CHARACTERIZATION OF *ESCHERICHIA COLI* O157:H7 FROM SUPER-SHEDDING FEEDLOT CATTLE

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DEDICATION

Dedicated to my first biology teachers –
Mr. David Leffelaar and Mr. Will Hunter
who instilled in me a love of science
and taught me that for every answered question
there are a thousand more unanswered

ABSTRACT

Escherichia coli O157:H7 is a major foodborne human pathogen that causes disease worldwide. Healthy cattle are the primary reservoir of this bacterium and the amount and frequency of *E. coli* O157:H7 shedding varies among individual cattle. The term "super-shedder" has been applied to cattle that are transiently high shedders ($\geq 10^4$ CFU/g feces). Targeting these cattle for mitigation strategies has been proposed as a means of reducing the incidence and spread of *E. coli* O157:H7 in the feedlot environment. The objectives of this study were to determine the frequency and duration of the super-shedding state in cattle; and to elucidate phenotypic or genetic differences among *E. coli* O157:H7 isolates recovered from super-shedding cattle and low-shedder cattle. It was found that the super-shedding state is short lived and lacks continuity and we were unable to successfully identify specific traits among super-shedder isolates that could be used to differentiate them from low-shedder isolates.

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value of "work hard, play hard" and keeping life fun. Finally a great big thank you goes to

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Munns, K.D., Selinger, L.B., Stanford K., Guan, L., Callaway T.R., and McAllister, T.A. 2015. New perspectives on super-shedding of *Escherichia coli* O157:H7 by cattle. *Foodborne Pathogens and Disease*. **12:** 12:89-103

Chapter 2

Munns K.D., Selinger L., Stanford K, Selinger L.B. and McAllister T.A. 2014. Are super-shedder feedlot cattle really super? *Foodborne Pathogens and Disease* **11:**329-331.

Chapter 3

Munns, K.D., Zaheer R., Xu Y., Stanford, K., Laing, C.R., Gannon, V.P.J, Selinger, L.B. and McAllister, T.A. 2015. What causes super-shedding: Comparative genomics of *Escherichia coli* O157:H7 isolated from super-shedder and low-shedder cattle. PloS ONE. In preparation

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Aga	N-Acetyl-D-Galactosamine
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
AUC	area under the curve
CFU	colony forming units
CLSI	Clinical and Laboratory Standards Institute
CT-SMAC	cefixime sorbital MacConkey agar
DNA	deoxyribonucleic acid
eae	intimin
EbgA	evolved- ß-galactosidase
gad	glutamate decarboxylase
GIT	gastrointestinal tract
HUS	hemolytic uremic syndrome
IMS	immunomagnetic separation
LEE	Locus of enterocyte effacement
LRC	Lethbridge Research Centre
LSPA-6	lineage typed using lineage-specific polymorphism assay
MAC-T	bovine mammary epithelial cell line
MPN	most probable number
mTSB	modified tryptic soy broth
NSF	nonsorbitol fermenting
NSS	non-super-shedder
nsSNPs	non-synonymous single nucleotide polymorphism
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PM	phenotype microarrays
PT	phage type
PTS	phosphotransferase system
Raf	raffinose
rafA	α-galactosidase
rafB	Raf permease
rafD	sucrose hydrolase
RAJ	recto-anal junction
RAMS	rectoanal mucosal swabs
REPCs	restriction endonuclease digestion pattern clusters
RNA-seq	ribonucleic acid sequencing

RR	repeat region
RSE	recto-anal junction squamous epithelial
SAM	S-adenosyl-MethionineATP
SBI	Shiga toxin-encoding bacteriophage insertion
SEM	standard error of the mean
SNPs	single nucleotide polymorphisms
SS	super-shedder
STEC	Shiga toxin-producing *E. coli*
Stx	Shiga toxin gene
Tir	translocated intimin receptor
TSB	tryptic soy broth
USD	United States Dollars
VFA	volatile fatty acids

CHAPTER 1: Perspectives on super-shedding of *Escherichia coli* O157:H7 by cattle[1]

1.1 *Escherichia coli* O157:H7

Escherichia coli O157:H7 is a Shiga-toxin-producing, non-sorbitol-fermenting human pathogen that asymptomatically colonizes cattle as a primary reservoir (Wells *et al.,* 1991; Chapman *et al*., 1993; Ferens and Hovde, 2011). Transmission to humans can occur through the consumption of contaminated food or water, person-to-person contact or direct-contact with animal faeces or an animal reservoir (Ferens and Hovde, 2011). Infection in humans may remain asymptomatic or result in gastroenteritis including bloody diarrhea (hemorrhagic colitis) which can progress to renal failure through hemolytic uremic syndrome (HUS) resulting in mortalities, especially in children. Research suggests that consumption of as few as 10 cells of *E. coli* O157:H7 can cause human infections (Schmid-Hempel and Frank, 2007), but individual strains differ in virulence (Baker *et al*., 2007). The pathogenicity of *E. coli* O157:H7 is attributed, in part, to the production of verotoxins or Shiga-like toxins named so because of their similarity to the toxins produced by *Shigella dysenteriae* (O'Brien *et al.,* 1992). Lysogenic bacteriophages carry the genes responsible for producing these toxins (stx_1 and stx_2) that inhibit protein synthesis in eukaryotic cells causing cell death (Rashid *et al.,* 2006). In the United States, *E. coli* O157:H7 infections cause over 73,000 illnesses and 61 deaths per year (Mead *et al*., 1999), costing the American healthcare system approximately $405

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[1] This chapter is an adapted version of the manuscript "New perspectives on super-shedding of *Escherichia coli* O157:H7 by cattle." 2015. Munns, K.D., Selinger, L.B., Stanford K., Guan, L., Callaway T.R., and McAllister, T.A. 2015. *Foodborne Pathogens and Disease*. **12:** 12:89-103

million (2003 USD dollars) (Frenzen *et al.,* 2005). If direct and indirect costs of Shiga

toxin-producing *E. coli* (STEC) illness are included, the total cost to the US economy

exceeds $1 billion USD annually (Scharff, 2010). These estimates include cost for

medical care, losses in productivity, and premature death. In Canada, the rate of infection

of *E. coli* O157:H7 in 2012 was 1.39 per 100,000 (Public Health Agency of Canada,

2013) and reached levels as high as 9.81 per 100,000 in 2000 (Public Health Agency of

Canada, 2009).

1.2 Defining a super-shedder

The frequency of shedding and number of *E. coli* O157:H7 shed in the feces varies

greatly among individual cattle (LeJeune *et al.,* 2004b; Stanford *et al.,* 2005a). Studies in

cattle typically report shedding of *E. coli* O157:H7 to be sporadic and of short duration

(Besser *et al*., 1997) ranging from 10 to 10^9 CFU/g feces (Chase-Topping *et al.,* 2008;

Stephens *et al.,* 2009). The term "super-shedder" has been applied to cattle that shed $\geq 10^4$

CFU/g feces of *E. coli* O157:H7 (Matthews *et al.,* 2006a, b). Early definitions of a super-

shedder were often blurred by the use of the term "super-spreader" which is more related

to the degree that individuals are responsible for the environmental spread of *E. coli*

O157:H7 as opposed to quantifying the absolute number of CFU shed in feces. A review

by Chase-Topping *et al*. (2008) defined a super-spreader as an individual who has more

opportunities to infect other host types with a pathogen than other individuals of the same

host type through direct or indirect contact. In contrast, a super-shedder was defined as an

individual that sheds more organisms than another individual within a population for a

defined period of time. Characteristically, many more infectious units are released from a

super-shedder compared to a super-spreader; super-shedders are still capable of being

super-spreaders. In the present review we will discuss super-shedding and its impact on the spread, or super-spread, of *E. coli* O157:H7.

