via a type III secretion apparatus and it is partially through the interaction of these two proteins that intimate adherence of *E. coli* O157:H7 to host cells is gained (Nataro and Kaper 1998). Non-intimin mediated adherence is controlled by *ler* (Ogierman et al. 2000), expression of which is up-regulated by the presence of NaHCO<sub>3</sub> in the lower intestine, partially explaining the affinity for *E. coli* O157:H7 to the lower intestine (Abe et al. 2002).

There are also a number of non LEE encoded virulence factors. Two important non-LEE virulence factors are the Shiga-like toxins. The Shiga toxins are potent cytotoxins which contribute to hemorrhagic colitis and HUS in humans (Moxley 2004) and Shiga toxin 1 (Stx1) causes apoptosis of human intestinal epithelial cells (Smith et al. 2003). Other important *E. coli* O157:H7 virulence factors exist whose functions are not fully understood. For instance, a large plasmid contained within almost all *E. coli* O157:H7 strains, known as the pO157 plasmid, contains 100 ORFs of which only approximately 20 have been characterized (Yoon and Hovde 2008). These characterized ORFs include genes that encode a type II secretory system and a possible adhesin (*toxB*) (Yoon and Hovde 2008). Similarly, flagella have also been shown to be important in colonization *in vitro* (Erdem et al. 2007; Mahajan et al. 2009).

### **1.3. *E. coli* O157:H7 colonization of cattle**

Ruminants have been identified as a primary reservoir of *E. coli* O157:H7 with numerous outbreaks being traced to meat (MacDonald et al. 2004), dairy products (Gillespie et al. 2003), fresh produce (Welinder-Olsson et al. 2004), drinking and recreational waters (Ackman et al. 1997; Centre for Infectious Disease Prevention and Control 2005) contaminated by bovine feces. Neonatal calves are well known to be susceptible to *E. coli* O157:H7 and infection causes A/E lesions, watery and/or bloody diarrhea and can often be fatal (Cray and Moon 1995; Dean-Nystrom et al. 1997; Dean-Nystrom et al. 1998; Dean-Nystrom et al. 1999). *E. coli* O157:H7 prevalence in cattle is seasonal and different studies have reported prevalence rates as low as 2% to as high as 80% (Cray and Moon 1995; Elder et al. 2000; Callaway et al. 2003; Callaway et al. 2009). It appears that prevalence rates of *E. coli* O157:H7 in cattle and feedlot environments have been increasing since the first description of the pathogen; however it is difficult to determine whether prevalence rates are increasing or if the detection methods have become more sensitive. Surveillance and mitigation strategies for *E. coli* O157:H7 are primarily aimed at the cattle reservoir and mathematical models have predicted that agents to reduce shedding, including vaccines, could reduce human infections dramatically (Jordan et al. 1999).

#### **1.3.1. *E. coli* O157:H7 virulence factors and cattle colonization**

The intimin-Tir interaction is thought to be essential in the effective colonization of calves (Dean-Nystrom et al. 1998; Vlisidou et al. 2006). When neonatal calves were infected with *eae* positive strains of *E. coli* O157:H7 there were increased symptoms of

infection, including death, and increased shedding periods compared to mutant strains lacking *eae* (Dean-Nystrom et al. 1998; Vlisidou et al. 2006). Similarly, calves infected with Tir-producing strains of *E. coli* O157:H7 had longer shedding periods and shed more bacteria than mutant strains lacking the *tir* gene (Vlisidou et al. 2006). It was also noted that calves inoculated with double mutant *E. coli* O157:H7 strains lacking both *eae* and *tir* had comparable shedding to single mutant strains lacking *eae*, but not single mutants lacking *tir*, suggesting that intimin is involved in non-Tir mediated colonization as well (Vlisidou et al. 2006).

The role of the Shiga toxins in cattle is not well understood. Cattle lack receptors for Stx1 (Pruimboom-Brees et al. 2000) and the toxin is degraded in the crypt cells of the colon (Hoey et al. 2003). Further, Shiga toxin 2 (Stx2), which is toxic to humans, does not kill epithelial cells from the jejunum or descending colon of cattle (Baines et al. 2008a). However, recently Stx2 has been observed to increase colonization of *E. coli* O157:H7 in disease-susceptible animals (Robinson et al. 2006) and cattle (Baines et al. 2008a), likely through an increase in expression of non-Tir colonization sites such as nucleolin (Robinson et al. 2006). While the mechanism whereby the H7 flagellin is able to increase adherence is poorly understood, it has been reported that immunization of calves with H7 flagellin reduced colonization rates (McNeilly et al. 2008) demonstrating the potential for vaccines to target virulence factors involved in colonization.

### **1.3.2. *E. coli* O157:H7 colonization site**

*E. coli* O157:H7 colonization of cattle primarily occurs within the large intestine, likely due to an affinity of intimin- $\gamma$  for receptors of the large intestine (Tzipori et al. 1995;

Naylor et al. 2003). Despite this knowledge, prior to 2003, detection of the pathogen within necropsy samples of the large intestine of cattle was not successful even when *E. coli* O157:H7 was present in feces of the animals (Naylor et al. 2003). Naylor et al. (2003) hypothesized that the pathogen may colonize within the terminal rectum or anal canal of cattle because these areas are not routinely processed for necropsy due to their intrapelvic location within the animal. Upon investigation, it was discovered that *E. coli* O157:H7 does indeed colonize cattle most heavily within the terminal rectum, approximately 1 to 5 cm proximal to the recto-anal junction (RAJ) (Naylor et al. 2003; Low et al. 2005). The RAJ is an area dense with lymphoid follicles which was hypothesized to create a previously undescribed niche that *E. coli* O157:H7 cells are capable of colonizing (Naylor et al. 2003; Low et al. 2005). This hypothesis was supported by data regarding human infection that showed the pathogen to have an affinity for the follicle associated epithelium of Peyer's patches in the human small intestine (Phillips et al. 2000).

Since the recognition of the terminal rectum as the primary location of *E. coli* O157:H7 colonization, attempts to detect *E. coli* O157:H7 within this location have been made using recto-anal mucosal swabs (RAMS). It has been reported that RAMS may be the most sensitive method for detecting *E. coli* O157:H7 carriage in cattle (Rice et al. 2003; Greenquist et al. 2005; Davis et al. 2006; Cobbold et al. 2007). It was noted that concentrations of *E. coli* O157:H7 on the surface of feces were approximately 1000-fold higher than in the core of feces and researchers suggested that as feces passes through the terminal rectum, the fecal surface gets coated with *E. coli* O157:H7 that is colonizing the area (Naylor et al. 2003). Low et al. (2005) compared the concentration of *E. coli*

O157:H7 isolated from tissues 1 and 15 cm proximal to the RAJ and from feces 1 and 15 cm proximal to the RAJ. These researchers found that *E. coli* O157:H7 concentrations were higher closer to the RAJ (1 cm proximal) and that high levels of mucosal carriage was associated with fecal excretion of *E. coli* O157:H7 (Low et al. 2005). Further, it appeared that there existed two populations of animals; cattle shedding high concentrations of *E. coli* O157:H7 ( $>10^3$  CFU/g) detectable by less sensitive dilution plating methods and cattle shedding low concentrations ( $<10^3$  CFU/g) detectable only by immunomagnetic separation (Naylor et al. 2003; Low et al. 2005).

Rectal administration of *E. coli* O157:H7 has allowed researchers to study factors that may affect *in vivo* colonization of cattle because this technique provides the ability to directly apply the *E. coli* O157:H7 to the RAJ (Sheng et al. 2004). In one of the first uses of the recto-anal administration technique, mutant strains of *E. coli* O157:H7 lacking functional *eae* and *tir* genes colonized cattle less efficiently than the wild type strain (Sheng et al. 2006). In the same study, Stx2 producing and Stx2 lacking *E. coli* O157:H7 strains had similar colonization of the recto-anal mucosa suggesting that this toxin does not increase adherence at the follicle-dense RAJ (Sheng et al. 2006). In contrast, the pO157 plasmid was found to be an important factor for *E. coli* O157:H7 adherence and colonization (Sheng et al. 2006). In another application of recto-anal inoculation of *E. coli* O157:H7 researchers observed that shedding of an O-antigen-deficient mutant *E. coli* O157:H7 strain was shorter in duration and smaller in magnitude compared to the wild type strain suggesting a role for the O antigen in the colonization of cattle (Sheng et al. 2008).

Following the identification of the RAJ as a location of important colonization within cattle, several other studies were aimed at characterizing the location of *E. coli* O157:H7 within persistently shedding cattle. In one study, three persistently shedding cattle were necropsied and the majority of culture positive samples were obtained from the RAJ (Lim et al. 2007), supporting this area as the key site for *E. coli* O157:H7 colonization. In another study, intestinal tissues from experimentally inoculated cattle were examined 1 to 3 months after shedding had ceased (Baines et al. 2008b). Focal petechiae (pinpoint hemorrhages) and mucosal hemorrhages were observed in tissues from non-persistently and persistently shedding animals (Baines et al. 2008b). These hemorrhages were more numerous in tissues from the persistently shedding animals and repair of the tissue was estimated to take approximately 3 months following the end of shedding (Baines et al. 2008b). While pathologies were observed to affect the mucosa of the jejunum, ileum, cecum and ascending colon, the most severe pathologies were associated with the jejunum (Baines et al. 2008b). These authors suggested that the jejunum and ileum may play a larger role than the RAJ in maintaining infection and that the RAJ may serve as an indicator of infection status but it is not responsible for persistent shedding (Baines et al. 2008b). Nart et al. (2008) examined 8 to 14 week old weaned calves for the presence of pathologies and immune response to *E. coli* O157:H7 infection. The investigators found A/E lesions associated with the terminal rectum and a localized production of IgA antibodies (Nart et al. 2008). The presence of pathologies and localized immune responses suggests that *E. coli* O157:H7 should not be considered a commensal organism in cattle but instead a pathogen of cattle (Baines et al. 2008b; Nart et al. 2008).

Recently, Baines et al. (2008b) used an *in vitro* organ culture (IVOC) adherence assay to compare *E. coli* O157:H7 colonization of intestinal tissues from both persistent and non-persistent shedding cattle. While the IVOC adherence assay did not reveal any tissue tropism for *E. coli* O157:H7 colonization, there was significantly greater adherence to tissues from persistently shedding than non-persistently shedding cattle (Baines et al. 2008b). Due to the lack of an immune response in the *in vitro* assay, an “undefined mucosal factor” was implicated for causing the difference between tissues from the different animals and further speculated that this mucosal factor could in fact be alternate non-Tir based colonization sites (Baines et al. 2008b).

Other areas in cattle have been identified to harbor *E. coli* O157:H7 including oral cavities and hide surfaces (Keen and Elder 2002). However, it is unlikely that the pathogen actually colonizes these areas and is likely present only on the surface in a transient state. The oral cavity of cattle has been suggested to contain *E. coli* O157:H7 due to the process of rumination (Callaway et al. 2009). Similarly, cattle hides are presumed to be contaminated by feces and soil containing *E. coli* O157:H7 due to the extended duration of survival in these substrates (Wang et al. 1996; Kudva et al. 1998; Jiang et al. 2002; Franz et al. 2008). Fecal prevalence of *E. coli* O157:H7 was found to be correlated to hide prevalence; cattle that were housed in pens that contained greater than 20% of the total fecal pats contaminated with *E. coli* O157:H7 had much higher hide prevalence than cattle that were housed in pens with less than 20% of the total fecal pats contaminated with *E. coli* O157:H7 (Woerner et al. 2006). Due to often high prevalence of *E. coli* O157:H7 on cattle hides, practices aimed at reducing this load have been implemented (Callaway et al. 2009). Simple and inexpensive hide washing cabinets

(Arthur et al. 2007b) using ozonated, electrolyzed (Bosilevac et al. 2005) or hot water (Bosilevac et al. 2006) have been shown to be effective at significantly reducing the *E. coli* O157:H7 load on cattle hides.

#### **1.4. Shedding of *E. coli* O157:H7 by cattle**

The cattle reservoir has been identified as the key target for mitigation strategies aimed at reducing human *E. coli* O157:H7 infections (Jordan et al. 1999) and efforts to understand and reduce fecal shedding of cattle have been a focal point of research. Diet modification has been implicated as a practical method of reducing the amount of *E. coli* O157:H7 shed by cattle; however relationships between feeding practices and shedding have been clouded by conflicting or inconsistent results (Callaway et al. 2003). There seem to be a number of different dietary factors which may affect *E. coli* O157:H7 populations within cattle including fasting, feed additives and probiotics. Before and during transport, cattle are often subject to fasting for up to 48 hours (Callaway et al. 2009). Dietary stress was found to make weaned calves more susceptible to infection by *E. coli* O157:H7 (Cray et al. 1998). However, while fasting has been shown to increase total *E. coli* populations (Cray et al. 1998) it is unclear whether fasting also increases *E. coli* O157:H7 shedding as previous studies have failed to find an increase in shedding following fasting (Kudva et al. 1997; Harmon et al. 1999).

The feed additive monensin is an ionophore commonly used to increase the efficiency of feed utilization (Russell and Strobel 1989) and because the use of monensin roughly coincided with the first cases of human infection, it has been proposed that this additive may have played a role in increasing the prevalence of *E. coli* O157:H7 (Bach et

al. 2002a). Monensin concentrations up to 25 µg/ml did not affect *in vitro* growth and survival rates for *E. coli* O157:H7 (Bach et al. 2002a). Only at very high concentrations of monensin (50 µg/ml) was survival impaired (Bach et al. 2002a). Similarly, ionophores including monensin were found to have no effect on *E. coli* O157:H7 *in vitro* (Edrington et al. 2003b) or *in vivo* (Edrington et al. 2003a). In contrast, Van Baale et al. (2004) found that adding monensin to a grain diet resulted in the shedding of lower concentrations of *E. coli* O157:H7 in the first 5 days following inoculation. When monensin was added to a forage diet, the duration of *E. coli* O157:H7 shedding was significantly reduced (Van Baale et al. 2004). Whether monensin and other similar ionophores reduce fecal shedding of *E. coli* O157:H7 remains unclear, however the original hypothesis that monensin could select for and increase *E. coli* O157:H7 shedding has been dismissed.

In 1998, the probiotic effect of other bacteria on *E. coli* O157:H7 was demonstrated when a cocktail containing one *Proteus mirabilis* and 17 *E. coli* strains was able to significantly reduce cattle carriage and shedding of *E. coli* O157:H7 (Zhao et al. 1998). Since then there has been extensive research into developing and assessing the effects of different probiotics on *E. coli* O157:H7. Lactic acid producing *Lactobacillus* strains have been observed to reduce *E. coli* O157:H7 growth *in vitro* (Ogawa et al. 2001) and shedding *in vivo* (Brashears et al. 2003; Younts-Dahl et al. 2004). Similarly, the probiotic effect of a three strain mixture of commensal *E. coli* was demonstrated when *E. coli* O157:H7 shedding was significantly reduced in weaned calves, presumably by competitive exclusion (Tkalcic et al. 2003). Recently, a commensal *E. coli* strain, which has the ability to significantly reduce the levels of Shiga toxins produced by various STEC strains, was discovered and characterized (Reissbrodt et al. 2009). While the exact

mechanism or combination of factors that resulted in this reduction in Shiga toxins remains to be elucidated, this *E. coli* strain has potential as an effective probiotic. The mechanism by which probiotics are able to affect pathogens has received much attention and it was found that a cell free supernatant of a probiotic strain was able to increase epithelial cell tight junction integrity and prevent tight junction damage following the administration of *E. coli* O157:H7 cell free supernatant (Putala et al. 2008). Similarly, other researchers observed that *L. acidophilus* cell extracts were able to increase expression of MUC2 mucin, which inhibited *E. coli* O157:H7 attachment to human intestinal epithelial cells (Kim et al. 2008).

### **1.5. *E. coli* O157:H7 survival in the environment**

*E. coli* O157:H7 is capable of surviving within the environment for extended periods of time. The most common source of *E. coli* O157:H7 within the environment is cattle feces and fecal slurries (Avery et al. 2004; Bach et al. 2005a; Franz et al. 2005; Scott et al. 2006). However, *E. coli* O157:H7 can also survive for durations in cattle feed, water (Avery et al. 2008), soil (Franz et al. 2008; Semenov et al. 2008) and on fresh fruits or vegetables (Aruscavage et al. 2008). A significant source of *E. coli* O157:H7 contamination is attributed to contact with cattle feces, through cattle mediated transfer of feces, runoff or deposition of feces onto farm land. It is worth noting that *E. coli* O157:H7 can be present in the saliva of infected cattle, and this is a possible route for the contamination of feed and water troughs (Keen and Elder 2002). Once in the environment, the pathogen is available to re-infect and spread throughout cattle populations within individual feedlots. Of major concern are feces as a source of *E. coli*

O157:H7 contamination during transportation to housing at beef-processing facilities because hide prevalence has been noted to be increased just prior to slaughter, likely due to fecal contamination (Arthur et al. 2007a; Arthur et al. 2008). Further, infections of humans from contaminated environments have also been reported. Human *E. coli* O157:H7 outbreaks have been traced back to fairs and petting zoos (Stirling et al. 2008; Centers for Disease Control and Prevention 2009; Goode et al. 2009).

The long term survival of *E. coli* O157:H7 in animal feces has long been known (Wang et al. 1996; Kudva et al. 1998; Fukushima et al. 1999). In early studies, *E. coli* O157:H7 survival in feces ranged from 4 weeks up to 21 months depending on the environmental conditions (Wang et al. 1996; Kudva et al. 1998; Fukushima et al. 1999). Further, it was observed that *E. coli* O157:H7 persistence was enhanced when feces were under environmental conditions compared to feces that were maintained under controlled conditions (Kudva et al. 1998). Various studies have attempted to elucidate the factors that may alter *E. coli* O157:H7 survival in feces and many factors have been implicated including temperature, fecal matrix, pH, volatile fatty acid (VFA) content and dry matter (DM) content of the feces. In one study, *E. coli* O157:H7 had greater survival at 22°C than -10°C and 4°C (Bach et al. 2005a). Similarly, Kudva et al. (1998) observed that *E. coli* O157:H7 in bovine feces had higher viable counts for at least 14 days when incubated at 23°C compared to -20°C and 4°C. Further, a rapid decline in viable cell count was observed in feces at 37°C (Kudva et al. 1998). In another study, survival of *E. coli* O157:H7 was enhanced at 4°C compared to 37°C (Echeverry et al. 2006).

Diet can also influence the persistence of *E. coli* O157:H7 as at -10°C it persisted longer in feces from cattle fed barley as compared to those fed corn (Bach et al. 2005a).