1.3 Colonization of *E. coli* O157:H7 in super-shedding cattle

Colonization of the intestinal epithelium by *E. coli* O157:H7 has been proposed as a necessary measure for populations to reach requisite densities for super-shedding. Naylor *et al.* (2003) hypothesized that super-shedders shed high levels of *E. coli* O157:H7 due to effective colonization of the distal few centimeters of the bovine rectum, termed the recto-anal junction (RAJ). In a follow up study, Naylor *et al.* (2005) reported the *LEE4* operon, encoding a type III secretion system translocon and associated proteins, was essential for colonization when a deletion mutant of *LEE4* failed to colonize the gastrointestinal tract (GIT) of cattle. Subsequent studies have described an association between RAJ colonization and super-shedding of *E. coli* O157:H7. For example, Low *et al.* (2005) reported 9 of 10 naturally-colonized cattle have $>10^3$ CFU/mL in tissue washes from the terminal rectum with the highest numbers of *E. coli* O157 found most frequently close to the RAJ region. Cobbold *et al.* (2007) reported that rectoanal mucosal swabs (RAMS) from super-shedders had higher populations of *E. coli* O157:H7 relative to commensal *E. coli* than RAMS from low-shedders. This suggests that the RAJ of super-shedders was colonized with *E. coli* O157:H7 to a greater degree.

Bacteria have the ability to attach, colonize, and form biofilms on a variety of surfaces (Uhlich *et al.*, 2006). Perhaps this biofilm formation at the intestinal epithelium that may lead to a high density of *E. coli* O157:H7 at the RAJ. Biofilms are formed in distinct stages: (i) initial reversible attachment of bacteria to a surface, (ii) production of exopolysaccharide material to form an extracellular polymeric matrix resulting in

irreversible attachment, (iii) early development of biofilm architecture, and finally (iv)

maturation (Chmielewski and Frank, 2003; Kim and Wei, 2007). However, when the

biofilm becomes too thick, even well adapted microbes will die, causing biofilm fragility,

and sloughing of portions of the biofilm architecture (stage v) (Shirtliff *et al.,* 2002).

Biofilm sloughing in the intestine could be responsible for the fecal densities of *E. coli*

O157:H7 that are required for the host to be designated as a super-shedder (i.e., 10^4

CFU/g feces). Intermittent sloughing of the intestinal biofilm could also account for the

sporadic nature of shedding and super-shedding in cattle. Biofilms are not static and the

sloughing off of viable bacteria promotes the colonization of downstream environments

(Van Houdt and Michiels, 2005). Recent research by Williams *et al.* (2014) has

demonstrated that five heifers with multiple super-shedding events also had a high

number of fecal samples from which *E. coli* O157:H7 was not detected. Specifically three

of these heifers showed intermittent and infrequent low levels of shedding with clusters

of high shedding levels, fitting a pattern of clearance and re-infection, typical of a biofilm

sloughing phenomenon. While the role of biofilms in colonization of the intestinal

epithelium by *E. coli* O157:H7 could account for the intermittent shedding of this

bacterium in super-shedders, the factors that trigger the mass release of this organism into

feces are unknown. As such, feces collected after a biofilm sloughing event may exhibit

super-shedder levels, with subsequent fecal samples being negative for *E. coli* O157:H7

until the next sloughing event occurs.

1.4 Prevalence of super-shedders and their role in the transmission of *E. coli* O157:H7

The reported prevalence of super-shedders is dependent on a variety of factors including size and method of sample collection (fecal or RAMS), consistency of feces/swabbing technique, laboratory protocols, season of sample collection (summer *vs.* winter), unit of measure (farm, pen, truck or animal), and method of enumeration. Additionally, some studies report percentage prevalence of super-shedding among all samples collected while others report only among samples that are positive for *E. coli* O157:H7. Consequently, super-shedder cattle have been reported to comprise between 0.48% - 71% of a given population (**Table 1.1**).

A limitation associated with detecting super-shedders is that CFU/g feces is commonly determined via direct plate counts, a process that involves serial dilution of sampled feces. This technique is time consuming, making it difficult to process large numbers of samples rapidly. Other methods of enumeration include the most probable number technique, which also requires a high degree of replication at the appropriate dilution to narrow confidence intervals. Quantitative PCR (or real-time PCR) can also be employed to obtain accurate counts of *E. coli* O157:H7, however this method requires the extraction of high-quality DNA. This step can be time limiting and expensive, and lacks sensitivity in terms of detecting *E. coli* O157:H7 if it is present at levels below super-shedding.

Enumeration of *E. coli* O157:H7 within feces has been shown to be a stochastic process because of the variability in the distribution of the bacteria within a single fecal sample. This was illustrated by the observation that when 120 fresh fecal pats from two

Table 1.1. Summary of ten studies, with diverse sampling and analysis methodology, examining the prevalence of super-shedding ($\geq 10^4$ CFU *E. coli* O157:H7 g/feces) cattle from Canada, United Kingdom and USA.

Location of study	Number of cattle (n)	Sampling details (dates, location, sampling frequency)	Number and type of samples	Percentage of super-shedders identified	Reference
Australia	52 heifers	▪ Oct to Feb 2013 ▪ Pasture ▪ Raised as replacement dairy animals ▪ 18 weekly sampling points	893 fecal sample (10g)	**3.6%** (32/893 of samples tested) **46.2%** (24/52 super-shedding cattle identified)	Williams *et al.*, 2014
Canada	160 (20 pens, eight head of cattle per pen)	▪ Jul to Oct 2003 ▪ Experimental research pens at commercial feedlot and slaughter operation ▪ Sampled twice per week, over a 14-week period	8960 (4480 from freshly passed manure and 4480 recto-anal mucosal swabs	**3.1%** (5/160 cattle from 3 pens were super-shedder)	Cobbold *et al.,* 2007
Canada	4185 [Feedlot X n=1357 (summer) n=1284 (winter); Feedlot Y n=779 (summer) and n=768 winter)]	▪ May to Aug 2007 (summer) ▪ Sept 2007 to Jan 2008 (winter) ▪ Two commercial feedlots	8370 (4185 fecal grabs and 4185 perineal swabs)	**7.16%** (153/2136 cattle were super-shedding during summer) **0.48%** (10/2052 cattle were super-shedding cattle during winter)	Stephens *et al.,* 2009; Stanford *et al.,* 2012

Country	Sample	Details	Samples	Results	Reference
Canada	400 cattle	▪ Jul 2011 ▪ Single commercial feedlot ▪ Single time point	400 rectal fecal samples (50 g)	**2.8%** (11/400) **23.9%** (11/46 positive for *E. coli* O157:H7) samples	Munns *et al.,* 2014; Xu *et al* 2014
Scotland	999 cattle	▪ May to Jul 2002 (summer) ▪ Jan – Mar 2003 (winter) ▪ Single abattoir	999 faecal sample (25 g) (545 summer and 454 winter)	**0.7%** (overall; 7/999) **0.7%** (summer sampling; 4/545) **0.66%** (winter sampling; 3/454) **6.9%** (7/101 super-shedding cattle identified among cattle positive for *E. coli* O157:H7)	Ogden *et al.,* 2004
Scotland	3,111 Scottish farms	▪ Mar 2002 and Feb 2004 ▪ No. of fecal pats sampled was determined from the no. of cattle/group ▪ It was assumed that 8% of the animals in positive groups would be shedding ▪ Sufficient fresh pat samples were taken to ensure a mean 90% probability of detecting *E. coli* O157:H7 if at least one shedding animal was present ▪ high-level shedders were identified using	481 farms (selected to ensure similar numbers were included from each region of Scotland)	**16.9%** (13/77 positive *E. coli* O157:H7 farms had a concentration of *E. coli* of $\geq 10^4$ CFU/g feces)	Chase-Topping *et al.,* 2007

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Country	Sample	Details	Sampling Method	Results	Reference
		mixture distribution analysis on counts of *E. coli* O157			
United Kingdom	721	▪ May to Jul 2002 ▪ local abattoir ▪ Weekly samples – 9 visits	589 rectal fecal grab (75g)	**9%** (4/44 cattle positive for *E. coli* O157:H7)	Omisakin *et al.,* 2003
USA	1503 cattle 50 truckloads from the Midwest United States	▪ May – Jun 2007 ▪ Weekly sampling for 5-wks ▪ Single commercial abattoir	1503 fecal samples obtained from intact rectums	**3.7%** (55/1503) **52.0%** (26/50 truckloads had at least one high-shedding animal)	Fox *et al.,* 2008
USA	319 cattle	▪ Sept 2004 – May 2005 ▪ Research feedlot ▪ Sampled once a month ▪ 10 sampling points	3,190 rectal fecal samples (10g)	**1.1%** (35/3190)	Arthur *et al.,* 2009
USA	168 steers in Ohio	▪ Oct 2005 – Apr 2006 ▪ Beef feedlot facility ▪ Bi-weekly sampling	1784 direct plated fecal grab (10g) samples	**2.2%** (at the sample level; 39/1784 super-shedding samples tested by direct plating) **22.6%** (animal level; 38/168 of the steers experienced at least one super-shedding event throughout the entire feeding period) **71%** of pens having at least one animal experiencing a super-shedding event	Cernicchiaro *et al.,* 2010; 2011

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feedlots were sampled multiple times, the fecal prevalence increased from 8.2% with one sample/pat to a plateau of 20% with five samples/pat (Echeverry *et al.,* 2005). Methods of *E. coli* O157:H7 enumeration in feces can also differ with regard to the total amount of feces collected. For example, Ogden *et al*. (2004) enumerated *E. coli* O157:H7 using 25 g of serially diluted feces. Others have enumerated *E. coli* O157:H7 using a 1 g subsample collected from a larger thoroughly mixed sample (Stephens *et al*., 2009; Munns *et al*., 2014) or the limited amount of feces (< 1g) collected on swabs (Cobbold *et al.,* 2007; Arthur *et al*., 2013). Future efforts to standardize sampling and diagnostic procedures are imperative to permit better comparison of the prevalence of super-shedders among studies. Currently there is no rapid means to identify super-shedders; however, if genetic markers specific to the super-shedding trait in either the animal or the pathogen could be identified, a PCR based diagnostic technique could be potentially developed.

Super-shedders have been reported to have a substantial impact on the on-farm prevalence and transmission of *E. coli* O157:H7 into the surrounding environments. In previous reports, anywhere from 47% (Stephens *et al.,* 2009) to >90% (Chase-Topping *et al.,* 2008) of the shedding of *E. coli* O157:H7 within feedlot pens has been attributed to super-shedders. Omisakin *et al.* (2003) estimated that only 9% of cattle were high-shedders of *E. coli* O157:H7 at concentrations of $\geq 10^4$ CFU/g. This 9% represented >96% of the total *E. coli* O157:H7 produced by all cattle tested (*n*=761). In another study, relating bacterial counts to infectiousness and fitting dynamic epidemiological models to prevalence data from a cross-sectional survey of cattle farms in Scotland, Matthews *et al.* (2006a) suggested that 80% of transmission arises from the 20% most infectious individuals.

A study carried out by McGee *et al.* (2004) examining the role of super-shedders in transmission within the feedlot showed that placing inoculated steers shedding *E. coli* O157:H7 at levels >500 CFU/g of feces for 48 h in pens resulted in contamination of the hides of 66% of the non-colonized cohorts. In another study, feedlot cattle that were negative for *E. coli* O157:H7 over the course of study were five times more likely to be housed in a pen that lacked super-shedders than housed in a pen with a super-shedder (Cobbold *et al.,* 2007). Placement of fecal pats inoculated with *E. coli* O157:H7 in pens to simulate the presence of a super-shedder resulted in 3% of hide samples from naïve cattle being positive for the inoculated strain within one day after deposition of the fecal pats (Stephens *et al.,* 2008). In contrast, one positive hide sample (0.45%) was detected in pens that received inoculated fecal pats with levels of *E. coli* O157:H7 that were below that of a super-shedder (Stephens *et al.,* 2008). In a subsequent study, Stanford *et al.* (2011b) assessed the impact of a model super-shedder interacting with pen mates on the spread of *E. coli* O157:H7 by spreading 100 g or 50 g of inoculated feces (10^6 CFU *E. coli* O157:H7/g) across the perineum of a single animal once daily in the morning. Control pens received 3,000 g of feces inoculated with the same level of *E. coli* O157:H7 in the form of 5 simulated fecal pats (300 g each) on the pen floor. Results revealed steers housed with the model super-shedder were 1.3 times more likely to shed *E. coli* O157:H7 in their feces than steers housed with simulated fecal pats. These results were obtained even though the number of *E. coli* O157:H7 cells introduced into the pens was similar, highlighting the importance of behavioral interaction in the transmission of *E. coli* O157:H7 among cattle (Stanford *et al.*, 2011b). Arthur *et al.* (2009) further demonstrated the importance of super-shedders in the transmission of *E. coli* O157:H7, whereby 95%

of pens containing one or more super-shedders housed herds with a hide prevalence of *E. coli* O157:H7 >80%, while only 29% of the herds in pens without a super-shedder exceeded a hide prevalence of 80% .