Similarly, reduced *E. coli* O157:H7 survival was observed in manure derived from cattle fed straw as compared to manure from cattle fed a mixture of grass and maize silage (Franz et al. 2005). Dehydration of feces was suggested to reduce *E. coli* O157:H7 survival in early studies (Wang et al. 1996; Kudva et al. 1998), however more recent research suggests that this may not be the case (Bach et al. 2005a; Hutchison et al. 2005). Both pH and VFA content have been suggested to affect the survival of *E. coli* O157:H7 and it is believed that rapid fermentation of grain diets in the rumen leads to increased VFA concentrations which in turn lowers ruminal pH (Leyer et al. 1995; Diez-Gonzalez et al. 1998). However, different studies have resulted in conflicting results regarding the effect of pH on *E. coli* O157:H7 survival. In one study, high VFA concentrations and a pH below 6.5 reduced *E. coli* O157:H7 survival (Bach et al. 2005a). In another study, feces with a high pH (7.8) from cattle fed straw reduced the survival of *E. coli* O157:H7 as compared to the lower pH (6.1) in feces from cattle fed a mixture of grass and maize silage (Franz et al. 2005). However, comparing across studies is often difficult due to differences in methodologies and Franz et al. (2005) suggested that the increased rate of decline could be related to fiber content as well. *E. coli* O157:H7 is often associated with relatively nutrient rich environments and slower release of readily available nutrients from feces with high fiber content may impair survival (Franz et al. 2005).

*E. coli* O157:H7 contaminated water can infect humans (Ackman et al. 1997) and this pathogen may also persist in water within feedlots (McGee et al. 2002; Avery et al. 2008). Duration of survival seems to vary with water source as it was observed that *E. coli* O157:H7 survived longer in lake waters than river waters and drinking troughs (Avery et al. 2008). Further, the mode of contamination seems to have an effect and

researchers observed that passage through the bovine gastrointestinal tract increased *E. coli* O157:H7 survival in untreated river water, possibly due to acclimatization to a more harsh environment (Scott et al. 2006).

There are a number of other surfaces on which *E. coli* O157:H7 can survive for extended periods of time and the roles of these surfaces in the maintenance and transmission of the pathogen have been partially elucidated. *E. coli* O157:H7 was observed to have extended survival in grass hay feed in the laboratory, outside and within barns, and survived up to 60 days in the laboratory (Davis et al. 2005). *E. coli* O157:H7 has also been found to grow and persist in wood chip bedding. At 25°C, *E. coli* O157:H7 counts in cedar chip bedding moistened with water decreased whereas on bedding moistened with undiluted urine they increased (Davis et al. 2005). *E. coli* O157:H7 can also persist in manure-amended soils for weeks and even months (Jiang et al. 2002; Franz et al. 2008). It appears that persistence can be influenced by soil composition.

Researchers observed that more oligotrophic conditions can reduce *E. coli* O157:H7 survival (Franz et al. 2008). Similarly, *E. coli* O157:H7 can survive for five to six months following the deposition of feces onto pasture (Avery et al. 2004). Due to a number of outbreaks linked to contaminated fruits and vegetables, there has been a focus on determining the fate of *E. coli* O157:H7 on contaminated fresh produce. In a study comparing survival of *E. coli* O157:H7 on a range of vegetables at different temperatures, there was considerable growth of the pathogen on lettuce (~3 log increase) and soybean sprouts (~2 log increase) when incubated at 8°C for 12 days, but no growth of *E. coli* O157:H7 was on these crops at 4°C (Francis and O'Beirne 2001). *E. coli* O157:H7 grew more readily on damaged as compared to intact lettuce (Aruscavage et al. 2008) and a

contaminated coring knife was able to contaminate 19 lettuce heads (McEvoy et al. 2009) demonstrating the importance of careful handling during harvesting.

### **1.6. *E. coli* O157:H7 lineage types**

In an effort to increase knowledge of the evolutionary history of *E. coli* O157:H7, a new method of performing genomic comparisons was devised. Using fluorescently labeled primers to amplify over-represented oligomers of the *E. coli* O157:H7 genome, non-specific amplification was used to sequence and piece together large fragments of the genome which allowed the detection of not only single nucleotide insertions or deletions but large-scale genome alterations as well (Kim et al. 1999). Using this novel method of genome comparison, termed octamer-based genome scanning (OBGS), a phylogenetic tree was constructed for a number of clinical *E. coli* O157:H7 strains from humans as well as strains from cattle and two separate phylogenetic branches, known as lineages, were identified (Kim et al. 1999). Further, due to the over-representation of the study strains associated with clinical human disease to cluster within the lineage I branch and the propensity of study strains isolated from healthy cattle to group within the lineage II branch, it was suggested that the lineage I branch may contain the strains which are the primary cause of human illness, while lineage II strains may be relatively benign (Kim et al. 1999). It was suggested that the higher association of lineage I strains with human illness was due to phage-specific polymorphisms which also clustered specifically with each lineage (Kim et al. 1999). Indeed, others have also observed the link between *E. coli* O157:H7 phage type and lineage type as well as the over-representation of lineage I strains as the cause of human illness. Recently, Zhang et al. (2007) found certain phage-

types to be specific or dominant to the different lineages while Ziebell et al. (2008) found a similar phage-type bias and also noted that 73.8% of their human and 49.5% of their bovine *E. coli* O157:H7 isolates studied were lineage I strains. However, a recent surveillance study conducted in southern Alberta noted that lineage I strains accounted for 72.2% of bovine and 90.1% of human *E. coli* O157:H7 isolates, indicating that geographic location may influence the prevalence of the lineages of *E. coli* O157:H7 (Sharma et al. 2009).

As a follow-up to their OBGS analysis, a more diverse set of *E. coli* O157:H7 strains from Australia and the USA were analyzed using OBGS and a more extensive phylogenetic tree was constructed (Kim et al. 2001). Again the same two lineages were identified from the diverse group of *E. coli* O157:H7 strains which suggested that the divergence of lineage I and II strains was an ancestral event prior to the dissemination of the pathogen to the geographically separate continents (Kim et al. 2001). However, with the increased number of geographically distributed strains of *E. coli* O157:H7, it has become apparent that regional subpopulations had arisen with the emergence of at least three independent lineage II branches (Kim et al. 2001).

#### **1.6.1. Lineage-specific polymorphism assay-6**

In an effort to devise a higher throughput method of lineage typing, Yang et al. (2004) screened 95 lineage-specific OBGS fragments for polymorphisms which were within selectively neutral genes or non-coding regions. A total of six fragments were selected based on these criteria and used to create the lineage-specific polymorphism assay-6 (LSPA-6), a multiplex PCR assay capable of categorizing *E. coli* O157:H7 strains into

OBGS lineages based on PCR allele length (Table 1.1) (Yang et al. 2004). For the LSPA-6 assay, allele sizes of OBGS lineage I strains are denoted as 1's while allele sizes of OBGS lineage II strains are denoted as 2's to give a six digit LSPA-6 genotype (Yang et al. 2004). The LSPA-6 codes *E. coli* O157:H7 lineage I strains as 111111 (for lineage I fragment lengths at all loci), lineage II strains as 222222 and also types a number of LSPA-6 lineage intermediates, such as 211111. It was suggested that many of these intermediate lineage types belong to OBGS lineage II strains (Yang et al. 2004). However, more recent characterization of the most common intermediate lineage type, LSPA-6 genotype 211111, suggests that this group shares some features with both lineage I and II strains and may represent a third distinct phylogenetic subset of *E. coli* O157:H7 strains, designated lineage I/II strains (Zhang et al. 2007; Laing et al. 2008; Ziebell et al. 2008).

The LSPA-6 alleles were compared to genome sequences of a commensal *E. coli* K-12 strain and a strain of uropathogenic *E. coli*. The *E. coli* K-12 strain contained five of the six alleles used in the LSPA-6 and four of these five had allele sizes which corresponded to OBGS lineage I strains. The uropathogenic *E. coli* strain only contained two of the six alleles used in the LSPA-6 but both of these were the same size as OBGS lineage I alleles (Yang et al. 2004). This suggests that the OBGS lineage I state is ancestral and that lineage II traits are derived (Yang et al. 2004). Twelve regions of genomic DNA were found to be conserved in most lineage I strains and in other *E. coli* strains such as K-12, but these were regions were present in only a few lineage II and intermediate lineage strains. This further supports the hypothesis that lineage I is the ancestral lineage (Steele et al. 2007).

**Table 1.1.** Allele sizes of lineage-specific polymorphisms in the LSPA-6

Primer/allele name	Lineage 1 expected amplicon size (bp)	Lineage 2 expected amplicon size (bp)
<i>fold-sfmA</i>	161	170
<i>Z5935</i>	133	142
<i>yhcG</i>	394	472
<i>rbsB</i>	218	209/214
<i>rtcB</i>	270	279
<i>arp-iclR</i>	315	333/324

### **1.6.2. Virulence and pathogenicity of *E. coli* O157:H7 lineages**

*E. coli* O157:H7 lineage I strains are more often associated with human clinical infection (Kim et al. 1999; Kim et al. 2001; Saridakis et al. 2004; Yang et al. 2004; Ziebell et al. 2008) suggesting that lineage I strains are more virulent or more readily survive in the human digestive tract than lineage II. Saridakis et al. (2004) compared the survival of lineage I and II strains challenged with different acids. Lineage I strains were found to have greater survival than lineage II strains after a six hour challenge in a cocktail of volatile fatty acids, while lineage II strains had greater survival after a three and six hour challenge in HCl (Saridakis et al. 2004). However, most of the lineage I strains were of human or porcine-origin whereas the lineage II strains were of bovine-origin, raising the possibility that the sensitivity of isolates to different acid challenges depends on the host from which they were derived. To our knowledge, further studies of *E. coli* O157:H7 lineage survival within acid challenges have yet to be completed.

There have been few comparisons of gene expression profiles between *E. coli* O157:H7 lineage I and II strains. Many have compared expression profiles between *E. coli* O157:H7 isolates of bovine and human-origin (McNally et al. 2001; Ritchie et al. 2003; Lejeune et al. 2004; Rashid et al. 2006; Baker et al. 2007), often suggesting that their results are related to lineage type. However, surveillance studies suggest that a large fraction of bovine isolates are still lineage I strains (Kim et al. 1999, Kim et al. 2001, Yang et al. 2004, Ziebell et al. 2008), making it difficult to solely equate lineage to host type. In 2006, microarray analysis was used to compare gene expression between *E. coli* O157:H7 lineage I and II isolates. It was found that of the 610 genes monitored, 73 differed in their level of expression between lineages (Dowd and Ishizaki 2006). Genes

that tended to be up-regulated in lineage I strains included *stx2A*, *stx2B* and some genes coding for proteins involved in the type III secretory system, an observation that may account for the increased virulence associated with lineage I strains (Dowd and Ishizaki 2006). Other genes of potential importance up-regulated in lineage I strains were involved in urease production, which might confer increased acid resistance (Dowd and Ishizaki 2006). In another study, twelve conserved regions within the genome of lineage I strains were absent in most lineage II strains and many of these regions were located within prophage or prophage-like elements related to virulence factors, including the shiga toxin genes, or contain ORFs with homology to virulence genes (Steele et al. 2007). Similarly, Zhang et al. (2007) found 132 out of 1751 variably absent or present (VAP) ORFs to be specific or dominant to either lineage I or II strains and noted that many of these occurred within prophage regions. Not surprisingly, there were VAPs located within the region encoding Stx2 as well as an absence of known effector genes (*nleH1-2*, *nleF* and *pchD*) in lineage II strains (Zhang et al. 2007). The VAPs located around Stx2 are likely related to results found by others where the Stx2c variant was found in 59 of 61 lineage II strains but only 2 of 112 lineage I strains (Ziebell et al. 2008) and an Stx2-specific ELISA found that lineage I strains produced more Stx2 than lineage II strains (Dowd and Williams 2008). Most recently, Laing et al. (2008) used 23 VAP regions to develop a comparative genomic fingerprinting method for typing *E. coli* O157:H7 strains. This method was found to be superior to both pulsed field gel electrophoresis and phage-typing and produced a phylogeny where LSPA-6 lineages I, II and I/II all grouped separately (Laing et al. 2008).

## CHAPTER TWO

### **Lineage type does not influence the survival of *Escherichia coli* O157:H7 in feces from cattle fed grain or grass hay diets**

#### **2.1 ABSTRACT**

Genetic comparisons of numerous *Escherichia coli* O157:H7 strains have resulted in the recognition of different phylogenetic branches of the pathogen and it appears lineage I strains cause the majority of human illnesses. Recently described differential acid resistance between *E. coli* O157:H7 lineages suggests that cattle feeding practices and warm summer months may alter fecal acidity, resulting potentially in increased transmission of lineage I strains to the food supply. The objective of this study was to document the impact of lineage type on the proliferation and survival of *E. coli* O157:H7 in feces from cattle fed grain or forage diets at different temperatures encountered in Alberta during peak shedding periods. Three strains of *E. coli* O157:H7 lineages I and II were inoculated into feces derived from cattle fed grain or hay and survival was assessed at 4°C, 12°C and 25°C over a period of 28 days. There were no significant lineage associated differences in survival in grain or hay feces at any temperatures studied. There was an interaction of temperature, feces type and day post inoculation on the survival of *E. coli* O157:H7 in feces. This was related to pH of the feces. Fecal pH increased with temperature for both feces types and hay feces always had a higher pH than grain feces, however this difference was smallest at 25°C. The highest volatile fatty acid accumulation was observed in the grain feces at 4°C and VFA content was higher in grain than hay feces. *E. coli* O157:H7, irrespective of lineage, survived better in grain than hay feces at 4°C but the reverse was observed for grain and hay feces at 25°C. The

current study concludes that lineages of *E. coli* O157:H7 do not differ in their persistence in feces, but temperature and fecal matrix do affect the survival of *E. coli* O157:H7.

## 2.2. INTRODUCTION

*Escherichia coli* serotype O157:H7 is a pathogenic, Gram-negative bacterium that belongs to the enterohemorrhagic *E. coli* (EHEC) group. *E. coli* O157:H7 infections cause diarrhea, hemorrhagic colitis and can lead to often fatal hemolytic uremic syndrome (HUS) in humans. There have been outbreaks and sporadic cases of *E. coli* O157:H7 infection since its first identification as a predominant pathogen associated with HUS in the early 1980's (Riley et al. 1983; Ratnam et al. 1988). In North America, *E. coli* O157:H7 causes thousands of infections and several deaths per year (Centre for Infectious Disease Prevention and Control 2005; Chase-Topping et al. 2008) costing national healthcare systems millions of dollars annually (Frenzen et al. 2005; United States Department of Agriculture 2006). Despite aggressive efforts to improve operational practices (Bach et al. 2002b) to reduce the incidence of transmission at the processing level, the incidence of *E. coli* O157:H7-related disease outbreaks has not significantly declined. Understanding how this pathogen behaves in the environment and host may provide new insight into control measures.

Ruminants have been identified as the primary reservoir of *E. coli* O157:H7 with numerous outbreaks being traced to contaminated meat (MacDonald et al. 2004) and dairy products (Gillespie et al. 2003). Other sources of infection include fresh produce (Welinder-Olsson et al. 2004) as well as drinking and recreational waters (Ackman et al. 1997; Centre for Infectious Disease Prevention and Control 2006) that have been contaminated by livestock manure. Controlling *E. coli* O157:H7 shedding in cattle has received much attention with an increasing focus on identification of strategies to reduce the post-shedding dissemination and proliferation of this pathogen (Bach et al. 2002b).

Previous work from our lab has indicated that animal diet may affect the survival of *E. coli* O157:H7 in feces as survival was enhanced in feces from cattle fed barley compared to those fed corn (Bach et al. 2005b), indicating that diet modifications could potentially reduce *E. coli* O157:H7 prevalence.

New molecular techniques, such as octamer-based genome scanning, have helped identify two distinct lineages of *E. coli* O157:H7, lineages I and II (Kim et al. 2001; Yang et al. 2004). Although both lineages have been recovered from cattle, lineage I is primarily associated with human infection (Sharma et al. 2009). At present, rapid lineage genotyping techniques such as the lineage specific polymorphism assay (LSPA-6) are valuable for discrimination of lineage types from different sources including humans, cattle and the environments (Yang et al. 2004). Assessing the differential survival of *E. coli* O157:H7 lineages in feces could provide useful insight into why there are differences in the prevalence and dissemination of *E. coli* O157:H7 lineages.

The objective of this study was to determine the *in vitro* growth and survival characteristics of *E. coli* O157:H7 lineages I and II in feces stored at 4°C, 12°C and 25°C from cattle fed barley-grain or hay diets. For this purpose, we used three representative strains from each lineage type. We monitored the pH, dry matter content (DM) and volatile fatty acid (VFA) content of the feces in order to gain information on how changes in chemical composition of feces with time may influence the persistence and survival of *E. coli* O157:H7.

## 2.3. MATERIALS AND METHODS

### 2.3.1. *E. coli* O157:H7 strains

Distinct lineage I (LSPA-6 genotype 111111) and II (LSPA-6 genotype 222222) strains were used for *in vitro* inoculation of feces and were typed using the LSPA-6 assay (Yang et al. 2004). Three *E. coli* O157:H7 strains from each lineage I (Sakai, A and B) and lineage II (E3081, C and D) were used. Strains A, B, C and D were isolated from feces of feedlot cattle, strain Sakai (RIMD 0509952) was obtained from the American Type Culture Collection (Rockford, MD) and E3081 was kindly made available by W. C. Cray, National Animal Disease Center, Ames, IA.

To circumvent problems associated with high coliform counts in cattle feces, nalidixic acid resistant (Nal<sup>R</sup>; 50 µg/ml nalidixic acid) strains of Sakai, A, B, C and D strains were produced; strain E3081 was naturally resistant to kanamycin/ampicillin (Kan<sup>R</sup>/Amp<sup>R</sup>; 100 µg/ml of kanamycin and ampicillin) and thus a Nal<sup>R</sup> mutant of this strain was not produced. Nal<sup>R</sup> strains were produced by stepwise selection on increasing concentrations (5, 10, 20, 40 and 50 µg/ml) of nalidixic acid (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) until strains resistant to  $\geq 50$  µg/ml were obtained. Once the desired level of resistance was achieved in liquid culture, Nal<sup>R</sup> strains were plated on sorbitol MacConkey agar supplemented with cefixime (0.05 mg/l), potassium tellurite (2.5 mg/l) (CT-SMAC) and nalidixic acid (50 µg/ml; CT-SMACnal; Dalynn, Calgary, Alberta, Canada) and incubated at 37°C for an additional 24 h. Single isolates were selected by re-streaking on CT-SMACnal plates. All parental and Nal<sup>R</sup> strains were stored in glycerol at -80°C for subsequent inoculations. The Nal<sup>R</sup> *E. coli* O157:H7 produced in this study have been denoted with subscript “n” (eg. Sakai<sub>n</sub>, A<sub>n</sub>, etc). We did

not find any differences in the growth kinetics between Nal<sup>R</sup> generated strains and their parental counterparts (data not shown) as noted by others (Duffy et al. 2006).

### **2.3.2. Feces collection**

Feces were collected from 16 British-Continental steers fed either barley-grain based diets (85% barley, 10% barley silage and 5% vitamin and mineral supplement on a dry matter basis) or 100% grass hay diets and designated as “grain” or “hay” feces, respectively. Composite samples were prepared from feces collected from four animals. Each replicate consisted of feces from four different animals. Feces were collected within 1 h after defecation. Homogenized feces were weighed (297 g) into sterile stomacher bags (Fisher Scientific, Ottawa, ON, Canada) prior to inoculation with *E. coli* O157:H7.

### **2.3.3. *E. coli* O157:H7 inoculation and sampling**

The lineage I (Sakai<sub>n</sub>, A<sub>n</sub> and B<sub>n</sub>) and II (C<sub>n</sub>, D<sub>n</sub> and E3081) strains were individually inoculated into feces originating from cattle fed grain or hay. For this purpose, strains were grown at 37°C in tryptic soy broth (TSB; Difco, Ottawa, Ontario, Canada) for 18 h. Growth was measured spectrophotometrically and the cell suspension was diluted with TSB to obtain an OD<sub>640</sub> of 0.5 which corresponded to  $\approx 10^8$  CFU/ml. Cells were further diluted to  $10^7$  CFU/ml using phosphate buffered saline (1× PBS).

For inoculation, 3 ml of *E. coli* O157:H7 ( $10^7$  CFU/ml) were inoculated into 297 g of pre-weighed feces to give a final concentration of  $10^5$  CFU/g. Each inoculated sample was mixed for 4 min at high speed using a laboratory stomacher 400 (Seward Ltd., Worthing, West Sussex, UK). Feces were scraped to the bottom of the stomacher

bag at the midway point and upon completion of stomaching. The inoculated feces were left in the stomacher bags with the tops spread open (Wang et al. 1996).

For each temperature and feces type, duplicate control inoculations were prepared whereby sterile inoculum (3ml, sterile TSB diluted 1:9 v/v in 1× PBS) was added to 297 g of pre-weighed feces. The control inoculations containing no *E. coli* O157:H7 were stomached, incubated and sub-sampled in the same manner as the inoculated feces.

Duplicate fecal samples for each diet and inoculated with each strain were incubated at 4°C, 12°C or 25°C to monitor the persistence of individual lineage types. These temperatures were selected as they represent those commonly encountered in southern Alberta during spring (4°C, 12°C) and summer months (25°C), seasons when the prevalence of *E. coli* O157:H7 is highest. The fecal samples were sub-sampled (10 g) on days 0, 1, 3, 7, 14, 21 and 28. The sub-samples were taken by collecting feces from multiple locations within each of the stomacher bags. The sub-samples were then mixed prior to bacterial enumeration and chemical analyses. During sampling, efforts were made to avoid dried feces.

#### **2.3.4. Enumeration and detection of *E. coli* O157:H7**

*E. coli* O157:H7 was enumerated from fecal samples by serially diluting 1 g of feces in 9 ml 1× PBS and plating 50 µl (in duplicate) of the appropriate dilutions (ranging from 10<sup>-1</sup> to 10<sup>-4</sup>) on CT-SMACnal for all strains except E3081. For E3081, CT-SMAC supplemented with kanamycin and ampicillin (100 µg/ml; CT-KASMAC, Dalynn) was used. Colonies were enumerated using a colony counter (Reichert, Depew, NY) and CFU/g wet weight calculated. Three representative non-sorbitol fermenting (NSF)

colonies from each sampling point were confirmed as *E. coli* O157 using the *E. coli* O157 latex test kit (Oxoid Ltd., Basingstoke, Hampshire, UK). During the course of incubations, representative individual NSF colonies were confirmed to retain their lineage characteristic using the described LSPA-6 assay.

### **2.3.5. Fecal dry matter, volatile fatty acid and pH determinations**

Fecal DM content and pH were measured 0, 7, 14, 21 and 28 d following inoculation as described previously (Bach et al. 2005b). VFA content was quantified 0, 7, 14, 21 and 28 d following inoculation. Due to processing limitations, VFA content was analyzed for one strain of each lineage and control feces on days 7, 14, 21 and 28 while all three strains of each lineage and control feces were analyzed for VFA content on day 0. Analysis of pH and DM content was performed on all three strains of each lineage and control feces on all days. All pH, DM and VFA analysis was performed in duplicate. Fecal samples for VFA analysis were weighed (0.2 g) into micro-centrifuge tubes, mixed with 1 ml 25% (w/v) *m*-phosphoric acid (Sigma-Aldrich Canada Ltd.) and frozen at -20°C for subsequent analysis by gas chromatography. The acidified samples were thawed overnight at 4°C and centrifuged at 14 000 × *g* for 5 min. A 0.6 ml aliquot of the supernatant was transferred to an autosampler vial and mixed with 0.1 ml of internal standard (0.25 mmol/l crotonic acid; Sigma-Aldrich Canada Ltd.). Concentrations of acetic, propionic, iso-butyric, butyric, iso-valeric, valeric and caprioc acids were quantified using an Agilent 6890 gas chromatograph fitted with a flame ionization detector (Agilent Technologies Canada Inc., Mississauga, Ontario, Canada). A ZB-FFAP capillary column (30 m × 0.32 mm × 1µm; Phenomenex, Torrance, CA) was used with

helium as a carrier gas (linear velocity ca 1 ml/min). The samples were split-injected (split ratio ca 10 : 1) with analysis performed using the following temperature programs: 170°C, 4 min; increasing 3.5°C per min to 180°C; increasing 30°C per min to 215°C and held for 3 min. The injector was maintained at 225°C and the flame ionization detector at 250°C. VFA concentrations were quantified by comparing the peak area ratios (acid/internal standard) against standard curves using the same concentration of internal standard.

### **2.3.6. Statistical analysis**

*E. coli* O157:H7 counts (CFU/g) and VFA concentrations (mmol/l) were calculated and log transformed to normalize the data. For viable count, DM, pH and VFA analysis, separate analyses were performed using the MIXED procedure (SAS Institute Inc. 2005) with temperature, feces, lineage, day and all their interactions in the model as fixed effects and the replication by feces by lineage interaction as the random effect. Lineage included *E. coli* O157:H7 lineages I and II for viable count analysis and lineages I, II and the control feces for DM, pH and VFA analysis. Day was treated as a repeated measure effect to account for potential correlations among the various days. Mean separation was performed using a LSD test. Additionally, viable count data were analyzed by strain for each temperature treatment using the MIXED procedure (SAS Institute Inc. 2005) with feces, strain, day and all their interactions in the model as fixed effects and the replication by feces by strain interaction as the random effect. Mean separations for the strain differences were performed using a LSD test with a Bonferroni correction. Various types of variance-covariance matrices were fitted and the one with the lowest AIC value was

used for the final analysis. The UNIVARIATE procedure was used to check the residuals for normality and for potential outliers. When an outlier was detected, it was removed before the final analysis was performed. Differences were considered significant where  $P < 0.05$ .

## **2.4. RESULTS**

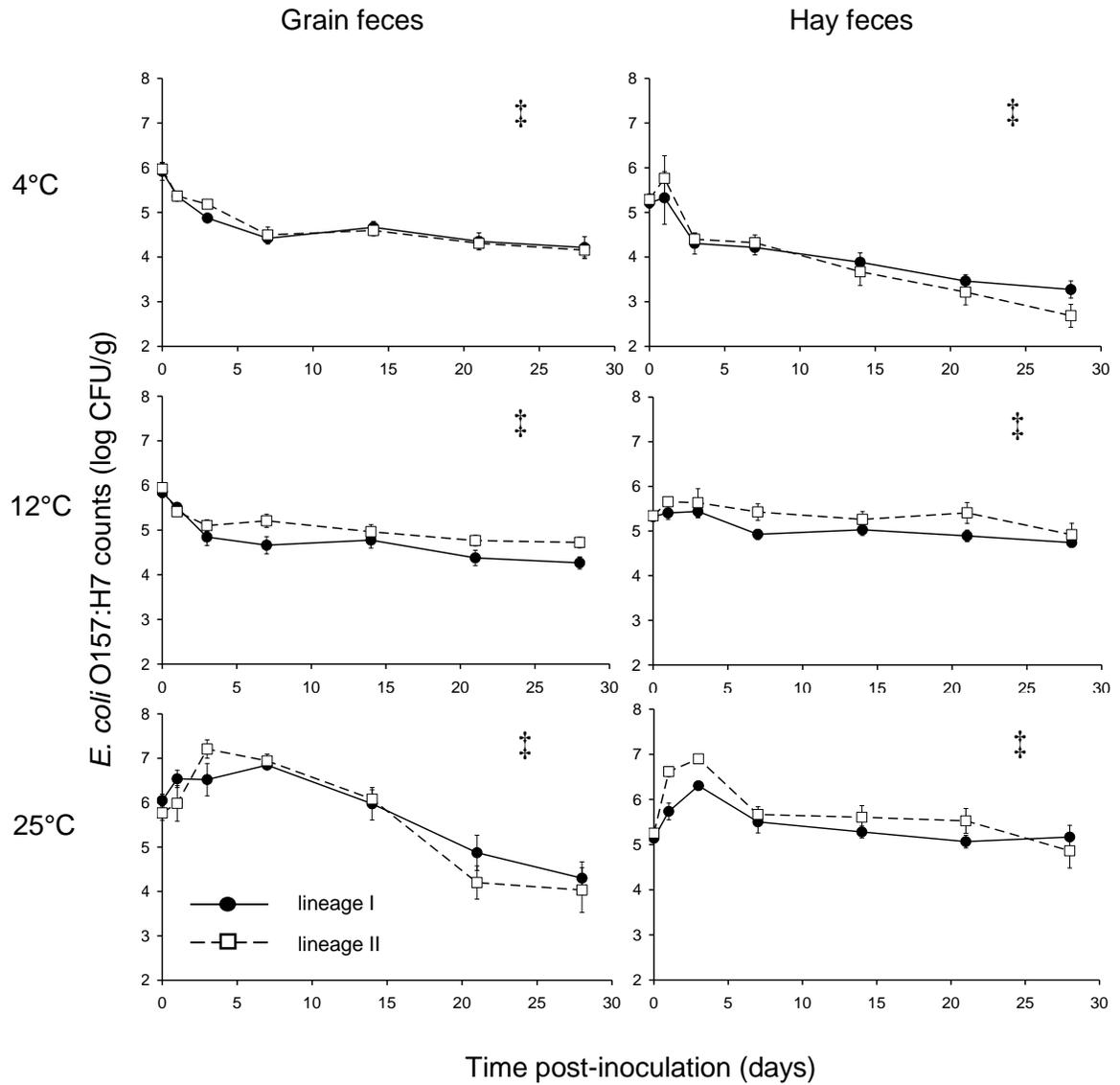
There was a progressive decline in viable *E. coli* O157:H7 after the inoculations reached peak cell densities (at 0 to 3 d; Fig. 2.1), the rate of which was dependant on the feces type and temperature of the incubation. No background *E. coli* O157:H7 or other nalidixic acid resistant (50 µg/ml) or kanamycin/ampicillin resistant (100 µg/ml) bacteria were detected in any of the control inoculations. Growth curves for all antibiotic resistant *E. coli* O157:H7 strains are shown in appendix one (Fig. A.1).

### **2.4.1. Thermostability of *E. coli* O157:H7 lineages**

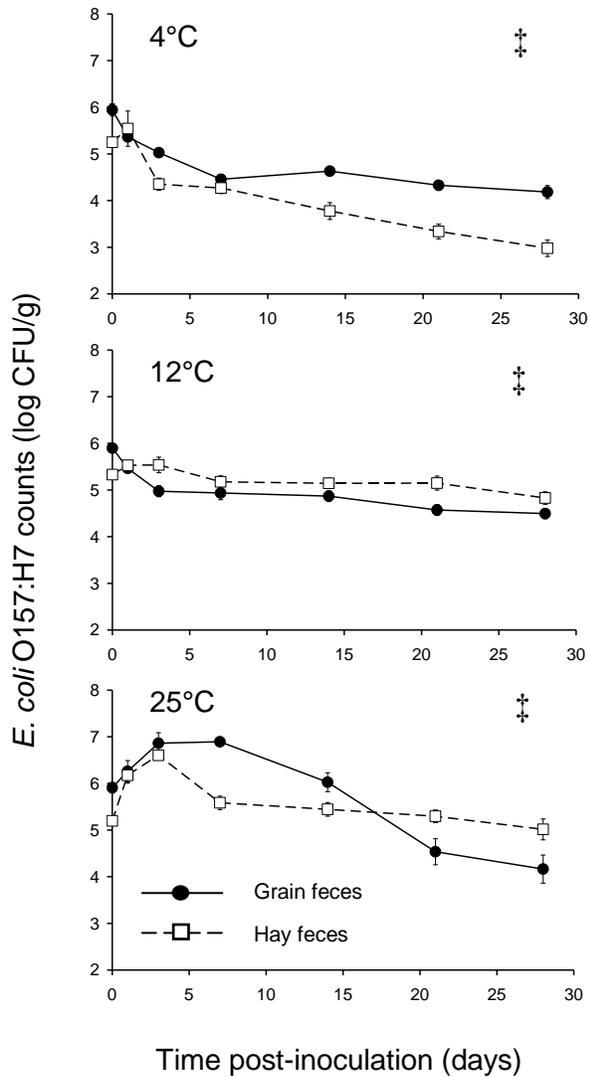
There was no effect of lineage type ( $P = 0.13$ ; Table 2.1) or interactions containing lineage type ( $P > 0.05$ ) on the counts of *E. coli* O157:H7 in feces (Fig. 2.1). There was a highly significant interaction of temperature, feces type and day ( $P = 0.001$ ) on the number of *E. coli* O157:H7 in feces. The interaction of temperature, feces and day resulted in the grain feces having higher counts than the hay feces on days 3, 14, 21 and 28 at 4°C (Fig. 2.2). At 12°C, there was only one day where the grain and hay feces had different *E. coli* O157:H7 counts; *E. coli* O157:H7 numbers were higher in the hay feces on day 21. At 25°C, the grain feces had higher *E. coli* O157:H7 numbers than the hay feces on days 7 and 14 but lower counts on days 21 and 28.

**Table 2.1.** Interactions and effects of lineage type, feces origin, temperature and time post-inoculation on viable *E. coli* O157:H7 counts, dry matter content, pH and volatile fatty acid content of feces.

<b>Variable</b>	<b>Effect/Interaction</b>	<b>P value</b>
Viable <i>E. coli</i> O157:H7 counts	lineage*feces*temp*day	0.82
	lineage*feces*temp	0.19
	lineage*feces*day	0.18
	lineage*temp*day	0.32
	lineage*feces	0.49
	lineage*temp	0.20
	lineage*day	0.07
	lineage	0.13
	feces*temp*day	0.001
	Fecal DM Content	lineage*feces*temp*day
lineage*feces*temp		0.30
lineage*feces*day		0.016
lineage*temp*day		0.23
lineage*temp		0.54
feces*temp*day		0.60
feces*temp		0.002
feces*day		0.001
Fecal pH	temp*day	0.001
	lineage*feces*temp*day	0.66
	lineage*feces*temp	0.16
	lineage*feces*day	0.004
	lineage*temp*day	0.18
	lineage*temp	0.30
	feces*temp*day	0.001
Fecal VFA Content	lineage*feces*temp*day	0.99
	lineage*feces*temp	0.88
	lineage*feces*day	0.92
	lineage*temp*day	0.93
	lineage*feces	0.05
	lineage*temp	0.92
	lineage*day	0.94
	feces*temp*day	0.03



**Figure 2.1.** Differences in the growth and survival of *Escherichia coli* O157:H7 lineages I and II when inoculated in feces from grain or hay fed cattle. Feces were incubated at 4°C, 12°C and 25°C. Mean  $\pm$  standard error values represent the average of three strains (in duplicate) for lineage I (Sakai<sub>n</sub>, A<sub>n</sub>, B<sub>n</sub>) and lineage II (E3081, C<sub>n</sub>, D<sub>n</sub>). ‡ denotes a significant interaction of temperature, feces type and day ( $P = 0.001$ ).



**Figure 2.2.** Differences in the growth and survival of *Escherichia coli* O157:H7 when inoculated in feces from grain or hay fed cattle. Feces were at 4°C, 12°C and 25°C. Mean  $\pm$  standard error values represent the average of six strains (Sakai<sub>n</sub>, A<sub>n</sub>, B<sub>n</sub>, E3081, C<sub>n</sub>, D<sub>n</sub>). Denoted values are means from two independent experiments. ‡ denotes a significant interaction of temperature, feces type and day ( $P = 0.001$ ).

#### 2.4.2 Survival of individual *E. coli* O157:H7 strains

At all temperatures there were significant differences between individual strains for *E. coli* O157:H7 survival (data not shown). At 4°C, there was a significant interaction of feces, strain and sampling day ( $P = 0.003$ ). In the grain feces, there were no differences between different strains on any sampling day ( $P > 0.05$ ). In the hay feces, viable counts were not different among strains on days 0, 7, 14, 21 and 28 ( $P > 0.05$ ). On day 1 of the hay feces, the strain hierarchy for viable counts was  $D_n > \text{Sakai}_n, A_n, C_n > \text{E3081} > B_n$  ( $P < 0.05$ ). On day 3 of the hay feces,  $\text{Sakai}_n$  and E3081 had higher counts than  $B_n$  ( $P < 0.05$ ) but there were no differences between the other strains.