Super-shedders transported and maintained in lairage with herd mates prior to slaughter have also been linked to carcass contamination in processing plants. In a previous study, contamination of the carcass was associated with the presence of a super-shedder within the trailer during transport to the abattoir (**Table 1.1**; Fox *et al.,* 2008). Similarly, Fegan *et al.* (2005) showed carcass contamination was highest in lots with the highest levels of *E. coli* O157:H7 in feces (7.5×10^5 MPN/g), resulting in carcasses from cattle that were not shedding the organism to become adulterated. A recent contamination of meat from a major southern Alberta meat processing facility resulted in 17 confirmed human cases of *E. coli* O157:H7 infection and the largest meat recall (>2400 meat items) in Canadian history (Bottemiller, 2012). Although the cause of the contamination was not identified, it was hypothesized that super-shedders may have contributed to "high event days", where levels of *E. coli* O157:H7 entering the plant overwhelmed the "critical control points" employed to prevent this pathogen from entering the food chain (Lewis *et al.,* 2013). Consequently, mitigating super-shedders has been proposed as a viable approach to reducing the transmission of *E. coli* O157:H7 among cattle, its dissemination into the environment and the risk of carcass contamination (Matthews *et al.,* 2006b).

1.5 Understanding the super-shedding phenomenon

1.5.1 How long is an animal a super-shedder?

As discussed previously, according to the definition of Chase-Topping *et al*. (2008), cattle that shed at $\geq10^4$ *E. coli* O157:H7 CFU/g feces are defined as super-shedders. However,

in this definition, the temporal pattern of super-shedding was not defined. A previous report noted that the number of *E. coli* O157:H7 shed varies with shedding duration (Davis, 2006), whereby heifers that were culture positive for 23 days or longer had higher fecal counts of *E. coli* O157:H7 than heifers that were positive for a shorter duration. Previous studies have defined cattle as super-shedders, or alternatively as persistent-shedders, if they shed *E. coli* O157:H7 for more than three consecutive months (Lim *et al.,* 2007; Carlson *et al.,* 2009).

In reality, the duration of super-shedding by an individual animal is largely unknown despite the fact that duration of super-shedding impacts the amount of *E. coli* O157:H7 released into the environment. For example, assuming that the average feedlot steer weighs 498 kg and excretes 36 kg/day of feces (Lorimor *et al.,* 2000), a super-shedder ($\geq 10^4$ *E. coli* O157:H7/g feces) that sheds for a day would excrete 3.6×10^8 cells of *E. coli* O157:H7 into the environment, a level that would result in 2.5×10^9 cells being released into the environment if shedding occurred continuously for 7 days (**Figure 1.1**). These numbers would reach or exceed 10^{10} cells per day if four or more super-shedders were within the same pen.

In the past, *E. coli* O157:H7 shedding profiles in cattle have been based predominantly on point estimates on the farm and typically comprise a cluster of samples with a high level of variation (Robinson *et al.,* 2009). Others have evaluated the incidence of super-shedding using single samples taken from a cross-sectional survey of multiple farms (Chase-Topping *et al.,* 2008). However, Robinson *et al*. (2009) reported that within-animal variation of shedding *E. coli* O157:H7 was greater than that among animals over time. With this in mind, we hypothesize that super-shedding may vary
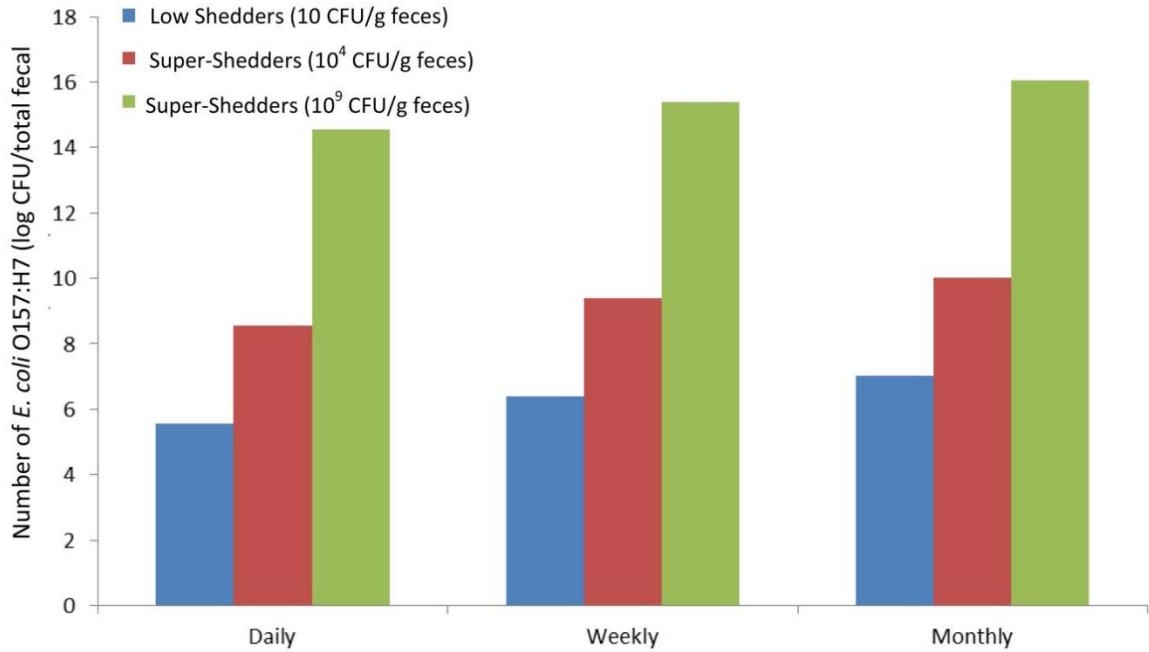
Figure 1.1. Potential amount of *E. coli* O157:H7 excreted into the environment from one feedlot steer if it were a low shedder (10 CFU/g), super-shedder (10^4 CFU/g feces), or excessive super-shedder (10^9 CFU/g feces) with the assumption the average steer weighs 498 kg and produces 36 kg/day of total wet manure (Lorimor *et al.,* 2000).

within an individual animal at any given time and that a single sample from animals on a single day is inadequate to accurately describe super-shedding events and impacts.

In a recent study, steers previously identified as super-shedders were sampled twice daily over 6 days after transport from a commercial feedlot to a research facility (Munns *et al.,* 2014). It was determined that super-shedding was a short-lived phenomenon and was not necessarily confirmed when multiple samples were collected from a single steer over the duration of a single day. Furthermore, changes in management practices such as increased handling, mixing with cohorts, or a change in diet failed to induce a return of the host to a super-shedding state. Williams *et al.* (2014) reported heifers that were detected as super-shedding were not identifiable as long term shedders and suggested that the use of 'super-shedding event' could be a better descriptor of the phenomenon as opposed to the term 'super-shedder' in reference to a specific animal. A number of factors other than the intermittent nature of shedding of *E. coli* O157:H7 by feedlot cattle could contribute to variability of shedding, including the feedlot selected, the sampling season or even year-to-year differences in pathogen prevalence (Munns *et al.,* 2014). Seasonality has been shown to play a role in shedding, with *E. coli* O157:H7 being more prevalent during summer than winter (Ferens and Hovde, 2011). Upon examining the literature we concluded there are very few studies that have examined the persistence of naturally occurring super-shedders in feedlots over time, an approach that should be the objective of future studies.