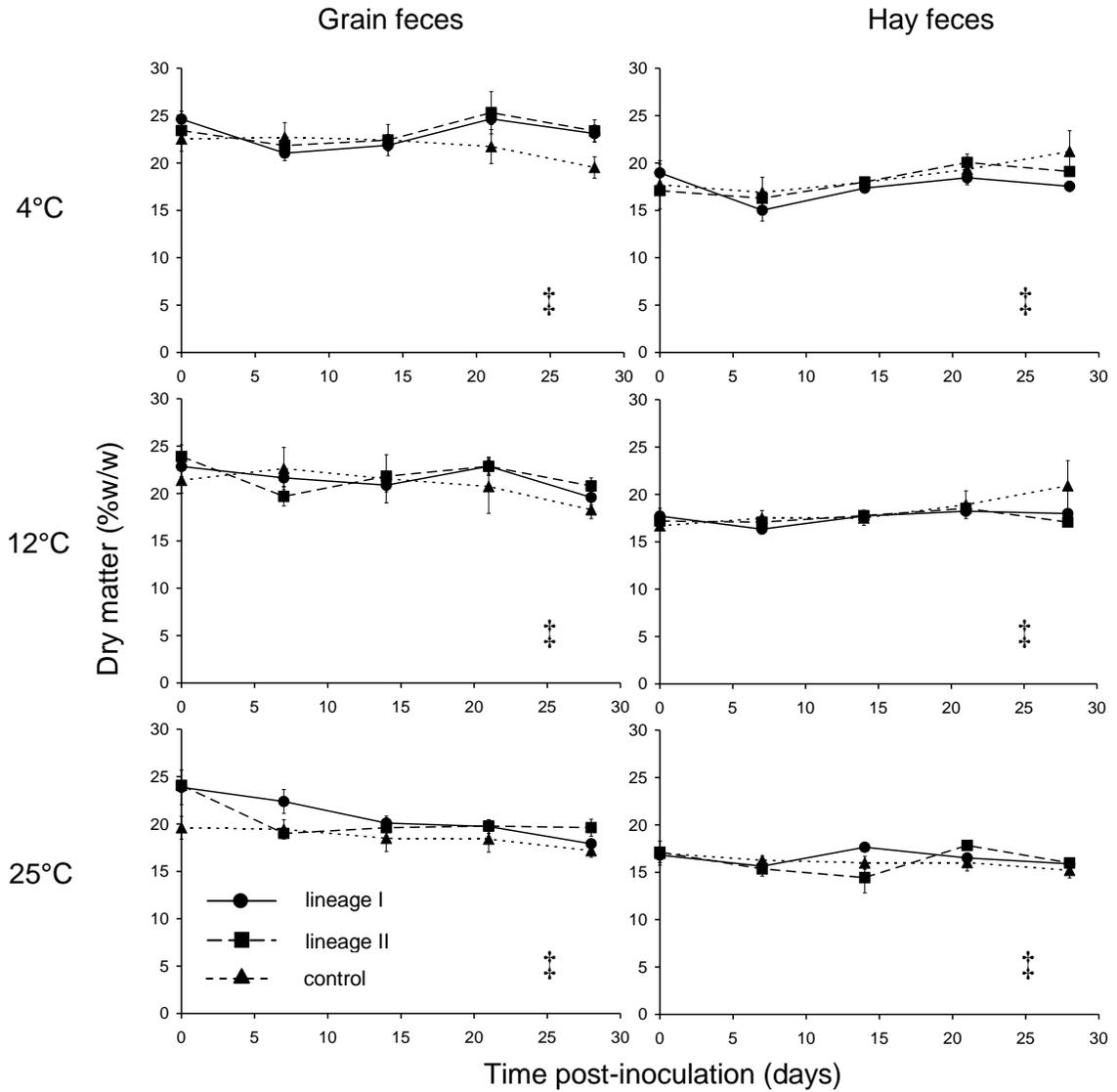
At 12°C, there was a significant interaction of feces and strain ( $P = 0.003$ ). This resulted in strains  $\text{Sakai}_n, B_n, \text{E3081}$  and  $C_n$  having higher viable counts than  $A_n$  in the grain inoculations ( $P < 0.05$ ) and strains E3081 and  $C_n$  having higher counts than  $A_n, B_n$  and  $D_n$  in the hay feces ( $P < 0.05$ ).

Similarly, at 25°C, a significant interaction of feces, strain and day was observed ( $P = 0.003$ ). No differences were observed between different strains on days 0, 1, 3 and 14 in the grain feces. On day 7 in the grain feces, the strain hierarchy for cell counts was  $\text{E3081}, C_n > \text{Sakai}_n, B_n > A_n, D_n$  ( $P < 0.05$ ). On day 21 in grain feces, the hierarchy for cell counts was  $\text{Sakai}_n > B_n, C_n > \text{E3081} > A_n > D_n$  ( $P < 0.05$ ). On day 28 of the grain feces,  $C_n$  had higher cell counts than  $D_n$  ( $P < 0.05$ ). In the hay inoculations, there were no differences between strains on days 0, 1, 3, 14, 21 and 28. On day 7 the observed hierarchy for cell counts was  $B_n, \text{E3081}, C_n > \text{Sakai}_n > D_n > A_n$  ( $P < 0.05$ ).

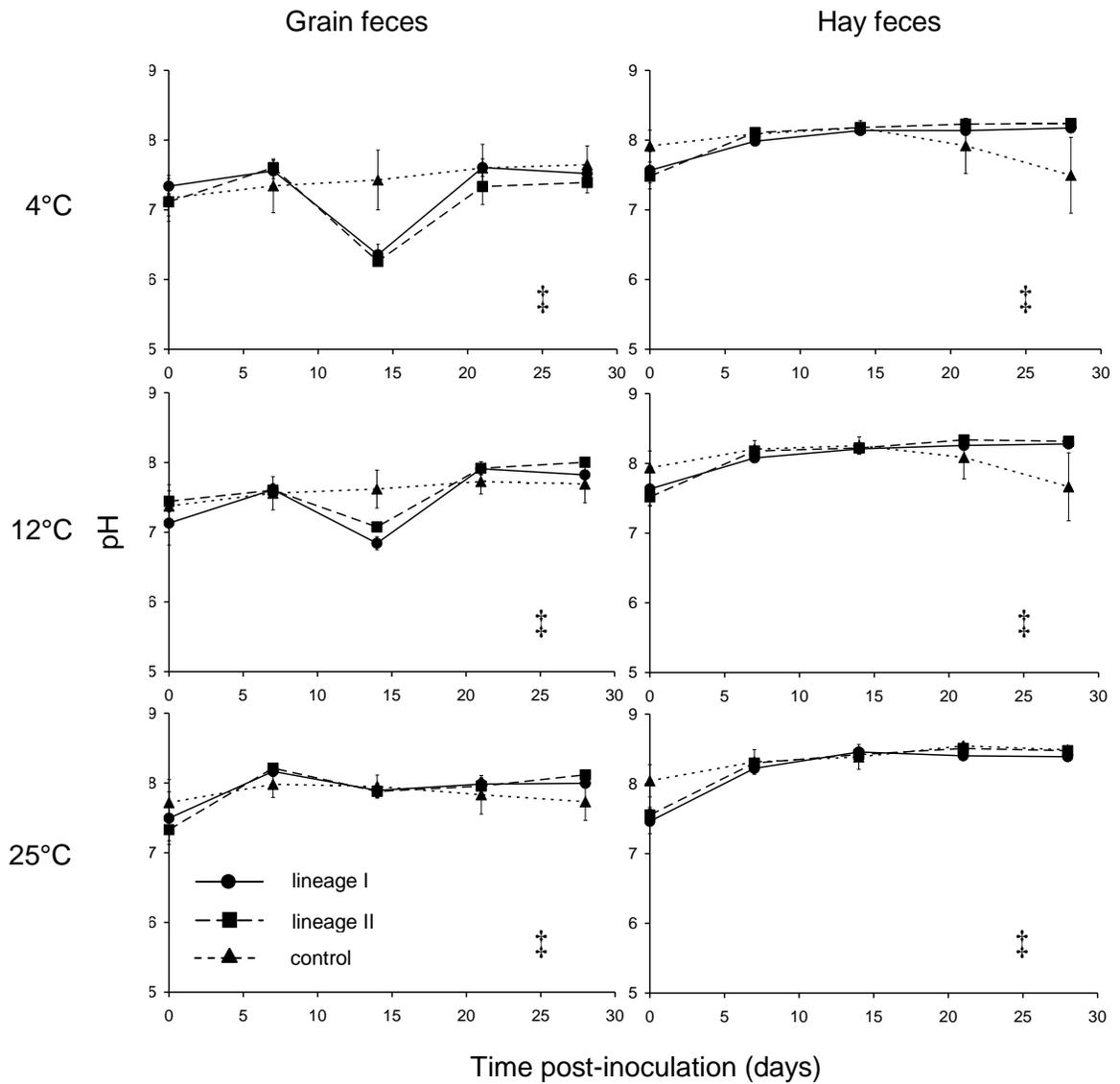
### **2.4.3. Fecal dry matter, volatile fatty acid and pH determinations**

There was a significant interaction of temperature and feces type on the DM content of the feces ( $P = 0.002$ ; Fig. 2.3). The DM content was lowest at 25°C for both feces types and likely represents a difference in relative humidity between incubation systems as well as efforts to avoid dried feces when sampling. For all temperatures, the grain feces had higher DM content than the hay feces. There was also a significant interaction of temperature and day ( $P < 0.001$ ). Despite the significant interaction of temperature and day, there were no trends of increasing or decreasing DM at any temperature. Finally, there was a significant interaction of feces, lineage type and day ( $P = 0.016$ ). On every day and for both lineage types and the controls, the grain feces had higher DM content than the hay; approximately 3-6% higher. On day 7 in the grain feces, the lineage I group had approximately 1.5% higher DM content than the lineage II group.

There was a significant interaction of temperature, feces type and day on the pH of the feces ( $P < 0.001$ ; Fig. 2.4). For both feces types, there were no differences between temperatures in pH of the feces on day 0. Following day 0, the pH was higher for the 25°C treatment than the 4°C treatment on every sampling day for both feces. The hay feces had a higher pH than the grain feces on every day for the 4°C and 12°C groups. At 25°C, the hay feces had a higher pH than the grain feces on days 14, 21 and 28. Both feces types had a trend of increasing pH over time for all temperatures. There was also a significant interaction of feces, lineage and day ( $P = 0.004$ ). On day 14, the control grain feces had a higher pH than the lineage I and II grain feces. On day 28, the control grain feces had a higher pH than the lineage I grain feces. On day 14, the control hay feces had a lower pH than the lineage I and II hay feces. Each of these differences between control



**Figure 2.3.** Changes in mean dry matter (DM) content in grain or hay feces during incubation at 4°C, 12°C and 25°C for 28 days following inoculation with *E. coli* O157:H7 lineages I and II. Mean  $\pm$  standard error values represent the average of three strains (in duplicate) for lineage I (Sakai<sub>n</sub>, A<sub>n</sub>, B<sub>n</sub>) and lineage II (E3081, C<sub>n</sub>, D<sub>n</sub>). Control values represent mean  $\pm$  standard error values of duplicate fecal inoculations containing no *E. coli* O157:H7. ‡ denotes a significant interaction of temperature and feces type ( $P < 0.05$ ).



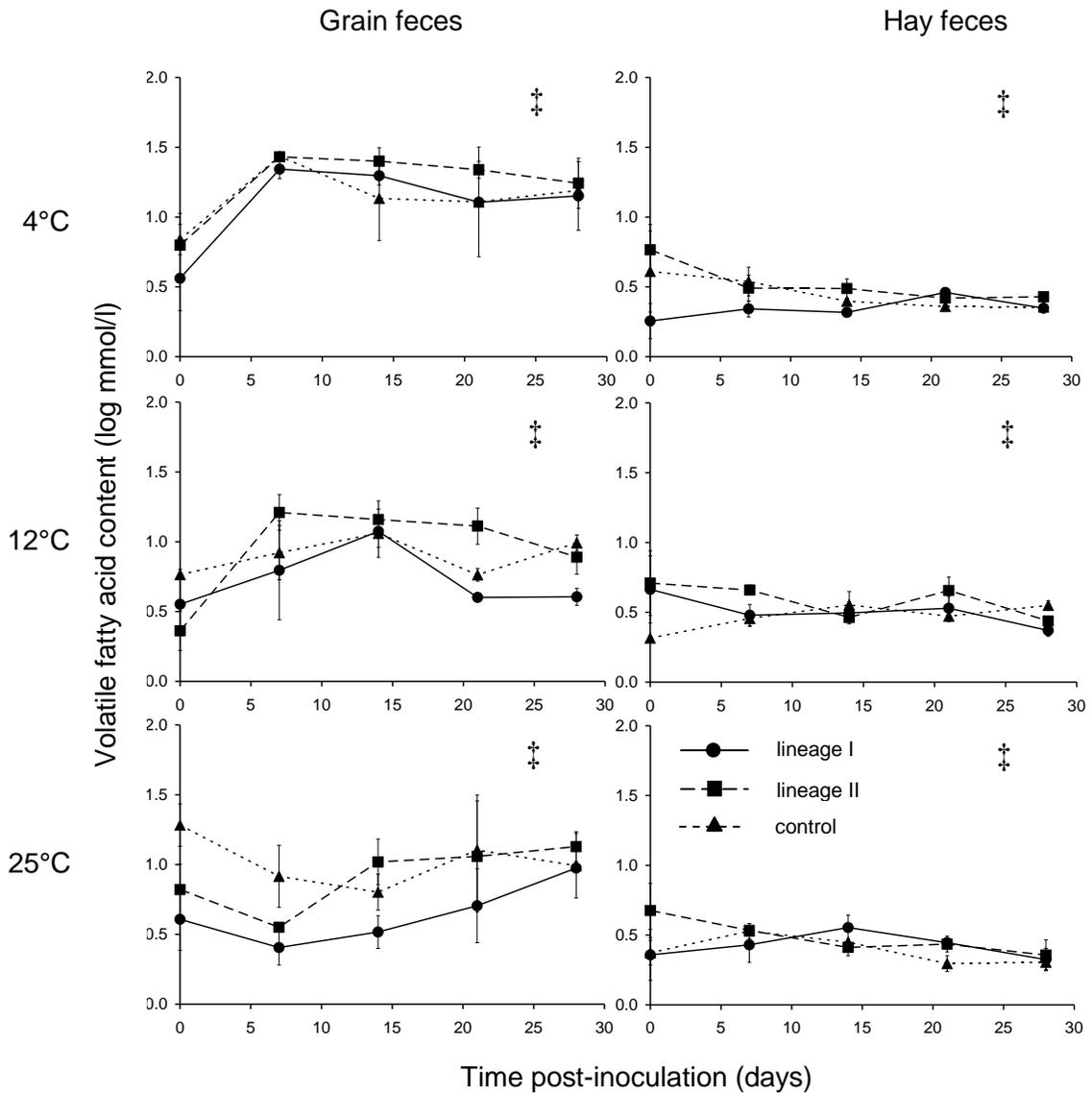
**Figure 2.4.** Changes in pH of grain or hay feces during incubation at 4°C, 12°C and 25°C for 28 days following inoculation with *E. coli* O157:H7 lineages I and II. Mean  $\pm$  standard error values represent the average of three strains (in duplicate) for lineage I (Sakai<sub>n</sub>, A<sub>n</sub>, B<sub>n</sub>) and lineage II (E3081, C<sub>n</sub>, D<sub>n</sub>). Control values represent mean  $\pm$  standard error values of duplicate fecal inoculations containing no *E. coli* O157:H7. ‡ denotes a significant interaction of temperature, feces type and day ( $P < 0.05$ ).

feces and feces containing *E. coli* O157:H7 were small in magnitude (approximately a 0.2 difference in pH) and likely a result of the smaller sample size of the control feces compared to the inoculated feces.

There was a significant interaction of temperature, feces type and day on VFA content ( $P = 0.03$ ; Fig. 2.5). In the grain feces, VFA content was highest at 4°C on days 7, 14, 21 and 28. In contrast, VFA content was not different across temperatures for the hay feces. At 4°C, the grain feces had higher VFA content than the hay feces on days 7, 14, 21 and 28. The same was true at 12°C on days 7 and 14 and at 25°C on days 21 and 28. VFA content data is broken down by its organic acid component for each feces type and temperature combination in Appendix Two (Fig. A.2). There was also a significant interaction of feces and lineage ( $P = 0.05$ ). In the grain feces, the *E. coli* O157:H7 lineage I feces had lower VFA content than the lineage II and control feces. However, this difference may reflect the variability and limited samples for which VFA content could be analyzed.

## **2.5. DISCUSSION**

Currently, there is limited information available regarding the effect of different temperatures and fecal matrices on the survival of lineages of *E. coli* O157:H7. Some studies have reported long-term survival of *E. coli* O157:H7 in feces (Avery et al. 2004; Bach et al. 2005b; Franz et al. 2005; Scott et al. 2006). The current study was not intended as a long-term survival study but instead aimed to define the proliferation and persistence characteristics of *E. coli* O157:H7 lineages I and II in the first few days and weeks at temperatures commonly encountered in Alberta during peak shedding periods.



**Figure 2.5.** Changes in volatile fatty acid content of grain or hay feces during incubation at 4°C, 12°C and 25°C for 28 days following inoculation with *E. coli* O157:H7 lineages I and II. Mean  $\pm$  standard error values represent the average of duplicate inoculations for lineage I (Sakai<sub>n</sub>) and lineage II (E3081). Control values represent mean  $\pm$  standard error values of duplicate fecal inoculations containing no *E. coli* O157:H7. ‡ denotes a significant interaction of temperature, feces type and day ( $P < 0.05$ ).

In addition, since cattle are fed high grain diets during the finishing process, the current study also examined the role that diet may play in the persistence of *E. coli* O157:H7 in bovine feces. Feces are a vector of transmission of *E. coli* O157:H7 (Wang et al. 1996; Bach et al. 2005b) and changes in cattle diets have been shown to affect *E. coli* O157:H7 survival in feces (Bach et al. 2005b; Franz et al. 2005). However, it is not known if the persistence of *E. coli* O157:H7 differs between lineages. Lineage associated differences in the growth or survival of *E. coli* O157:H7 in feces shortly after excretion could greatly alter the transmission potential to other animals and the environment. Further, if one feed type provides a selective survival advantage to *E. coli* O157:H7 lineage I strains, surveillance and mitigation strategies would need to include this factor into consideration because increased survival would also likely increase prevalence at the processing plant and in turn increase contamination of the food supply.

The current study describes, for the first time, that different lineage types of *E. coli* O157:H7 do not differ in survival in feces arising from cattle fed grain or forage diets. Previously reported differences in acid tolerance between lineages of *E. coli* O157:H7 (Saridakis et al. 2004) suggested that conditions which could alter fecal acidity, such as animal diet and temperature, may also influence the survival of lineages in feces, however this was not observed. The lack of differential lineage survival in either grain or hay feces at any of the three temperatures examined suggests that feeding practices and warm summer temperatures do not contribute to lineage I strains being a more predominant contaminate of the food supply than lineage II strains of *E. coli* O157:H7. Therefore the bias for *E. coli* O157:H7 lineage I to be implicated in human disease more often than lineage II is likely due to increased virulence and not prevalence. A recent

modeling study reported heterogeneity in human cases of *E. coli* O157:H7 infection where exposure dose was not correlated with the percentage of exposed individuals becoming infected (Teunis et al. 2008), supporting the hypothesis that *E. coli* O157:H7 lineage I strains are more virulent but not necessarily more prevalent. Studies have found that some strains of *E. coli* O157:H7 survive better in the environment than others, with acid resistance being one of the factors responsible for this differential survival (Arnold and Kaspar 1995; Bach et al. 2005a; Bach et al. 2005b). Alkaline tolerance is thought to be provided by the stationary-phase sigma factor (RpoS), which affects cellular responses to a variety of stresses (Bhagwat et al. 2006). Mutant alleles for RpoS have been identified (Notley-McRobb et al. 2002; King et al. 2004) and it was possible that different RpoS alleles or differences in RpoS regulation among *E. coli* O157:H7 lineages could have affected lineage survival in the moderately alkaline hay feces. However, comparing tolerances to alkaline conditions via the RpoS system was beyond the scope of this study. Further, the lack of lineage associated differences in survival within the feces suggests that RpoS systems may not differ among *E. coli* O157:H7 lineages. It has been previously demonstrated that different RpoS alleles were correlated with alkaline tolerance but these differences were at much higher pHs than those observed in the current study (Bhagwat et al. 2006). Without further study it is difficult to speculate how the RpoS system may have influenced *E. coli* O157:H7 survival. However, if RpoS plays a role under slightly alkaline conditions, it could account for any strain differences in survival that were observed. Indeed, based on our results it does seem that of the strain set used, certain strains had a tendency to survive better than others, such as Sakai<sub>n</sub>, E3081 and C<sub>n</sub>. The

current study used a limited strain set and it is possible that with a larger set of strains, lineage associated differences in survival could become more apparent.