1.5.2 Factors contributing to super-shedding

There are several possible factors that may influence the ability of an individual to achieve super-shedding status, but it is logical to propose that they involve complex

interactions among the microbes, host and environment (**Figure 1.2**). For example, survival of *E. coli* O157:H7 in the environment influences its likelihood of being acquired by a host. Its ability to colonize the RAJ may also influence the degree that it will be transmitted among cattle. Once the strain is acquired, its ability to survive within the digestive tract, out compete other microbiota, grow within digesta, colonize the RAJ and undergo a mass release from the intestinal epithelium may influence its ability to achieve levels that would enable the host to be defined as a super-shedder.

Potential factors that may influence the likelihood of an individual host being a super-shedder include innate and adaptive immune responses, susceptibility to intestinal colonization and behavioural attributes that influence animal-to-animal transmission of *E. coli* O157:H7. In addition, potential environmental factors including seasonality, pen hygiene, and diet may also play a role in the super-shedding phenomenon. Recent research associated with examining factors that may contribute to super-shedding have focused on the microbial strain-specific factors of *E. coli* O157:H7 with very little research focusing on the contribution of the host or the environment to this phenomenon. Specific characteristics of *E. coli* O157:H7 that such as phenotypic traits, including antimicrobial resistance, phage type, substrate metabolism and/or genetic differences including clonal relatedness, lineage type and whole genome similarities may be associated with super-shedding.

1.5.2.1 Microbial factors in super-shedding

Phenotypic similarities of super-shedding isolates vs. low-shedding isolates

Although there are very little phenotypic data associated with *E. coli* O157:H7 isolates recovered from super-shedders, previous studies have proposed that *E. coli* O157:H7

Animal factors
- Immune system response
- Susceptibility to intestinal colonization
- Animal to animal transmission
- Stress response

A

Presence of phage species in the digestive tract

B

Ability of *E. coli* O157:H7 to colonize the rectal anal junction

Esophagus

Rumen

Large intestine

Anus

Small intestine

Cecum

Ability of *E. coli* O157:H7 to proliferate in rectal contents

Acquisition from the environment

Ability of *E. coli* O157:H7 to survive in digestive tract

Competiveness of super-shedder *E. coli* O157:H7 *vs.* generic *E. coli*

Potential for mass release of *E. coli* O157:H7 from intestinal epithelium

Survival of *E. coli* O157:H7 in the environment and its potential for reinfection

Environmental factors
- Season
- Pen Hygiene
- Diet

Figure 1.2. Possible factors that influence the development of super-shedder cattle (Munns *et al*., 2015). **A)** Transmission electron micrograph of AKFV33 phage negatively stained with uranyl acetate. Scale bar represents 100 nm. (Niu *et al.,* 2012). **B).** Scanning electron micrograph of the terminal rectum colonized with *E. coli* O157:H7 Dark staining represents the lipopolysaccharide on *E. coli* O157:H7 cell surface (Chase-Topping *et al.,* 2008).

phage type 21/28 (PT21/28) are associated with super-shedders in Scotland (Chase-Topping *et al.,* 2007; 2008). These authors proposed that the type III secretion system of PT 21/28 strains may be altered in a manner that enables these bacteria to colonize the intestinal epithelium at higher levels. Previous research has shown five different PTs (PT8, PT14a, PT21, PT33, PT34) were identified among the *E. coli* O157:H7 isolates within Canada with the two most common PTs being PT14a and PT8 (Jokinen *et al.,* 2011). Arthur *et al*. (2013) examined super-shedder isolates (*n*=102) from the United States and identified 19 different phage types with PT4 accounting for 30% of the isolates. PT4 is also the most common phage type associated with *E. coli* O157:H7, infections in humans in the United States, suggesting that super-shedders may frequently harbour strains associated with human illness. Thus, the impact of super-shedding may be disproportionately greater on public health.

The total number of generic *E. coli* and specific *E. coli* serotypes fluctuates within individual animals (Smith, 1962; Hartl and Dykhuizen, 1984). Presumably, *E. coli* O157:H7 must compete with commensal strains of *E. coli* for substrates and colonization sites within the bovine GIT. It has been suggested that the ability of *E. coli* O157:H7 to use alternative carbon sources within the GIT gives it an advantage over generic *E. coli*. Accordingly, Durso *et al*. (2004) found that *E. coli* O157:H7 had the capacity to utilize 19 carbon sources that were not used by strains of generic commensal *E. coli*. These substrates included dulcitol, sucrose, and l-galactonic acid δ-lactone, but it is unknown if there is differential utilization of carbon sources between super-shedding and low-shedding strains of *E. coli* O157:H7.

Genetic strain-specific characteristics of E. coli O157:H7

Pulsed-field gel electrophoresis (PFGE) is currently considered the "gold standard" for DNA fingerprinting and investigating the epidemiology of *E. coli* O157:H7 (Goering, 2010). Previously, isolates of *E. coli* O157:H7 from cattle shedding low, medium, or super-shedder levels displayed identical PFGE patterns suggesting that they were the same subtype (Jeon *et al.,* 2013). Furthermore, in pens of 8 cattle, Cobbold *et al.* (2007) observed that isolates of *E. coli* O157:H7 were similar among super-shedders and low shedders. Changes in shedding levels could reflect the natural proliferation of *E. coli* O157:H7 within the GIT with low to moderate levels of shedding associated with population establishment and expansion, while the super-shedder status is achieved as the population climaxes and biofilms are sloughed. Subsequently, populations of *E. coli* O157:H7 decline and the animal becomes negative or continues to shed at low levels. If certain strains of *E. coli* O157:H7 exhibit this pattern of population dynamics, they may be more likely to give rise to super-shedders, but the identification of an isolate with these properties would depend heavily on the time and method of sampling.

Cattle originating from the same feedlot have been shown to exhibit *E. coli* O157:H7 isolates with indistinguishable PFGE profiles (LeJeune *et al.,* 2004b; Sanderson *et al.,* 2006). However, genotypes of *E. coli* O157:H7 that are shed at super-shedder levels have been shown to differ within and among feedlots. Stanford *et al.,* (2012) collected isolates from super- and low-shedders from two commercial feedlots and the subtypes that were associated with super-shedding often differed between the two feedlots. However, it was uncommon that PFGE profiles were identical between low- and super-shedder isolates collected from the same pen with 77% of super-shedder isolates being distinct from those

collected from low-shedders. These results are in agreement with Dodd *et al*. (2010) where high and low-shedding cattle in truck-loads at slaughter shared identical PFGE subtypes in feces less than 25% of the time. In addition, Arthur *et al*. (2013) reported that super-shedding isolates (*n*=102) represented 52 unique PFGE patterns with no *E. coli* O157:H7 genotypes clustering specifically in relation to the super-shedder phenotype. The diversity of *E. coli* O157:H7 isolates obtained from super-shedders suggest that no single strain is responsible for the super-shedder phenomenon within a population of cattle. This raises the possibility that all strains of *E. coli* O157:H7 may have the ability to proliferate to super-shedding levels. However, within a feedlot at a given sampling time, there is clear evidence that the same strain of *E. coli* O157:H7 may be commonly shared among super-shedders. At this point it is unknown if this reflects transmission of this strain among individual cattle or exposure to common environmental conditions that enable this isolate to proliferate to super-shedding levels in a number of individual cattle.