The strong interaction of temperature, feces type and day suggests that feeding practices and changing temperatures from spring to summer months may alter the chemical composition of bovine feces in a manner that alters *E. coli* O157:H7 growth and survival. Feces from steers fed grain versus grass hay were selected as the substrates for assessing *E. coli* O157:H7 lineage survival because these diets are routinely given as finishing and backgrounding diets, respectively. Previous studies have demonstrated that fecal matrix plays a role in *E. coli* O157:H7 survival. Reduced *E. coli* O157:H7 survival in corn based feces as compared to barley based feces has been previously observed at -10°C (Bach et al. 2005b). In another study, Franz et al. (2005) found that *E. coli* O157:H7 survived longer in manure from cattle fed a mixture of grass and maize silage as compared to feces from cattle fed straw. Both these groups correlated *E. coli* O157:H7 survival with pH; however, the former positively correlated the rate of decline with a pH below 6.5 while the latter positively correlated rate of decline with a high pH (~7.8). In the current study, there were differences in the survival of *E. coli* O157:H7, which were independent of lineage type. We found that *E. coli* O157:H7 survival was enhanced in grain feces compared to hay feces at 4°C. At this temperature, a more neutral pH in the grain feces (pH ranging from 7.1 to 7.6) versus a more alkaline pH of the hay feces (pH ranging 7.6 to 8.3) may have contributed to the increased survival. In contrast, at 12°C survival of *E. coli* O157:H7 in grain feces (pH ranging from 7.2 to 7.9) was comparable to hay feces (pH ranging from 7.6 to 8.3). Finally, at 25°C, the grain feces (pH ranging from 7.4 to 8.1) allowed a longer period of growth (~3 days of growth in grain feces

compared to ~1 day of growth in hay feces) followed by an increased rate of decline compared to the hay feces (pH ranging from 7.6 to 8.5). The current study supports previous reports and suggests a possible role of pH and temperature in the survival of *E. coli* O157:H7 in feces. The pH of grain feces did increase with increases in incubation temperature (a pH range of 7.1 to 7.6 at 4°C compared to 7.4 to 8.1 at 25°C) while the pH of the hay feces remained more stable (a pH range of 7.6 to 8.3 at 4°C compared to 7.6 to 8.5 at 25°C). In addition, below ~12°C *E. coli* O157:H7 survives better in grain feces than hay feces, while above ~12°C the reverse is true.

Volatile fatty acid content has also been previously correlated with *E. coli* O157:H7 survival in feces (Bach et al. 2005b). Differences in pH between feces derived from different feed types have been previously attributed to the rapid fermentation of grain based feeds within the rumen increasing the VFA concentration and it has been suggested that this process could favor an increased acid tolerance of commensal and pathogenic *E. coli* (Leyer et al. 1995; Diez-Gonzalez et al. 1998; Bach et al. 2005b). Higher VFA concentrations were observed in the feces from cattle fed grain than in feces from cattle fed hay supporting reports that grains can increase the VFA content of feces (Shabtay et al. 2009; Spiels and Varel 2009). The higher VFA concentrations of the grain feces were likely responsible for their lower pHs compared to the hay feces as the VFA content of the grain feces decreased with increasing temperature and pH. VFAs may accumulate to a higher degree in grain feces at low temperatures (< 12°C) and create a neutral environment that favors the survival of *E. coli* O157:H7. The pH reducing effect of increasing VFA concentrations does not seem to favor the survival of *E. coli* O157:H7

lineage I over lineage II or vice versa. However, the relationships between fecal matrix, pH, VFA content and temperature are complex.

Fecal dehydration has been implicated as a factor affecting *E. coli* O157:H7 survival because survival of the pathogen in feces has been found to be temperature dependent (Fremaux et al. 2008). However, conflicting results surrounding the issue of fecal dehydration have been reported. Wang et al. (1996) reported *E. coli* O157:H7 survival in feces to vary with temperature of incubation and suggested that this was related to lower water activity ( $a_w$ ) and dry matter content of samples at 22°C and 37°C as compared to 5°C. It was noted that the long term survival of the pathogen at such low  $a_w$  and dry matter content was unexpected because *E. coli* O157:H7 had not been documented to survive at low  $a_w$ . Others have also concluded that dehydration of feces may affect pathogen survival at increased temperatures (Himathongkham et al. 1999). In contrast, it was more recently reported that *E. coli* O157:H7 survival was not affected dehydration of the feces (Bach et al. 2005b; Hutchison et al. 2005). In the current study, we did not find a trend of feces dehydration over time at any temperature. This was likely due to sampling methods aimed at decreasing *E. coli* O157:H7 count variability by sampling from the moist portions of the pat and avoiding dry areas. Despite not finding a trend of dehydration, *E. coli* O157:H7 did decline over the course of this short term study, supporting reports that factors other than dehydration influence the survival of *E. coli* O157:H7 in feces (Bach et al. 2005b; Hutchison et al. 2005). It should be noted that fecal dehydration may take on a role as time progresses in longer duration studies. However, we did find differences in growth and survival at the three temperatures with

feces at 4°C having the lowest numbers of *E. coli* O157:H7 counts on the final sampling day.

This is the first study of its kind aimed at assessing *in vitro* growth and survival dynamics of selected strains of *Escherichia coli* O157:H7 lineages I and II in fecal inoculations. Differences in the survival of *E. coli* O157:H7 based on the fecal matrix and temperature was observed. However, no lineage associated differences in growth or survival in bovine feces were observed at any of the temperatures examined. This is a critical initial piece of knowledge regarding *E. coli* O157:H7 lineages as it suggests that factors other than growth and survival on the feedlot are responsible for differences in the number of human illnesses that the different *E. coli* O157:H7 lineages cause.

## CHAPTER THREE

### *Escherichia coli* O157:H7 strain origin, lineage and Shiga toxin 2 expression affects colonization of cattle<sup>1</sup>

#### 3.1. ABSTRACT

Enterohemorrhagic *Escherichia coli* O157:H7 has evolved into an important human pathogen with cattle as the main reservoir. The recent discovery of *E. coli* O157:H7-induced pathologies in challenged cattle has suggested that previously discounted bacterial virulence factors may contribute to the colonization of cattle. The objective of the current study was to examine the impact of lineage type, cell extract activity and cytotoxin expression on the amount of *E. coli* O157:H7 colonization of cattle tissue and cells *in vitro*. Using selected bovine and human-origin strains, we determined that lineage type predicted the amount of *E. coli* O157:H7 strain adherence of cattle intestinal cells: lineage I > intermediate lineages > lineage II. All *E. coli* O157:H7 strain colonization was dose-dependent with threshold colonization at  $10^3$ - $10^5$  CFU and maximum colonization at  $10^7$  CFU. We also determined that an as of yet, unknown factor, strain-origin, was the most dominant predictor of the amount of strain colonization *in vitro*. The amount of *E. coli* O157:H7 colonization was also influenced by strain cell extract activity and the inclusion of cell extracts from lineage I or intermediate lineage strains increased colonization of a lineage II strain. Human-origin strains had greater levels of Shiga toxin 1 (*stx1*) mRNA than bovine-origin strains. In addition, lineage I strains had higher levels of Shiga toxin 2 (*stx2*) mRNA. The current study supports a role for strain origin, lineage

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<sup>1</sup> Chapter published in Applied and Environmental Microbiology; Lowe, R.M.S., Baines, D., Selinger, L.B., Thomas, J.E., McAllister, T.A. and Sharma, R. (2009) *Escherichia coli* O157:H7 strain origin, lineage, and Shiga toxin 2 expression affect colonization of cattle. *Appl Environ Microbiol* **75**, 5074-5081.

type, cell extract activity and *stx2* expression in modulating the amount of *E. coli* O157:H7 colonization of cattle.

### 3.2. INTRODUCTION

Enterohemorrhagic *Escherichia coli* O157:H7 is a bacterium that causes serious human disease outbreaks through the consumption of contaminated food or water (Wales et al. 2005). Mature cattle are considered the primary reservoir for *E. coli* O157:H7 and historically were reported to have no symptoms or pathologies (Grauke et al. 2002; Van Baale et al. 2004; Lim et al. 2007) which was attributed both to a lack of receptors for a critical *E. coli* O157:H7 virulence factor, Shiga toxin1 (Stx 1) (Pruimboom-Brees et al. 2000) and a differential expression of type III protein secretion system effector molecules such as EspA, EspD and Iha (McNally et al. 2001; Rashid et al. 2006), in cattle compared with humans. In 2008, it was established for the first time that *E. coli* O157:H7 causes mild to severe intestinal pathology in persistent shedding cattle (Baines et al. 2008b; Nart et al. 2008) and that the cell extracts enhanced *E. coli* O157:H7 colonization of intestinal tissues of cattle (Baines et al. 2008c). This suggested that cattle were susceptible to *E. coli* O157:H7 infection and that previously discounted virulence factors could influence the colonization in cattle.

Three distinct *E. coli* O157:H7 lineages have been identified based on the lineage specific polymorphism assay (LSPA-6) that reflects the evolutionary history of the strain and their propensity to be present among animals, the environment and clinical human isolates (Kim et al. 1999; Kim et al. 2001; Yang et al. 2004; Manning et al. 2008; Ziebell et al. 2008; Sharma et al. 2009). Typically two predominant lineages have been described, lineages I and II (Kim et al. 2001; Yang et al. 2004) and more recently, intermediate lineages with characteristics of both lineage I and/or II, have been reported at higher frequency among cattle (Sharma et al. 2008). While all *E. coli* O157:H7

lineages have been isolated from feedlot cattle, the predominant recovery of lineage I from clinical human illnesses suggests that this particular lineage type has unique expression patterns that may contribute to its preferential colonization of humans. There is some evidence to suggest that lineage I strains do not express certain virulence factors in bovine hosts while other factors such as cytotoxins are expressed equally irrespective of host (Rashid et al. 2006). One virulence factor associated with all lineages is the bacterium's ability to form intimate A/E lesions or colonization sites in the ileum of susceptible animals (Phillips et al. 2000). The amount of colonization is enhanced by the expression of Shiga toxin 2 (Stx2) through both an increase in the expression of alternative non-Tir colonization sites (Robinson et al. 2006) and toxicity to the absorptive epithelial cells (Schuller et al. 2004). In cattle, A/E lesions are also formed (Baines et al. 2008b) and Stx2 increases colonization but is not cytotoxic to epithelial cells from the jejunum and descending colon of cattle (Baines et al. 2008a). Differential expression of *stx2* among *E. coli* O157:H7 lineages is also linked to the increased pathogenicity of lineage I strains in humans (McNally et al. 2001) and may affect cattle similarly. Together, this information suggests that at least some similar virulence factors affecting *E. coli* O157:H7 colonization in humans, function in cattle.

To gain a better understanding of the factors modulating *E. coli* O157:H7 colonization in cattle, we compared the ability of lineage I, lineage II and intermediate lineages isolated from human sources to colonize the jejunum tissue and a colonic cell line from cattle. We hypothesized that the bovine colonic cell line could be used as a model system to reflect *E. coli* O157:H7 colonization of tissue. To confirm the value of this model, the role of strain origin in colonization of cattle was examined. In order to

understand differences in colonization associated with lineage and strain-origin, we assessed cytotoxin mRNA production, cell extract activity and cell extract-induced changes in *E. coli* O157:H7 colonization. Given the known lack of Stx1 activity in cattle, we examined the effect of LSPA-6 genotype, strain origin (human vs. bovine) and cell extract activity on *E. coli* O157:H7 colonization of cattle.

### **3.3. MATERIALS AND METHODS**

#### **3.3.1. *E. coli* O157:H7 strains and culture conditions**

*E. coli* O157:H7 lineage I (Sakai, 24), lineage II (ECI 1717, 25) and intermediate lineages (84, 12, 86, 138, 197, H3-2R) used in this study (Table 3.1.) were obtained and characterized from a previous study (Sharma et al. 2009). These strains were maintained at -80°C in 25% glycerol:75% TSB (Becton Dickinson, Oakville, Ontario, Canada) and were grown overnight at 37°C in LB broth (Fisher Scientific, Ottawa, Ontario, Canada) when required. Each strain was serially diluted to the desired concentration with phosphate buffered saline (PBS). Bacterial cell counts were determined by plating on sorbitol MacConkey agar (SMAC; Dalynn, Calgary, Alberta, Canada) and examined for non-sorbitol fermenting (NSF) colonies that appeared as colorless colonies.

#### **3.3.2. Cell extract isolation**

*E. coli* O157:H7 cell extracts were isolated and concentrated using a combination of centrifugation and ultrafiltration as described previously (Baines et al. 2008c). Briefly, the strains were grown in 15 ml of M9 media (47 mM Na<sub>2</sub>HPO<sub>4</sub>, 22 mM KH<sub>2</sub>PO<sub>4</sub>, 8 mM NaCl, 19 mM NH<sub>4</sub>Cl, 44 mM NaH<sub>2</sub>CO<sub>3</sub> supplemented with 13.2 ml/l of 1 M glucose and

**Table 3.1.** Host source, lineage type, LSPA-6 genotype and virulence gene characteristics of the strains used in the study.

Strain	Source	Lineage; LSPA-6 genotype	Virulence gene characterization <sup>1</sup>				
			<i>stx1</i>	<i>stx2</i>	<i>ehxA</i>	<i>eae</i>	<i>tir</i>
Sakai	human	I; 111111	+	+	+	+	+
24	bovine	I; 111111	+	+	+	+	+
ECI 1717	human	II; 222222	+	+	+	+	+
25	bovine	II; 222222	+	+	+	+	+
84	human	intermediate lineage; 211111	+	+	+	+	+
12	bovine	intermediate lineage; 211111	+	+	+	+	+
86	human	intermediate lineage; 222212	+	+	+	+	-
138	bovine	intermediate lineage; 222212	+	+	+	+	-
197	human	intermediate lineage; 110111*	+	+	+	+	+
H3-2R	bovine	intermediate lineage; 100111*	+	+	+	+	+

\*Isolates of the selected intermediate lineage type were not available from both bovine and human hosts.

<sup>1</sup>Presence or absence of the designated gene represented by + or - respectively.

10 ml/l 30 % casamino acid solution, Sigma-Aldrich, Oakville, Ontario, Canada) at 37°C without shaking for 5 days. Following growth, the cultures were centrifuged at 5 049 ×g for 20 min, the supernatant was collected and the pellet discarded. The supernatant was centrifuged for an additional 20 min at 9 300 ×g and then added to an Amicon® Ultra-10K filter unit (Fisher Scientific). The supernatant was centrifuged at 5 049 ×g for 20 min and the concentrated solution (500 µl) was collected. The protein content of the cell extracts was assessed using a Quick Start Bradford Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). The concentrated cell extracts were then serially diluted with M9 media in a 1:1 ratio for eight dilutions.

### **3.3.3. *In vitro* organ culture (IVOC) *E. coli* O157:H7 adherence assay**

Necropsy samples were obtained from eight Hereford × Angus steers using standard methods (Van Baale et al. 2004; Baines et al. 2008b; Baines et al. 2008c). Jejunum tissues (30 cm) were removed within 2 min of release of the intestinal tract from the carcass and each piece was placed in 200 ml oxygenated Dulbecco's Modified Eagle's Medium (DMEM; high glucose, Hyclone, Logan, UT) supplemented with bovine albumin. The tissue was maintained at 4°C and transported back to the laboratory. Upon arrival, the tissue was cut open, washed using PBS at 4°C and 2.5 cm<sup>2</sup> pieces were excised. Excess fat or connective tissue was removed and the complete organ piece (mucosa and muscle) was used in order to maintain mucosal structure. Care was taken while cutting not to press down on the tissue and distort size. The tissue pieces were placed in PBS at 4°C until all sites were processed (about 20 min/animal). Experiments were started within 2 h of collecting the tissue sample.

The IVOC adherence assay was conducted as previously described (Baines et al. 2008c) using the human-origin *E. coli* O157:H7 strains. This assay has been established as representing *in vivo* colonization (Baehler and Moxley 2000; Phillips et al. 2000; Dibb-Fuller et al. 2001; Cobbold and Desmarchelier 2004). Briefly, each 2.5 cm<sup>2</sup> tissue piece was drained using a paper towel and placed in approximately 3 ml of DMEM at 15°C, adjusted to minimally cover the surface of the mucosa. This was critical for maintaining tissue viability for the duration of the experiment. To compare *E. coli* O157:H7 lineage adhesion to cattle intestinal tissue, varying concentrations (final exposure doses of 10<sup>3</sup>, 10<sup>4</sup>, 10<sup>5</sup>, 10<sup>6</sup>, 10<sup>7</sup> and 10<sup>8</sup> CFU/ml) of each *E. coli* O157:H7 strain was applied to the mucosal surface. The tissue pieces were incubated for 4 h under standard culture conditions (37°C, 95% humidity and 5% CO<sub>2</sub>). After incubation, each tissue piece was washed six times with 10 ml of PBS to remove any unattached bacteria. The tissue was then turned mucosa-side down in 3 to 4 ml of PBS supplemented with 1% TritonX-100 (Sigma-Aldrich) and incubated at 4°C overnight. The next day the released bacteria were quantified by plating serial dilutions on SMAC agar and counting the non-sorbitol fermenting colorless colonies. The assay was replicated eight times using the jejunum from eight different animals.

#### **3.3.4. *In vitro* cell culture (IVCC) *E. coli* O157:H7 adherence assay**

A colonic cell line was developed from a persistent *E. coli* O157:H7 shedding steer (F1112) in 2006 and has since been maintained in our laboratory. The cell line was cultured in 75 cm<sup>2</sup> tissue culture flasks (Fisher Scientific) in DMEM supplemented with 10% fetal bovine serum (FBS; Hyclone) and gentamicin (50 µg/ml, Sigma-Aldrich)

under standard culture conditions. Cells were sub-cultured by trypsinization of the monolayer with 5-10 ml of 0.25% trypsin-EDTA solution (Sigma-Aldrich) for 2-5 min, collecting and then centrifuging the cell suspension at  $2\ 087 \times g$  for 10 min. The supernatant was decanted and the cell pellet re-suspended in the culture medium. Cells were added to 6-well multi-well Falcon plates (Fisher Scientific) at a density of approximately  $10^3$  cells. Cells were cultured until confluent (approximately 1 week) and the culture medium changed every three days.

The IVCC adherence assay was performed with all human- and bovine-origin *E. coli* O157:H7 strains. The same methodology was used as described above for the IVOC *E. coli* O157:H7 adherence assay with a few modifications to accommodate the use of the colonic cell line. To compare *E. coli* O157:H7 strain colonization to the colonic cell line, each well of confluent cells was washed once with DMEM following which 3 ml of DMEM was added to each well to remove any residual FBS or antibiotic that could affect bacterial adherence. To each well, a 100  $\mu$ l aliquot of each *E. coli* O157:H7 strain was added to deliver final exposure doses of  $10^3$ ,  $10^4$ ,  $10^5$ ,  $10^6$ ,  $10^7$  and  $10^8$  CFU/ml. The cell cultures were incubated for 4 h under standard culture conditions. The medium was replaced for the highest dose after 2 h to maintain pH. At the end of the experiment, cell monolayers were washed four times with 10 ml of PBS to remove any unattached bacteria. To release the bacteria from the cells, 2 ml of PBS supplemented with 1% Triton X-100 was added to each well and incubated overnight at 4°C. Serial dilutions of the released bacteria were performed the next day and quantified by plating onto SMAC plates. Plates were incubated at 37°C overnight and *E. coli* O157:H7 was quantified by counting the non-sorbitol fermenting colonies that appeared colorless. The IVCC assay

was replicated 5 times for each strain with a minimum of 3 different culture dates for the colonic cell line.

The cell extracts from a human-origin *E. coli* lineage I strain have been shown to enhance the colonization of a bovine-origin *E. coli* lineage I strain that had significantly lower colonization on its own (Baines et al. 2008c). To understand the role of secreted materials (ie. cytotoxins) or materials released from the cell surface (ie. Surface proteins, endotoxins), the colonic cell line was exposed to about 80 ng of cell extract from each of the *E. coli* O157:H7 strains in conjunction with a  $10^8$  CFU/ml of the least adherent *E. coli* O157:H7 strain, ECI 1717. This assay was replicated three times for the cell extracts produced from all strains except for ECI 1717.

### **3.3.5. Lawn assay**

The lawn assay was used to compare the toxicity of cell extracts from the *E. coli* O157:H7 strains to epithelial cells. Epithelial cells were extracted from the jejunum and the lawn assay performed using the cell extracts from human and bovine *E. coli* O157:H7 strains as described previously (Baines et al. 2008d). Briefly, each tissue loop was opened and washed with epithelial cell saline (ECS; 137 mM NaCl, 2.7 mM KCl, 1 mM CaCl<sub>2</sub>, 0.4 mM NaH<sub>2</sub>PO<sub>4</sub>, 5.6 mM glucose, 44 mM NaH<sub>2</sub>CO<sub>3</sub>, pH 7.4; Sigma-Aldrich) until all luminal contents were removed. A mucosal strip was scraped away from the underlying tissues, finely diced, added to 200 ml of chelating buffer at 4°C (ECS supplemented with 17 g/l D-glucose, 8.8 g/l sodium citrate, 4.2 g/l citric acid and 2.9 g/l EDTA, pH 7.4; Sigma-Aldrich) and stirred at 600 rpm in a beaker for 2 to 5 min. The supernatant was decanted and replaced with fresh chelating buffer followed by stirring for another 20 min.

The supernatant containing cells was collected, centrifuged at  $1\ 262 \times g$  for 6 min. The resulting pellet was washed three times with DMEM containing 50  $\mu\text{g/ml}$  gentamicin. The final epithelial cell pellet was re-suspended in ECS. A 1% SeaKem Agarose (Mandel Scientific, Guelph, Ontario, Canada) support gel was poured into a petri dish and left to gel for 30 min. Next, the lawn agarose [3 ml of 3.7% SeaPlaque agarose (Mandel Scientific)] was mixed with 3 ml of cell suspension and poured over the support agarose. Each toxin dilution (3  $\mu\text{l}$ ) was applied and the treated lawn was incubated for 4 h under standard culture conditions. The amount of total cell extract applied was 0.1 to 120 ng. The lawn was stained with 0.1% trypan blue (Sigma-Aldrich) and de-stained using 1.84% KCl. Plates were scored the same day and the amount of cell extract activity was defined as the threshold dose (ng) of cell extract in the dilution series to cause areas of cell death as observed by the presence of a blue spot on the lawn.

### **3.3.6. Real time PCR analysis**

To determine which cytotoxins may be expressed by the strains according to origin and lineage type, levels of *stx1* (Shiga toxin 1), *stx2* (Shiga toxin 2) and *ehxA* (enterohemolysin) mRNA were quantified from all strains using real time PCR. Cells were cultured to log phase in LB broth and total RNA was extracted using RNeasy Mini Kit (Qiagen, Inc., Mississauga, Ontario, Canada) using RNAProtect bacteria reagent (Qiagen, Inc.). Extracted RNA was quantified using Quant-iT Ribogreen RNA assay kit (Invitrogen, Burlington, Ontario, Canada) on a Nanodrop 3330 fluorometer (ThermoScientific, Wilmington, DE). Analysis was performed using one-step Brilliant II QRT-PCR Master Mix Kit (Stratagene, La Jolla, CA). Relative quantification was

performed on an Mx3000p Real-time PCR system (Stratagene) with previously described primers, probes and cycling conditions for *stx1* (Sharma and Dean-Nystrom 2003), *stx2* and *ehxA* (Rashid et al. 2006). Each plate setup included a standard curve generated by reverse transcribing known concentrations of Sakai RNA (50, 25, 12.5, 6.25, 3.125 ng). Relative gene mRNA levels were analyzed using the built-in comparative threshold cycle (*Ct*) methods ( $\Delta\Delta CT$ ) of MxPro Real-Time software (version 4.01) normalized using the housekeeping gene *gnd* and calibrated to strain Sakai. All real-time PCR reactions were conducted in triplicate.

### **3.3.7. Statistical analysis**

The IVOC adherence assay and IVCC adherence assay data were log transformed to normalize the data prior to analysis. All data were analyzed using ANOVA followed by a posthoc Tukey's test for comparison of the means. For the IVOC, the model consisted of two factors, lineage type and dose. For the IVCC, the model consisted of two factors, strain origin and lineage type. Individual strain colonization was analyzed with dose as a factor. Threshold adherence, which was defined as the lowest exposure dose to have significantly higher adherence ( $P < 0.05$ ) than a dose of 10 times fewer bacteria, was determined. For all data analysis,  $P < 0.05$  was considered significant and  $P > 0.05$  was non-significant.

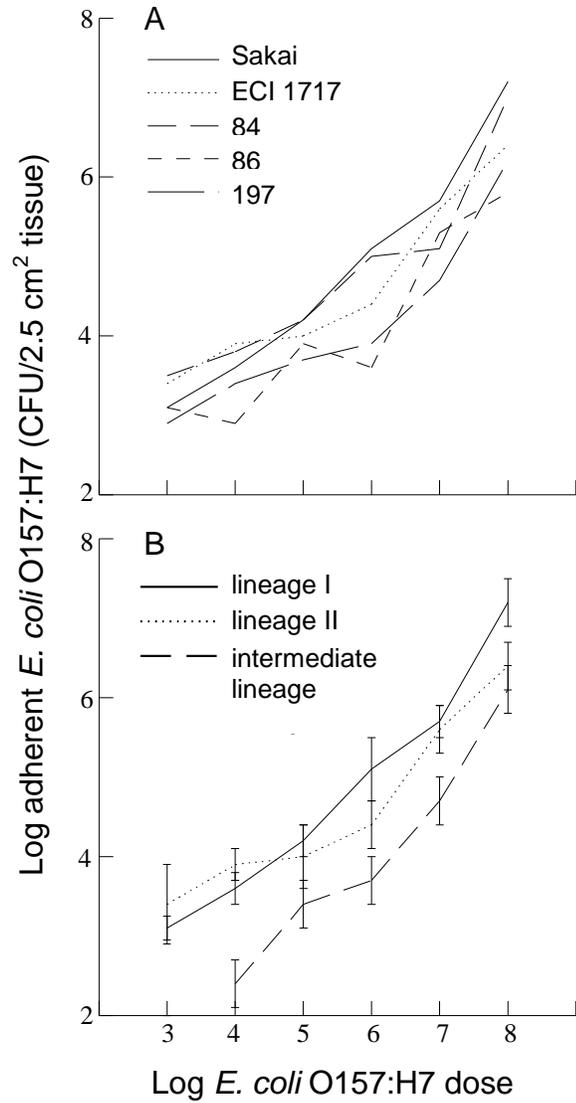
### 3.4. RESULTS

#### 3.4.1. IVOC *E. coli* O157:H7 adherence assay

There was a significant difference (Table 3.2) in colonization of the jejunum by *E. coli* O157:H7 human-origin strains ( $P = 0.001$ , Fig. 3.1A) and a significant dose-dependent response to *E. coli* lineage colonization ( $P = 0.001$ , Fig. 3.1B). The strain hierarchy for colonization of intestinal tissue was Sakai, 84, ECI 1717 > 86, 197. The strains Sakai, ECI 1717 and 84 colonized equally ( $P = 0.653$ ), while strains 86 and 197 had lower colonization levels ( $P = 0.502$ ). Strains Sakai and 84 had significantly higher colonization compared with strains 86 and 197 ( $P < 0.05$ ). Strain ECI 1717 had significantly higher colonization than strain 86 ( $P = 0.01$ ) but ECI 1717 was not significantly different from strain 197 ( $P > 0.524$ ). This apparent contrast between strains ECI 1717 (lineage II) and Sakai (lineage I)/84 (intermediate lineage) was related to a consistent but not significantly lower colonization in all assays. At the highest doses, strains Sakai and 84 had approximately ten times higher colonization than the rest of the strains (Fig. 3.1A). At  $10^3$ - $10^5$  CFU, strain 84 had the highest colonization, approximately ten times higher than the next highest strain, ECI 1717. Throughout the dose range, the lineage I strain, Sakai, was the most adherent strain while the lineage II strain, ECI 1717, provided variable adherence depending on the replicate. This variability was also observed for the intermediate lineage strains but the extent of variability was dependent on the exposure dose. The removal of this variable by using cultured cells allows for an examination of the ability of strains to colonize colonic cells thus supporting a role for a cell culture model in understanding *E. coli* O157:H7 infections in cattle. In addition, the intermediate lineages (84, 86, 197) when averaged had about a ten-

**Table 3.2.** Interactions and effects of lineage type, strain origin and *E. coli* O157:H7 exposure dose on *E. coli* O157:H7 adherence to bovine tissue and cells, cytotoxin activity, and delected virulence gene mRNA levels.

<b>Variable</b>	<b>Effect/Interaction</b>	<b>P value</b>
<i>E. coli</i> O157:H7 adherence to tissue	strain	0.001
	dose	0.001
<i>E. coli</i> O157:H7 adherence to cells	strain*dose	0.001
	lineage	0.001
	strain origin	0.001
	dose	0.001
Cytotoxin activity	lineage	0.05
	strain origin	0.05
<i>stx1</i> mRNA levels	lineage	0.70
	strain origin	0.001
<i>stx2</i> mRNA levels	lineage	0.007
	strain origin	0.33
<i>ehxA</i> mRNA levels	lineage	0.15
	strain origin	0.70



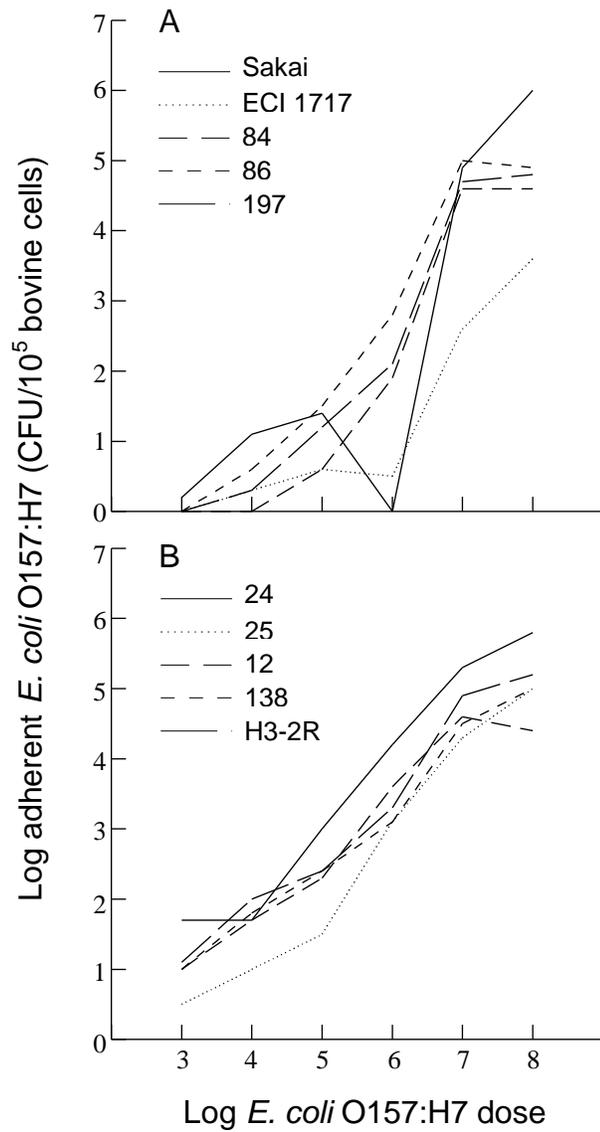
**Figure 3.1.** *Escherichia coli* O157:H7 lineage and strain colonization of the jejunum from cattle ( $n=8$ ) using an *in vitro* organ culture (IVOC) adherence assay. Lineage I (Sakai), lineage II (ECI 1717) and intermediate lineages (84, 86, 197) were assessed for colonization. A. Comparison of individual strains. B. Comparison of individual lineage types.

fold lower adherence relative to lineage I (Sakai) and lineage II (ECI 1717) strains. Within environmental exposure dose ranges, the range of *E. coli* O157:H7 density that cattle would be exposed to under natural circumstances (typically  $10^3$ -  $10^5$  CFU/ g) (Naylor et al. 2003), there was a significant but low colonization of the jejunum by all lineages (Fig. 3.1B). Further, at the lowest exposure doses, detection and enumeration of attached *E. coli* O157:H7 was interfered with by the presence of endogenous non-O157:H7 *E. coli*. The effect of the endogenous non-O157:H7 *E. coli* prevented the ability to measure *E. coli* O157:H7 adherence at doses lower than  $10^3$  CFU. The lineage II strain had the equivalent of a lineage I or much lower colonization depending upon the replicate or animal tissue used.

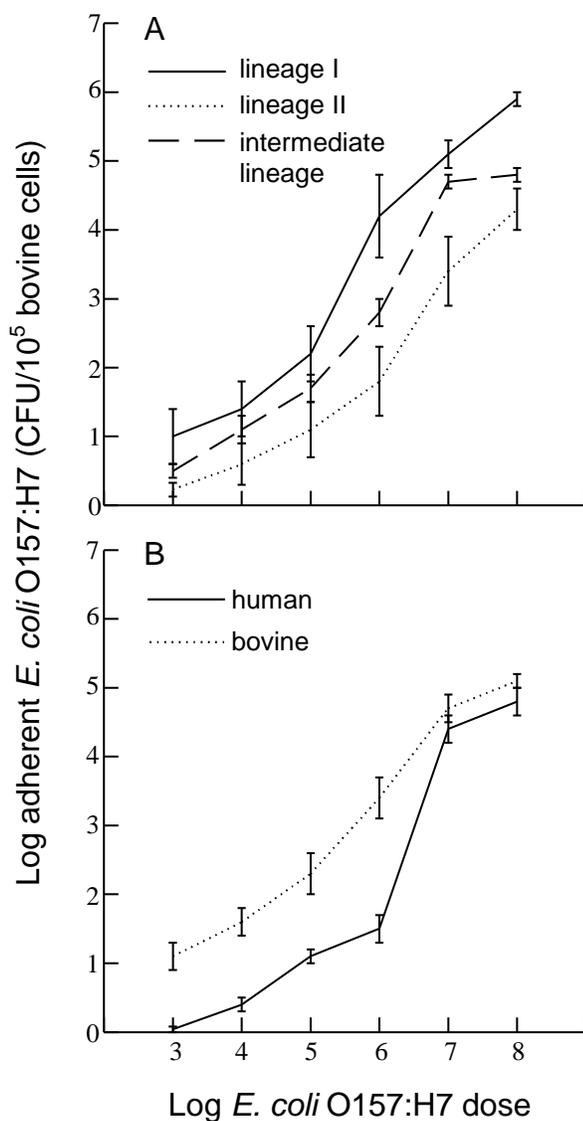
#### **3.4.2. IVCC *E. coli* O157:H7 adherence assay**

A similar hierarchy for *E. coli* O157:H7 lineage adherence was obtained with the colonic cell line as observed for the jejunum tissues. To ensure that this hierarchy was not related to strain origin, five additional *E. coli* O157:H7 strains were selected, four of which represented previously used human LSPA-6 genotypes but were of bovine origin. The strain hierarchy for colonization of the colonic cell line was 24, H3-2R > Sakai, 25, 12, 138 > 86, 197 > 84 > ECI 1717. However, this type of strain hierarchy is difficult to interpret due to differences in the low dose colonization between the human and bovine-origin strains. For example, the interaction of lineage type, strain origin and dose resulted in Sakai having lower adherence than H3-2R at doses below  $10^5$  CFU/ml but higher adherence at the highest dose.

We observed a significant dose-dependent response to *E. coli* O157:H7 colonization of the colonic cell line ( $P = 0.001$ ). The human-origin lineage I strain Sakai had a threshold adherence below  $10^3$  CFU/ml (Fig. 3.2A). The human-origin strains ECI 1717, 86 and 197 had threshold adherence at  $10^4$  CFU/ml (Fig. 3.2A). The human origin intermediate lineage strain 84 had a threshold adherence at  $10^5$  CFU/ml (Fig. 3.2A). This suggests that among human-origin strains of *E. coli* O157:H7, lineage I strains may have a higher probability of colonizing at low environmental exposure doses ( $10^3$ - $10^5$  CFU/ml). All bovine-origin strains had a threshold adherence below  $10^3$  CFU/ml (Fig. 3.2B). All strains had a maximum adherence at  $10^7$  CFU/ml; there were no significant differences in adherence between the  $10^7$  and  $10^8$  CFU/ml doses. There was a significant difference in *E. coli* O157:H7 lineage adherence to the colonic cell line ( $P = 0.001$ , Fig. 3.3A). The lineage I strains had greater adherence than the remaining lineages regardless of their origin, human or bovine (Fig. 3.3A). The lineage II strains had the lowest adherence compared with the other lineages, while intermediate lineages had an adherence that was between the lineage I and II strains (Fig. 3.3A). Finally, there was a significant difference in *E. coli* O157:H7 strain adherence to the colonic cell line based on strain origin ( $P = 0.001$ , Fig. 3.3B). The bovine-origin strains had higher adherence at environmental exposure doses ( $10^3$ - $10^6$  CFU/ml). In contrast, at higher exposure doses ( $10^7$ - $10^8$  CFU/ml), there were no differences between human and bovine origin strains (Fig. 3.3B). This suggests that bovine-origin *E. coli* O157:H7 strains have a higher probability of colonizing cattle than human-origin strains. There was a significant strain/dose interaction ( $P = 0.001$ ) that supports the differences in the slope of the dose/response curves for the bovine strains compared with the human strains.