Octamer-based genome scanning has revealed three distinct *E. coli* O157:H7 lineages based on the lineage specific polymorphism assay (Kim *et al.,* 1999; Kim *et al.,* 2001). Typically, two predominant lineages have been described, lineages I and II (Kim *et al.,* 2001; Yang *et al.,* 2004), but intermediate lineages (lineage I/II) have also been reported (Sharma *et al.,* 2009). While all three lineages have been found in cattle, only lineage I and the intermediate lineage are typically associated with human disease (Sharma *et al.,* 2009). In a broad study, 102 isolates collected using rectal swabs from super-shedders in processing plants or feedlots revealed that these isolates were equally distributed among the three lineages (Arthur *et al.,* 2013). This result suggests that *E. coli* O157:H7 isolates from all three lineages can achieve the densities associated with super-shedders.

Other molecular markers have been used to differentiate between strains of *E. coli* O157:H7 based on their ability to cause disease in humans. A non-synonymous base change (either an A or T allele) at position 255 in *tir* (Bono *et al.,* 2007) was evaluated as this gene is an important virulence factor associated with the type III secretion system and plays a role in adherence of *E. coli* O157:H7 to intestinal epithelial cells (Kaper *et al.,* 2004). Their study revealed that more than 99% of 108 human isolates harboured the *tir* 255 T>A T allele and lacked repeat region 1-repeat unit 3 (RR1-RU3), whereas of 77 bovine isolates, the tir 255 T>A T allele and RR1-RU3 absence were found in 55% and 57% of the isolates, respectively. Interestingly, Arthur *et al.,* (2013) reported that isolates of *E. coli* O157:H7 from super-shedders harbored the T allele more often (71%) than the A allele (29%). Others have reported that *E. coli* O157:H7 isolates with the T substitution in *tir* are more commonly associated with human outbreaks and are more likely to cause disease in humans (Bono *et al.,* 2007). In another study that did not differentiate super-shedders from low shedders, isolates from cattle were shown to have equal distribution of *tir* A allele (54.8%) and *tir* T allele (45.2%), while isolates from humans were dominated by the *tir* T allele genotype (92.9%) (Franz *et al.,* 2012). The reason for the specific association of clinical isolates from humans with the T allele is unknown. Adherence to epithelial cells is a major function of the Tir protein; allele polymorphisms could affect adherence and consequently the likelihood of these isolates causing infection in humans (Bono *et al.,* 2007). As such, it is possible that the T allele polymorphism also plays a role in attachment of *E. coli* O157:H7 within the GIT of cattle, but may not be an absolute prerequisite for cattle to become a super-shedder.

Arthur *et al.* (2013) also examined difference in lambdoid bacteriophage insertion

sites between *E. coli* O157:H7 isolates from super-shedders and low-shedders. Shiga

toxins including Stx_1, Stx_{2a}, and Stx_{2c} are encoded by these bacteriophages (Melton-Celsa

et al., 1998) with Stx_1-, Stx_{2a}-, and Stx_{2c}-associated bacteriophages typically inserted

within or adjacent to conserved chromosomal loci *yehV*, *wrbA* or *argW*, and s*bcB*,

respectively (Shaikh and Tarr, 2003; Kotewicz *et al.,* 2008; Strauch *et al.*, 2008). The

Shiga toxin-encoding bacteriophage insertion (SBI) assay consists of six individual PCRs

that amplify the Shiga toxin genes and their corresponding genomic insertion sites

(Besser *et al.,* 2007). Arthur *et al.,* (2013) found that super-shedding isolates clustered

into 12 groups with more than half (55.9%) of the isolates belonging to SBI genotypes 1-

3. Interestingly, Besser *et al.* (2007) observed four different SBI genotypes among human

isolates from the United States, with 92.5% of all isolates being represented by genotypes

1 (30.1%) and 3 (62.4%), genotypes which corresponded to the isolates most commonly

obtained from super-shedders (Arthur *et al.,* 2013). This suggests that SBI genotypes

from super-shedders correspond to those SBI genotypes that are most often associated

with human illness. However, 48.8% of *E. coli* O157:H7 isolates from cattle of undefined

shedding status in the northwestern United States and western Canada exhibited SBI

patterns that were rarely found among human clinical isolates (Besser *et al.,* 2007).

The determination of SBI for a particular strain is not only dependent on where the

stx genes are inserted, but also which *stx* genes are present. Arthur *et al.* (2013) examined

the presence of these genes in isolates from super-shedders and found that they carried

stx_{2a} or stx_{2c} separately more frequently than they carried stx_{2a} and stx_{2c} together. The

Shiga toxin 1 gene was carried by half (51%) of the super-shedder strains while all

isolates from low shedders were found to possess the collective set of stx_{2a}, stx_{2c}, and stx_1 genes.

Finally, Arthur *et al*. (2013) examined the antiterminator Q gene alleles (Q_{933} and Q_{21}) in isolates from super- and low shedders. These alleles have been found to be distributed differently between human and bovine *E. coli* O157:H7 isolates (LeJeune *et al.,* 2004a) and may serve as a genetic marker for isolates associated with super-shedding. The Q_{21}/ Q_{933} assay targets two different allelic variations of the antiterminator gene Q upstream of the prophage stx_2 region (LeJune *et al.,* 2004a). Allele Q_{933} is responsible for a strong anti-terminator activity resulting in relatively high expression levels of stx_2, while allele Q_{21} represents a weak anti-terminator activity resulting in lower stx_2 expression (Wagner *et al*., 2001). The Q gene may indirectly affect the colonization of bovine GIT through differences in Shiga toxin expression. Previous research has shown increased levels of Stx2 enhances adherence of *E. coli* O157:H7 to intestinal tissues of cattle (Baines *et al.,* 2008). *Escherichia coli* O157:H7 strains harboring the Q_{933} variant of the anti-terminator gene produced significantly higher levels of Stx2 toxin than strains with the Q_{21} variant or strains harboring both Q_{933} and Q_{21} (LeJune *et al.,* 2004a; Ahmad and Zurek, 2006). Interestingly, 58.8% of super-shedding isolates examined by Arthur *et al.,* (2013) harboured the Q_{933}/ Q_{21} combination (18.6%) or Q_{21} alone (40.2%) suggesting that Q_{933} may play a role in enabling *E. coli* O157:H7 to be shed at super-shedder levels through increased Stx2 expression.

In 2001, Hayashi *et al*. reported the complete chromosome sequence of an O157:H7 strain isolated from the Sakai outbreak and compared it to a benign laboratory strain, K-12 MG1655. Results illustrated that O157:H7 strain carries 1.4 Mb of sequence that is

absent from the K-12 strain. Specifically, the *E. coli* O157:H7 chromosome encoded 1632 proteins and 20 tRNAs that were not present in the K-12 genome. Among these, at least 131 proteins were assumed to have virulence-related functions (Hayashi *et al.,* 2001). Similarly, Perna *et al.,* (2001) sequenced the *E. coli* O157:H7 strain EDL933 and compared it to K-12, identifying 1,387 genes that were specific to the EDL933 strain. With this in mind, isolates recovered from super-shedders may possess genetic traits that differ from those isolated from low-shedders. This same approach could be applied to compare protein annotations or expression levels of isolates from super-shedders and low-shedders and should be considered in future studies examining strain-to-strain differences. However, the lack of bovine intestinal cell lines could make it challenging to elucidate the role that gene expression may have in the establishment of isolates in cattle that lead to the super-shedder phenomenon.