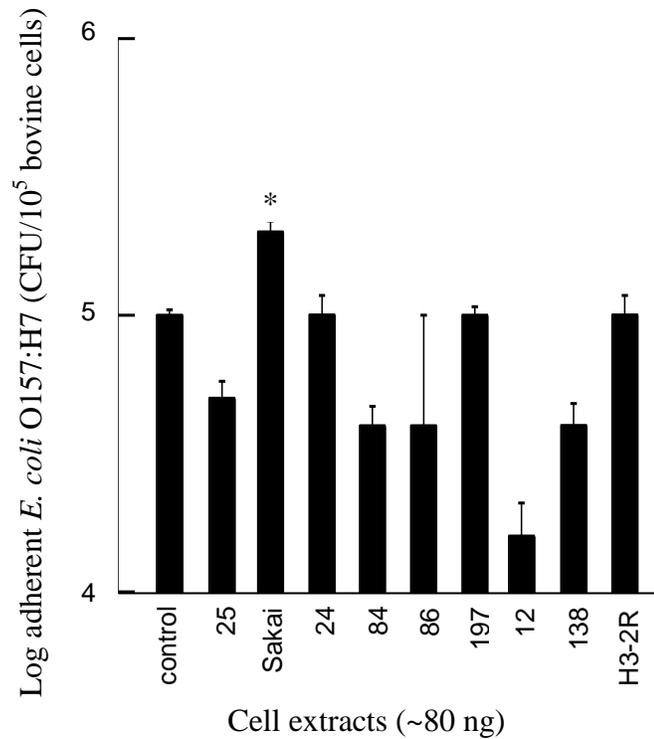
**Figure 3.2.** *Escherichia coli* O157:H7 strain colonization of the colonic cell line from cattle ( $n=5$ ) using an *in vitro* cell culture colonization assay. Lineage I (Sakai, 24), lineage II (ECI 1717, 25) and intermediate lineages (84, 86, 197, 12, 138, H3-2R) were assessed for colonization. A. Comparison of individual human-origin strains. B. Comparison of individual bovine-origin strains.



**Figure 3.3.** *Escherichia coli* O157:H7 lineage and strain origin effect on colonization of a colonic cell line from cattle ( $n=5$ ). A. Comparison of individual lineage types. Lineage I (Sakai, 24), lineage II (ECI 1717, 25) and intermediate lineage (84, 86, 197, 12, 138, H3-2R) strains. B. Comparison of different strain origins. Human-origin strains (Sakai, ECI 1717, 84, 86, 197) and bovine-origin strains (24, 25, 12, 138, H3-2R).

Sakai had significantly lower adherence than strains 24 and H3-2R at the  $10^3$  - $10^6$  CFU/ml doses ( $P < 0.01$ ) yet similar adherence at the  $10^7$  - $10^8$  CFU/ml doses (Fig. 3.2A, B). Sakai also had significantly higher adherence than ECI 1717 ( $P = 0.001$ ). In contrast, Sakai was not significantly different from strains 84, 86, 197, 25, 12 and 138 ( $P > 0.142$ ). ECI 1717 had significantly lower adherence than strains 86, 197, 24, 25, 12, 138 and H3-2R ( $P < 0.02$ ). In contrast, ECI 1717 was not significantly different from strain 84 ( $P = 0.14$ ). Strain 84 had significantly lower adherence than strains 24, 12, 138 and H3-2R ( $P < 0.05$ ). Strain 84 was not significantly different from strains 86, 197 and 25 ( $P > 0.35$ ). Strain 86 had significantly lower adherence than strain 24 at the four lowest doses ( $P = 0.001$ ). Strain 86 was not significantly different from strains 197, 25, 12, 138 and H3-2R ( $P < 0.06$ ). Strain 197 had significantly lower adherence than strains 24, H3-2R ( $P < 0.01$ ). Strain 197 was not significantly different from strains 25, 12 and 138 ( $P > 0.06$ ). Strain 24 had significantly higher adherence than strain 25 ( $P = 0.001$ ). Strain 24 was not significantly different from strains 12, 138 and H3-2R ( $P > 0.139$ ). Strains 25, 12, 138 and H3-2R were not significantly different from each other ( $P > 0.47$ ).

Cell extracts from the strains differentially affected the colonization of ECI 1717 to a colonic cell line ( $P = 0.001$ , Fig. 3.4). The addition of cell extracts to the same strain from which the toxins were derived was previously found to have little effect on adherence (data not shown); therefore the strain with the lowest adherence was selected for this assay as it would be expected to have the largest increase in adherence to the cell line. The cell extract from the human-origin lineage I strain Sakai ( $P = 0.05$ ) increased colonization as compared to the cell extracts from all other strains. The cell extracts from the human-origin intermediate lineage strains (84, 86 and 197), bovine origin lineage II



**Figure 3.4.** Effect of *Escherichia coli* O157:H7 cell extracts on ECI 1717 colonization of a colonic cell line ( $n=3$ ). The colonic cell line ( $10^5$  cells) was exposed to ~80 ng of cell extracts from each of the *E. coli* O157:H7 strains in conjunction with  $10^8$  CFU of the least adherent *E. coli* O157:H7 strain, ECI 1717. ECI 1717 without the cell extract addition is denoted by “control”. \* denotes a significant increase in *E. coli* O157:H7 adherence.

strain (25) and bovine-origin intermediate lineage strains (12, 138 and H3-2R) did not significantly increase the adherence relative to ECI 1717 alone.

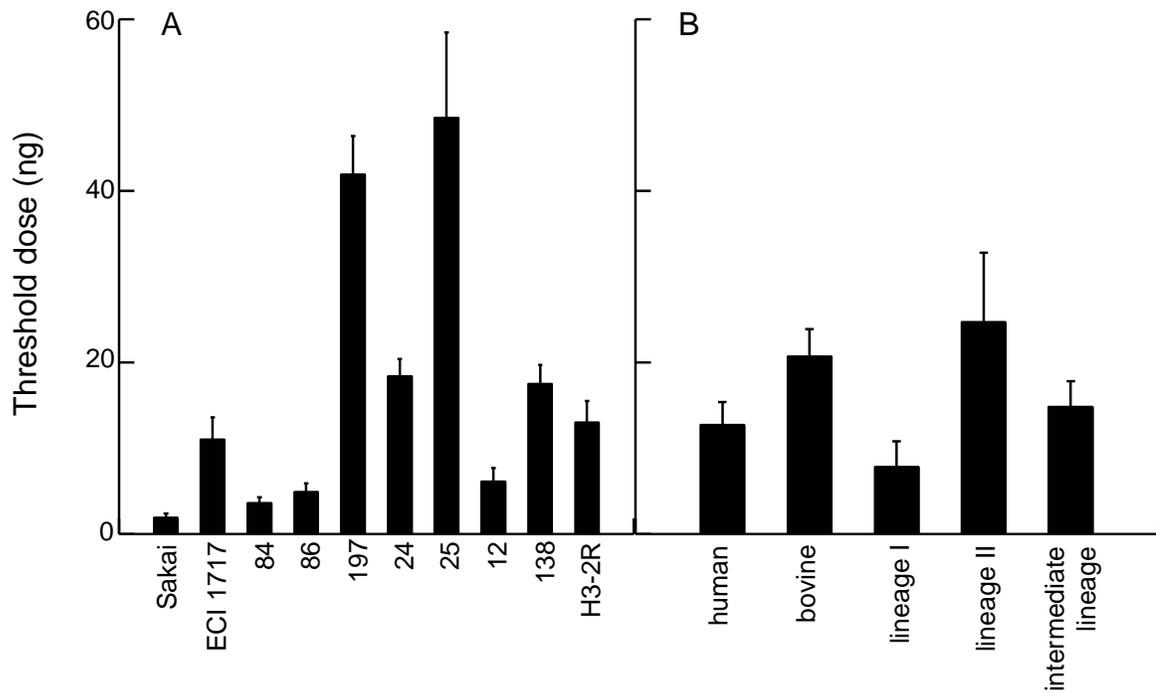
### **3.4.3. Lawn assay**

The lawn assay provides a threshold cell extract dose that causes epithelial cell death as determined by the uptake of trypan blue which is visualized as a blue spot. The hierarchy for the threshold doses of the cell extract activity was human strains < bovine strains ( $P = 0.05$ ); lineage I < intermediate lineages < lineage II ( $P = 0.05$ ). The strain hierarchy for the threshold dose (ng) for the cell extract activity was Sakai < 84, 86, 12 < H3-2R, ECI 1717 < 138 < 24 < 197, 25 ( $P < 0.05$ , Fig. 3.5).

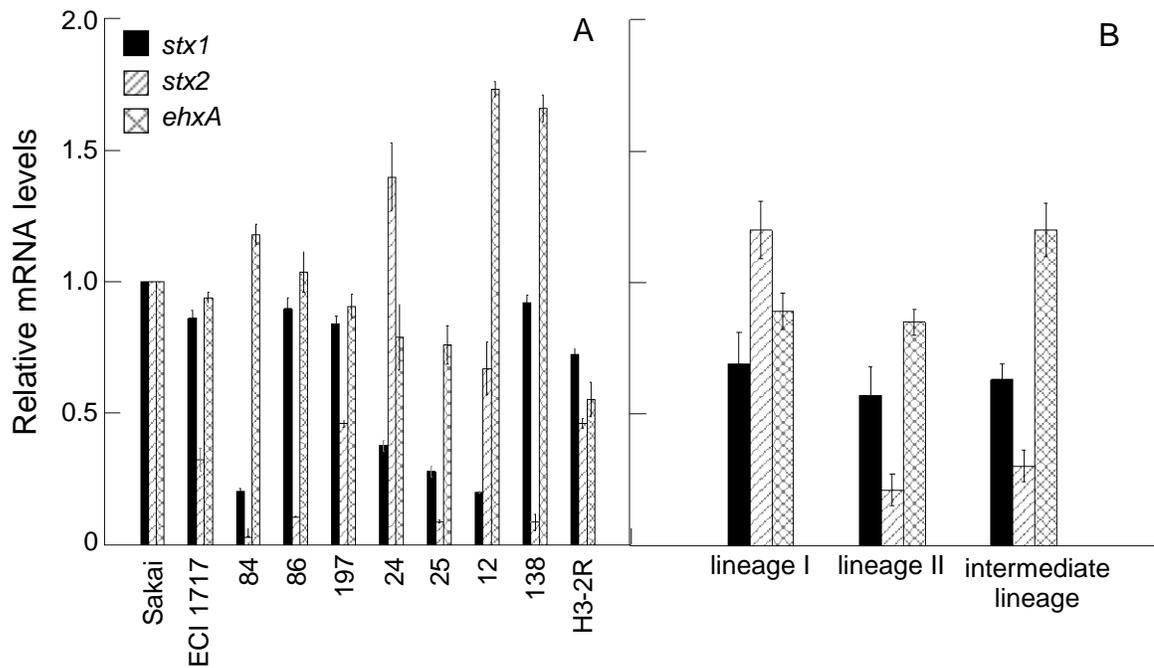
### **3.4.4. Real time PCR analysis**

*In vitro* mRNA levels of genes for *stx1* (Shiga toxin 1), *stx2* (Shiga toxin 2) and *ehxA* (enterohemolysin) were quantified and data were described relative to the mRNA levels of the human-origin lineage I strain, Sakai. The strain hierarchy for average virulence gene mRNA levels was Sakai > 24, 12, 138 > 86, 197 > H3-2R > 84 > 25 (Fig. 3.6).

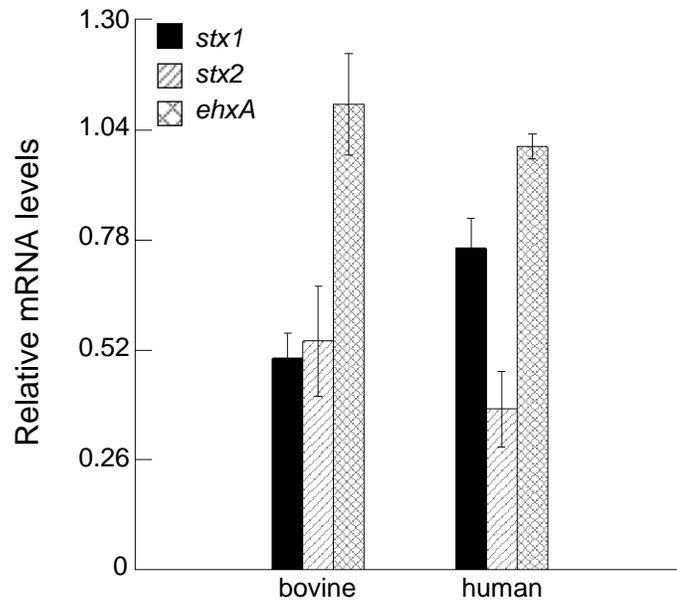
There was a significant lineage type  $\times$  strain origin interaction for levels of *stx1* mRNA ( $P = 0.033$ , Fig. 3.7) whereby human-origin strains had significantly higher mRNA levels than bovine-origin strains ( $P = 0.001$ ). There were no differences in levels of *stx1* mRNA between lineage types ( $P > 0.700$ ). We did not find a significant interaction of lineage type  $\times$  strain origin for levels of *stx2* mRNA ( $P = 0.059$ ). For *stx2*, there was no difference between human and bovine-origin strains ( $P = 0.330$ ), but the lineage I strains had significantly higher mRNA levels than lineage II and intermediate lineage strains ( $P$



**Figure 3.5.** Threshold dose (ng) for cell extract activity produced by *E. coli* O157:H7 strains using the absorptive epithelial cells from the jejunum of cattle ( $n=7$ ). A. Comparison of strains. B. Comparison of strain-origin (human-origin strains: Sakai, ECI 1717, 84, 86, 197; bovine-origin strains: 24, 25, 12, 138, H3-2R) and lineage types (lineage I strains: Sakai, 24; lineage II strains: ECI 1717, 25; intermediate lineage strains: 84, 12, 86, 138, 197, H3-2R).



**Figure 3.6** Relative levels of *stx1*, *stx2* and *ehxA* mRNA from different *Escherichia coli* O157:H7 strains ( $n=10$ ) and lineages ( $n=6$ ). A. Comparison of strains. B. Comparison of individual lineage types (lineage I strains: Sakai, 24; lineage II strains: ECI 1717, 25; intermediate lineage strains: 84, 12, 86, 138, 197, H3-2R).



**Figure 3.7.** *Escherichia coli* O157:H7 strain-origin effect on the relative production of *stx1*, *stx2* and *ehxA* mRNA ( $n=5$ ). Human-origin strains (Sakai, ECI 1717, 84, 86, 197), bovine-origin strains (24, 25, 12, 138, H3-2R).

< 0.007). There was no difference in *stx2* mRNA levels between lineage II and intermediate lineage strains ( $P = 0.992$ ). We did not find a lineage type  $\times$  strain origin interaction for mRNA levels of *ehxA* ( $P = 0.269$ ). There were no differences in mRNA levels of *ehxA* based on strain origin ( $P = 0.697$ ) or lineage type ( $P = 0.147$ ).

### **3.5. DISCUSSION**

Mature cattle challenged with *E. coli* O157:H7 respond with heterogeneous shedding periods, the duration and magnitude of which are affected by exposure dose (Besser et al. 2001), animal age (Cray and Moon 1995) and an uncharacterized mucosal factor (Baines et al. 2008b). However, it is still relatively unclear why one animal becomes more heavily colonized than another when exposed to the same dose and strain composition. One possibility is that alternative binding sites exist in some animals that allow for greater colonization. Stx2 enhances *E. coli* O157:H7 colonization in cattle (Baines et al. 2008a) and a similar impact has been documented in a less disease-susceptible animal model (Robinson et al. 2006). In the latter system, the Stx2 enhances the expression of non-Tir based colonization sites, nucleolin, and inclusion of a Stx2 expressing strain with a non-expressing strain results in an equivalent and higher colonization by both strains resulting in a longer duration of shedding. Generally, cattle shedding studies have used lineage I strains associated with clinical human disease cases and typically cattle challenges result in about a ten day shedding period (Bach et al. 2002b). In multi-strain studies using lineage I (human origin), intermediate lineage (human origin) and lineage I (bovine origin) strains, the shedding period expands to about forty-five days (Bach et al. 2005a) suggesting that the bacteria may be using a similar mechanism of alternate

binding sites in cattle to facilitate prolonged colonization. Earlier studies using the same multiple strains suggested that cell extract activity reflects *E. coli* O157:H7 strain colonization (Baines et al. 2008c; d), however a limited number of lineage and origin types were used. In the current study, we examined this question in more detail and determined that bovine-origin strains had less active cell extracts, as defined by the threshold cell extract dose to cause cell death in the lawn assay, compared with human-origin strains, confirming the previous results. However, bovine-origin *E. coli* O157:H7 strains had much higher colonization than human-origin strains at environmental doses, which is defined as the range of *E. coli* O157:H7 density that cattle would be exposed to under natural circumstances (typically  $10^3$ -  $10^5$  CFU/ g) (Naylor et al. 2003), suggesting that unknown factor(s) other than secreted cell extracts are important for cattle colonization. In addition, we examined the role of lineage type in *E. coli* O157:H7 colonization in cattle and found that lineage I strains more readily colonize the intestinal tract of cattle than other lineages. The hierarchy for lineage cell extract activity was lineage I > intermediate lineages > lineage II strains. This is in agreement with the previously mentioned studies (Baines et al. 2008d; c), but expands the comparison to include lineage II *E. coli* O157:H7 strains. Further, the same lineage hierarchy was observed for adherence with the IVCC assay, suggesting that cell extracts may be important to *E. coli* O157:H7 colonization of cattle and should be investigated further for their role in colonization.

Shiga toxins are key virulence factors of human disease with both lethal and sublethal effects on target cells (Valdivieso-Garcia et al. 1996; Ferens and Hovde 2000; Schuller et al. 2004; Robinson et al. 2006; Ferens and Hovde 2007). Only a few studies

are available that have examined the role of lineage or origin on Shiga toxin activities or the promotion of *E. coli* O157:H7 colonization of cattle. Inclusion of Stx2 enhances the colonization of a human-origin lineage I strain by a factor of 100 (Baines et al. 2008a) and in another study (Baines et al. 2008c), inclusion of the cell extract mixture from a human-origin lineage I strain increased colonization of a bovine-origin strain 10 fold compared with a 4 fold change for a human-origin lineage I strain. This suggested that the bovine-origin strains could utilize the cell extracts to enhance their own colonization. In the current study, we examined the *in vitro* mRNA levels of *stx*'s and *ehxA* to determine whether the amount or type of cytotoxin expressed could explain differences in *E. coli* O157:H7 strain origin and lineage colonization of cattle. We found that human-origin strains had higher mRNA levels of *stx1* than bovine-origin strains indicating that this toxin may be more important to the infection process in humans than in cattle. In cattle, Stx1 is readily degraded in the crypts of the colon (Hoey et al. 2003) and there are no apparent receptors present in the vascular tissue (Hoey et al. 2002) confirming that this toxin is unlikely to be critical for *E. coli* O157:H7 colonization of cattle. Previously, other researchers were unable to find differences in *stx2* mRNA levels in feces from clinical human cases and cattle that were experimentally inoculated with a human-origin strain of *E. coli* O157:H7 (Rashid et al. 2006). Similarly, in the current study, bovine and human-origin strains had similar levels of *stx2* mRNA suggesting that this toxin is of equal importance for colonization of cattle and humans. Finally, *ehxA* mRNA levels did not differ between human- and bovine-origin strains making it unclear whether this toxin contributes to the *E. coli* O157:H7 colonization process of cattle. All lineages had similar *stx1* mRNA levels, while *stx2* mRNA levels were higher in lineage I strains than lineage

II or intermediate lineages and this toxin has been shown to increase *E. coli* O157:H7 colonization (Baines et al. 2008a) confirming a link between Stx2 activity and higher strain colonization of cattle. In contrast, *ehxA* mRNA levels were highest in intermediate lineages with lineage I and II having similar levels. This supports a role for *stx2* expression in the higher lineage I colonization of cattle tissue. In our study, *in vitro* relative mRNA quantification data may have limitations for application to *in vivo* systems and we recognize that expression can be affected by numerous factors such as growth in the presence of epithelial cells (Jandu et al. 2009). Nevertheless, *in vitro* differences observed between strains of various origins or lineage types should not be overlooked as it is plausible that they would also be witnessed *in vivo*. Others too have previously used *in vitro* mRNA quantification to model expression that may occur within the cattle intestinal tract (Dowd and Ishizaki 2006; Baker et al. 2007; Dowd and Williams 2008). Future *in vivo* expression studies are crucial for inferring how expression may affect *E. coli* colonization of cattle. Another limitation of this study was the small strain set used; despite this, the highly significant interactions obtained provide novel insight into *E. coli* O157:H7 pathogenicity.

Tissue- and cell line-based *E. coli* O157:H7 colonization assays did have differences in lineage and strain colonization. There was significant variability in the colonization of lineage II and intermediate lineage strains in any given tissue where the colonization could be as high as a lineage I strain or as low as a lineage II strain. This variability is possibly due to as yet undefined mucosal factors, such as alternate binding sites (Baines et al. 2008b) which necessitated increased replication to detect differences. This limitation is particularly present at environmental dose ranges (Baines et al. 2008c)

and further supports the efficacy of a bovine cell culture model which provides more consistent replication. In addition, the lower environmental dose ranges used in this study gave greater variability than higher doses in *E. coli* O157:H7 colonization of the cattle tissue. This was associated with a difficulty in detecting the pathogen in the presence of the endogenous non-O157 *E. coli*. Again, the IVCC colonization assay did not have this problem as there are no endogenous bacteria allowing for better separation of strain differences. This allowed us for the first time, to detect the significance of strain origin to *E. coli* O157:H7 adherence of cattle. We conclude that the IVCC assay is a rapid, easy and sensitive method for evaluating the contribution of *E. coli* O157:H7 virulence factors to colonization of host-specific cells.

The *E. coli* O157:H7 lineage I and II strains are thought to cluster as separate phylogenetic branches and lineage I is the ancestral form, while lineage II is derived through changes to or loss of virulence genes (Kim et al. 1999; Kim et al. 2001; Yang et al. 2004; Steele et al. 2007). *E. coli* O157:H7 lineage I strains cause increased human illness (Kim et al. 1999; Ziebell et al. 2008) and colonize bovine tissues at a higher level than lineage II strains. The relative abundance of *E. coli* O157:H7 lineage I strains across different geographic areas has been attributed to the rapid dissemination of the newly evolved pathogen into a previously unoccupied ecological niche within human and bovine populations (Yang et al. 2004). However, different geographic subpopulations of lineage II strains have been identified and even though the changes from lineage I to lineage II strains are thought to predate the spread of the lineage II strains (Kim et al. 2001), it is likely that the genetic changes separating these different groups of lineage II strains are due to losses or rearrangements of virulence factors. Indeed, it has been

suggested that such genomic changes could have a high frequency due to the relative abundance and homology of numerous prophage-like genomic inserts encoding virulence and regulatory genes within the *E. coli* O157:H7 genome (Zhang et al. 2007). The likelihood of these different lineage II strains becoming or remaining successful within bovine populations despite decreased individual colonization capabilities is increased dramatically due to the previously unknown niche that is supplied by highly colonizing *E. coli* O157:H7 lineage I strains. This mechanism of virulence factor mutation followed by “piggy-back” colonization may have contributed to the different lineage II subgroups that are presently common within feedlot environments and may explain how multiple branches of a lesser colonizing phenotype evolved. The current study is the first of its kind to determine that strain origin, lineage type and levels of *stx2* mRNA greatly influence the amount of *E. coli* O157:H7 colonization in the jejunum and a colonic cell line from cattle.

## GENERAL DISCUSSION

Yearly outbreaks of *E. coli* O157:H7 infection is the source of considerable morbidity and mortality via hemorrhagic colitis and HUS (Centre for Infectious Disease Prevention and Control 2005; Centers for Disease Control and Prevention 2007) and modeling studies have predicted that controlling the pathogen in the cattle reservoir prior to cattle processing will have the greatest impact on food safety (Jordan et al. 1999). As such, mitigation of *E. coli* O157:H7 in the feedlot is a primary focus of research. However, despite increasing knowledge of the potential benefits of vaccination (Smith et al. 2009; Thornton et al. 2009), feeding (Cray et al. 1998; Brashears et al. 2003; Bach et al. 2005a), transportation (Cuesta Alonso et al. 2007; Dewell et al. 2008) and slaughtering (Bosilevac et al. 2005; Bosilevac et al. 2006) procedures, there has not been a decline in the number of clinical cases of *E. coli* O157:H7 in humans.

The recent description of different *E. coli* O157:H7 lineages that cause varying degrees of human illness (Kim et al. 1999; Ziebell et al. 2008) suggests that variation exists with regard to how these lineages persist within cattle. Such variation could explain some of the conflicting ideas regarding *E. coli* O157:H7 infection, shedding and survival as well as the lack of success in controlling it within the feedlot. While *E. coli* O157:H7 lineage based studies exist, their focus has been on surveillance (Kim et al. 2001; Sharma et al. 2009) or genetic comparisons (Dowd and Ishizaki 2006; Zhang et al. 2007; Ziebell et al. 2008). This thesis encompasses preliminary comparisons of different *E. coli* O157:H7 lineages in regards to their survival in feces and their colonization of the bovine digestive tract.

A comparison of *E. coli* O157:H7 lineages I and II for growth and survival in bovine feces was examined in order to observe possible differences which could be indicative of the different lineages abilities to re-infect animals within the feedlot and possibly the lineages abilities to survive long enough to enter the food supply. It was hypothesized that feeding practices combined with the warm summer months may lead to increased survival of *E. coli* O157:H7 lineage I strains compared to lineage II strains, increasing the dissemination of lineage I strains into the food supply. This situation could in part account for the apparent bias of *E. coli* O157:H7 lineage I strains being more associated with human illness. However, upon investigation there were no differences between the two lineages of *E. coli* O157:H7 in growth or survival in feces over a range of temperatures. This suggests that the higher incidence of *E. coli* O157:H7 lineage I strains causing illness is due to another factor or combination of factors, such as increased colonization of cattle or humans, increased shedding by cattle or increased virulence as compared to lineage II strains.

Diets and temperatures used in the study were selected to mimic commonly encountered conditions within southern Alberta during peak shedding periods. These two factors had a larger effect on *E. coli* O157:H7 survival than lineage type and affected both lineage types equally. *E. coli* O157:H7 populations were most stable at 12°C in both feces from cattle fed either grain or hay but reached their highest concentrations at 25°C. However, ambient conditions fluctuate around all of these temperatures; therefore, *E. coli* O157:H7 may experience periods of stability, decline or even growth at different times. Fecal composition also affected growth and survival of *E. coli* O157:H7. It is important to note these differences because cattle are commonly switched to high grain diets just

prior to slaughter and this could cause an increased prevalence and concentration at the processing plant. This effect was observed to be temperature dependant and correlated to pH of the grain feces.

After determining that *E. coli* O157:H7 lineages I and II had similar growth and survival characteristics in feces, it was hypothesized that lineage I strains may have an increased ability to colonize cattle and/or humans. *E. coli* O157:H7 lineage colonization of cattle was investigated because if differences exist, pre-slaughter intervention strategies should account for them. Further, intermediate lineage types were investigated as well as the more common lineage I and lineage II types in order to determine if the relatively rare intermediate lineage types might be of interest for further study. *E. coli* O157:H7 lineage colonization of cattle was compared using two *in vitro* models, a previously described organ culture model (Baines et al. 2008c) and a novel cell culture model. Results demonstrate the utility of the cell culture model because it has neither the tissue to tissue variability nor the indigenous bacteria that limit the organ culture model, yet this cell culture model is able to provide comparable trends to the organ culture model. Both of these models demonstrated significantly greater adherence for lineage I strains than lineage II and intermediate lineage strains. This result has multiple implications. First, enhanced colonization would allow *E. coli* O157:H7 lineage I strains to re-infect cattle within the feedlot at a higher rate than other lineages. Effective colonization is also likely to be a pre-requisite for persistent colonization; therefore, it is feasible to speculate that lineage I strains would be better able to reach threshold adherence levels to become persistently colonized. Further, increased colonization by lineage I strains likely results in increased shedding, increasing the dissemination of these

strains to the food supply and further increasing their transmission to other animals on the feedlot. Finally, this result suggests that the increased pathogenicity of lineage I strains to humans could be related to increased colonization through similar mechanisms.

Interestingly, there was a significant effect of an unexpected factor, strain origin. Bovine-origin strains of *E. coli* O157:H7 were observed to have much greater colonization than human-origin strains at common environmental doses but the two groups had similar colonization at high doses. This suggests that the pathogen may become acclimatized to a particular host type and be better able to colonize it, possibly through changes in gene expression patterns.

In an attempt to better understand these differences in lineage and strain origin colonization, comparisons of cell extract activity and cytotoxin gene transcription were made among the lineages. Using the lawn assay, it was observed that the cell extracts from lineage I strains had a lower threshold dose for causing intestinal epithelial cell death than other lineages and were capable of increasing the adherence of a less effective colonizing strain. However, it was also observed that human-origin *E. coli* O157:H7 strains produced cell extracts that were more toxic than those produced by bovine-origin strains despite the increased adherence of the bovine-origin strains, suggesting that cell extract activity may only be a predictor of adherence for a subset of *E. coli* O157:H7 strains. This is supported by previous reports that Stx2 is not toxic to epithelial cells from the jejunum and descending colon of cattle (Baines et al. 2008a) but does increase colonization of *E. coli* O157:H7 (Robinson et al. 2006; Baines et al. 2008a). Transcription of mRNA of selected virulence genes was also quantified and lineage I strains had higher levels of *stx2* than lineage II and intermediate lineage strains

supporting the idea that this toxin may play an important role in colonization. With the relatively limited comparison of human and bovine-origin *E. coli* O157:H7 strains, it was also observed that human-origin strains had higher levels of *stxI* mRNA and while this does not explain the enhanced colonization of bovine-origin strains to the bovine colonic cell line, it is evidence of differential gene expression between the two origin types. Further, lower levels of *stxI* mRNA in bovine-origin strains would be expected if expression patterns are host specific because cattle lack receptors for Stx1 (Pruimboom-Brees et al. 2000) and it is instead degraded in the crypt cells of the colon (Hoey et al. 2003).

This thesis has been aimed at further understanding the roles of different lineages of *E. coli* O157:H7 within the feedlot environment and represents an initial stage of research within a recently defined and unexplored gap of *E. coli* O157:H7 knowledge. Future studies of *E. coli* O157:H7 lineage dynamics should further explore the mechanism(s) and implications of enhanced *E. coli* O157:H7 lineage colonization. Such studies could include *in vivo* colonization and shedding studies. Further, the role of strain origin should be examined to determine if *E. coli* O157:H7 strains exhibit host specificity or differential expression in different host types. With a better understanding of those *E. coli* O157:H7 groups which pose a greater risk to human health, it may be possible to focus mitigation strategies on a subpopulation of *E. coli* O157:H7 in an effort to reduce the risk of this pathogen to the food supply.

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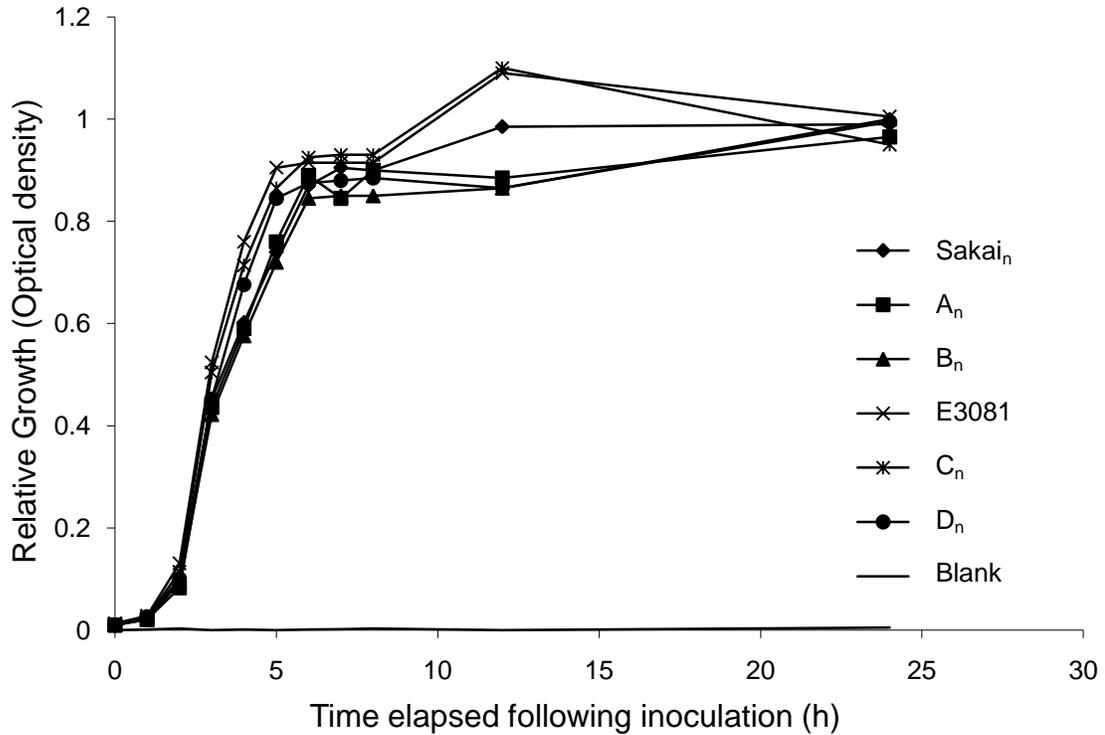
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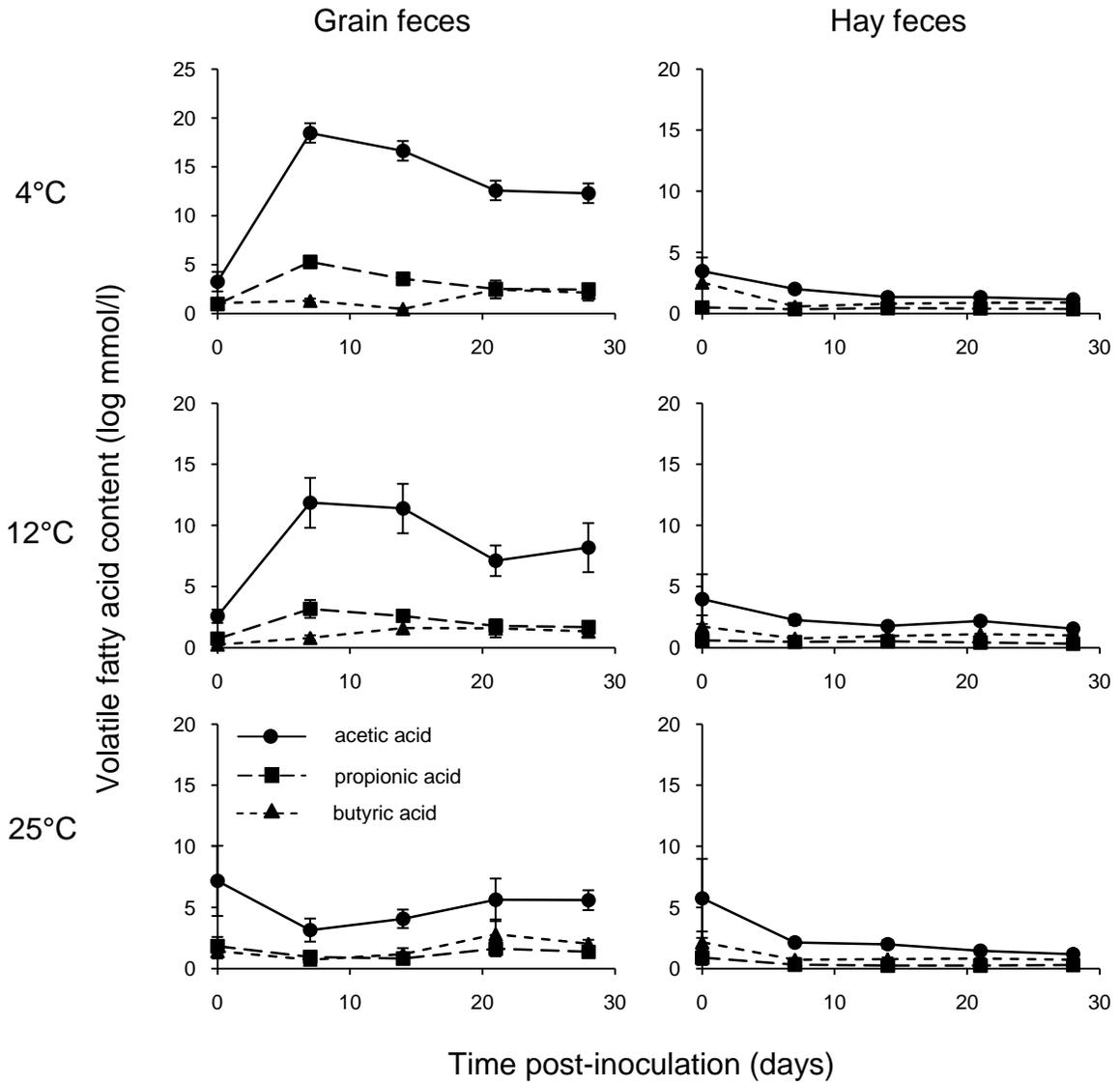
## APPENDICES

### Appendix One



**Figure A.1.** Relative growth comparison of *E. coli* O157:H7 strains used for bovine feces inoculations. For all strains, 100  $\mu$ l of overnight growth (18 hours at 37°C) was inoculated into 10 ml of pre-warmed TSB followed by incubation at 37°C. Cell density was monitored spectrophotometrically (OD<sub>640</sub>) for 24 hours. Blank refers to 10 ml of pre-warmed TSB inoculated with 10  $\mu$ l of sterile TSB and incubated at 37°C.

Appendix Two



**Figure A.2.** Volatile fatty acid content of grain and hay feces incubated at 4°C, 12°C and 25°C separated into its organic acid components. Values for iso-butyric, iso-valeric, valeric and caprioc acids were below limits of reliable detection and are therefore not included. Mean  $\pm$  standard error values represent average acid contents from duplicate Sakai<sub>n</sub>, E3081 and control feces inoculations.