1.5.2.2 Host factors contributing to super-shedding

Possible role of microbial imbalance in super-shedding

The incidence and shedding of *E. coli* O157:H7 is also affected by the nature and composition of the microbiome (i.e., bacteria, fungi and protozoa) within the host's GIT. The GIT microbial community is critical to host health and well-being and even minor changes in these populations are thought to cause dramatic shifts that affect livestock productivity (Callaway *et al.,* 2009). The microbiota associated with the bovine GIT not only produces necessary nutrients (i.e., vitamins and short chain volatile fatty acids; Flint, 2004; Flint *et al.,* 2007) but also has been implicated in the development of a healthy immune system and exclusion of enteric pathogens (Guarner and Malagelada, 2003). Recent studies examining cattle feces have revealed a high degree of variation among

bacterial community composition among individuals (Durso *et al.,* 2010; Shanks *et al.,* 2011). Xu *et al*. (2014) sequenced the bacterial 16S rRNA genes to characterize fecal bacterial communities in 11 super-shedders and 11 non-shedders that were fed the same high barley grain diet within a Canadian feedlot. Their dataset supported variations in microbiome composition between super-shedders and non-shedders. Interestingly, it was found that super-shedders exhibited higher bacterial richness and diversity and bacterial populations clearly clustered separately between super-shedders and non-shedders. In a related study by Dugat-Bony *et al.* (2013), a total of ~350,000 pyrotags were produced using pyrosequencing of 16S rRNA from digesta samples from the duodenum, jejunum (proximal, mid and distal), cecum, colon (descending and spiral) and RAJ, as well as epithelium scraped from the RAJ from 6 cattle (3 super-shedder and 3 *E. coli* O157:H7 negative). The bacterial communities for digesta samples taken from the small intestine (duodenum to jejunum) were not significantly different between super-shedders and cattle negative for *E. coli* O157:H7. In contrast, there were significant differences between the two study groups in their large intestine (i.e., cecum to the terminal RAJ) whereby as per the first study, super-shedders had more diverse communities than non-shedders (Dugat-Bony *et al.,* 2013). Both studies raise the possibility that the microbiomes in super-shedders are unique from non-shedders, whereby conditions are such that some strains of *E. coli* O157:H7 can proliferate to the super-shedder state. It is not known at this time if super-shedding is a cause or an effect of such microbial differences, but changes in microbial communities in super-shedders could potentially alter substrate utilization or end product production in a manner that makes this environment more favorable for the proliferation of *E. coli* O157:H7.

It is important to note that these results could also be related to differences in native lytic phage populations between low and super-shedders. In a recent study, Hallewell *et al*. (2014) examined the total prevalence of lytic phages among super-shedding ($\geq 10^4$ CFU/g feces) and low shedding ($<10^4$ CFU/g feces) steers. Interestingly, the total number of fecal samples positive for phage was higher in low shedders (20.9%) as compared to super-shedders (8.3%; *P*=0.004) suggesting that super-shedding may be influenced by the presence of lytic phage. Interestingly, T4-like phages were more frequently isolated from low shedders whereas O1 and T1-like phages were more frequently isolated from super-shedders (Hallewell *et al.,* 2014). Differences in the activity of lytic phage against *E. coli* O157:H7 could also contribute to sporadic shedding in super-shedders.

Mechanisms of competitive inhibition among *E. coli* strains (Riley and Wertz, 2002; Sawant *et al.,* 2011) could also result in differential proliferation of *E. coli* O157:H7 within the GIT of cattle. Some *E. coli* strains are able to inhibit the growth of other *E. coli* strains and shifts in *E. coli* populations could affect the proliferation of *E. coli* O157:H7 within the digestive tract of cattle (Reissbrodt *et al*., 2009). In recent years there has been increased interest in the use of probiotics including: direct-fed microbials, competitive-exclusion cultures, and prebiotics as a pre-harvest mechanism to reduce *E. coli* O157:H7 populations in cattle, a topic which has been reviewed extensively (Callaway, 2010; Callaway *et al.,* 2013). These modes of action, including epithelial site attachment, unfavorable intestinal fermentation profiles, production of metabolites, and/or bacterial antagonism, interrupt the cycle of transmission and colonization of *E. coli* O157:H7 in cattle and have been used as complementary interventions to improving

food safety at the live animal level. A closer examination of bacterial relationships among

E. coli may identify members of the intestinal microbiome that play a role in super-

shedding dynamics. For example, colicins are antimicrobial proteins that are produced by

bacteria and are toxic to some strains of *E. coli*, including *E. coli* O157:H7 (Callaway *et*

al., 2004; Schamberger *et al.,* 2004). They are produced by strains of *E. coli* carrying a

colicinogenic plasmid that bears the genetic determinants for colicin synthesis, immunity,

and release (Cascales *et al.,* 2007). At this point in time there has been no research

examining whether or not microbial populations within super-shedders exhibit

differences in the presence or absence of this plasmid, a possibility that could be

investigated in future research.

The innate and adaptive immune response of the host

Characterizing the host immune response in individuals that transmit zoonotic bacteria is

essential as differences in immune status may contribute to the heterogeneity of shedding

of *E. coli* O157:H7 within cattle. Furthermore, such studies could lead to the

identification of biomarkers that could be potentially associated with individual cattle that

are prone to becoming super-shedders.

Shiga toxins produced by *E. coli* O157:H7 do not cause physical symptoms in cattle

as cattle lack receptors for these toxins (Pruimboom-Brees *et al.,* 2000). However,

colonization of *E. coli* O157:H7 in the GIT elicits an innate immune response. The

profound alteration of intestinal cells associated with attachment and effacing lesions has

been reported to be accompanied by an increase in neutrophils and eosinophils in several

areas of the GIT (Dean-Nystrom *et al.,* 1997; Woodward *et al.,* 1999; Stoffregen *et al.,*

2004). Currently there is little evidence to suggest that *E. coli* O157:H7 are able to impact

the host immune response and influence changes in the microbial community. A study conducted by Nart *et al.,* (2008) investigated the pathological changes and associated immune responses in calves orally inoculated with 10^9 CFU of *E. coli* O157:H7 per animal. Samples of rectal mucosa were collected from three calves that shed *E. coli* O157:H7 at levels $\geq 10^4$ CFU/g of feces for at least 2 weeks. In these samples, IgA antibodies that bound to antigens on the surface of *E. coli* O157:H7 cells were detected. Furthermore, intestinal cells elicited a quantifiable neutrophilic response suggesting that those individuals that shed high numbers of *E. coli* O157:H7 experienced a pathological change within intestinal cells at the RAJ. These findings are important as they suggest that cattle with the potential to shed higher levels of *E. coli* O157:H7 may generate an increased number of antibodies against *E. coli* O157:H7. Such a response may also contribute to intermittent shedding of *E. coli* O157:H7, but it is not known if a similar response occurs in cattle that achieve super-shedding status under commercial production conditions.

1.5.2.3 The role of dietary factors and animal husbandry practices on super-shedding

It is well known that diet composition and feed processing impact the fecal shedding of *E. coli* O157:H7 (Callaway *et al.*, 2009; 2010). The bactericidal properties of ruminal and volatile fatty acids (VFA) impact the survival of intestinal *E. coli* populations (Wolin, 1969) and changes in diet that influence VFA production can influence shedding. For example, barley is more rapidly fermented in the rumen than corn, and studies have shown that barley-fed cattle shed more *E. coli* O157:H7 than those fed corn (Berg *et al.*, 2004; Buchko *et al.*, 2000). Likewise, compared to dry-rolling, steam flaking increases the ruminal fermentation of corn and the shedding of *E. coli* O157:H7 (Fox *et al.*, 2007).

Of the super-shedder studies in **Table 1.1**, only a few report the diet at the time of sampling. Most studies have identified super-shedders in cattle fed high-grain finishing diets (Stephens *et al*., 2009, Xu *et al*., 2014), and to our knowledge no studies have investigated the impact of diet on shedding in previously identified super-shedders. Higher shedding has been associated with cattle housed at high densities within feedlots as compared to low densities within pastures (Gunn *et al*., 2007). Feed additives including seaweed (Tasco-14) (Braden *et al*., 2004), orange peels, citrus pellets (Callaway *et al*., 2011) as well as ß-agonists (Edrington *et al*., 2006) and ionophores (Van Baale *et al*., 2004) have all been shown to effect the shedding of *E. coli* O157:H7. Further research is needed to determine if such additives or changes in feeding practices affect the prevalence or duration of super-shedding.

1.6 Strategies to control super-shedding cattle in the feedlot

The majority of mitigation practices developed to date have focused on preventing the adulteration of meat and other foods with *E. coli* O157:H7 during slaughter, processing and retail handling (Karmali *et al.,* 2010). Alternatively, targeting super-shedders for mitigation has been proposed as a pre-harvest means of reducing the incidence and spread of *E. coli* O157:H7 to pen-mates and the feedlot environment (Matthews *et al.,* 2006a,b). The primary limitation associated with this notion is the lack of an effective method for identifying super-shedders within a herd. If a simple method for identifying super-shedders could be developed, then pre-harvest mechanisms for the reduction of super-shedders may be applied including probiotics, vaccination, dietary shifts, and inclusion of other potential inhibitors such as essential oils, tannins and other phenolics. Several of these approaches to mitigating *E. coli* O157:H7 in cattle have been reviewed

in detail elsewhere (Stevens *et al.,* 2002; Loneragan and Brashears, 2005; LeJeune and Wetzel, 2007; Sargeant *et al.,* 2007; Callaway *et al.* 2013).

Bacteriophages have been assessed for their ability to control *E. coli* O157:H7 associated with human infection and contaminated processed foods and other agricultural products (Brüssow, 2005; Greer, 2005) due to their high specificity, ubiquity and ability to self-replicate in bacterial host cells (Sulakvelidze *et al.,* 2001). Phages have been used to reduce shedding of *E. coli* O157:H7 in artificially inoculated models that shed at super-shedding levels, though these were not natural super-shedders (Callaway *et al.,* 2008). Previous work has shown phages exhibited activity against the majority of bovine and human STEC O157:H7 isolates (Niu *et al.,* 2009); however, each specific phage may exhibit differences in host range and lytic capability (i.e., multiplicity of infection), a trait that could impede the effectiveness of phage therapy. These differences in host range and lytic capability, along with phage distribution may also account for the observed intermittent nature of super-shedding and shedding in general (Callaway *et al.,* 2003; Raya *et al.,* 2006). To account for these differences, Niu *et al.* (2009) reported that a four-phage cocktail is the logical approach to efficacious on-farm therapy.

Vaccines also have been developed as a means to reduce the amount of *E. coli* O157:H7 in cattle; however, because this organism is a natural resident within the intestinal tract of cattle vaccination can be difficult. Currently, vaccines have had variable results depending on the number of vaccinations required to elicit full immunity (Callaway, 2010). In an attempt to reduce this variability, vaccine production has specifically targeted aspects that influence the adhesion of *E. coli* O157:H7 to the intestinal epithelium or its metabolic activity. Many vaccines currently target proteins

involved in iron transport within the bacterium, or are crude bacterial extracts and require two or more immunizations to elicit a potent immune response (Callaway, 2010). There is no evidence to support that these vaccines would be more effective against super-shedders, especially if the super-shedder phenotype was triggered by the host. Furthermore, such vaccines may not be effective in super-shedders owing to the intermittent nature of this phenomenon or if super-shedding is associated with compromised intestinal immunity. However, Matthews *et al*. (2013) modelled the effect of vaccinating super-shedders and showed the risk of transmission of *E. coli* O157:H7 to humans could be reduced by 50% to 85% if the vaccine was effective. Although vaccines may reduce numbers of high-shedders of *E. coli* O157:H7 (Cull *et al.,* 2012; Stanford *et al.,* 2014), vaccination does not prevent cattle from becoming super-shedders (Stanford *et al.,* 2014). Development of next generation vaccines that target bacterial proteins that play an integral role in the development of the super-shedding state is warranted to advance this promising tool.

1.7 Conclusions and Hypothesis

Super-shedders are thought to play a key role in the dissemination and risk of adulteration of beef, and resultant human illnesses, but there is still no simple means to identify these individuals within a population of cattle. Evidence that super-shedders share a commonality with isolates associated with human illness, makes it more imperative that the etiology of this phenomenon be characterized. Recent studies have suggested that the super-shedding state is short lived and lacks continuity. Perhaps the sporadic nature of super-shedding is a result of intermittent sloughing of *E. coli* O157:H7 biofilms from the intestinal epithelium and their subsequent release into feces. This theory merits further

research so as to better understand the factors that trigger the mass release of *E. coli* O157:H7 into feces. Although there is regional evidence of strain specific characteristics of *E. coli* O157:H7 associated with super-shedding, these traits appear to be inconsistent within super-shedder isolates collected from various geographical locations. More research on the nature of microbial communities in the host is required to clarify the roles of bacterial richness and how the predator-prey relationship among bacteriophage and *E. coli* O157:H7 influence super-shedding. As well, a better understanding is required of the innate immune response specifically within super-shedders. Once these factors are better understood, improved methods for identifying and mitigating super-shedders within cattle populations may be developed.

Hypothesis 1: If the super-shedding phenomenon in cattle is short-lived and lacks continuity, then *E. coli* O157:H7 will be transiently detected when super-shedder cattle are repeatedly sampled over time.

Hypothesis 2: Genomic traits within some strains of *E. coli* O157:H7, including lineage, clade and phage type, as well as virulence gene profiles, enable them to achieve cell densities within the feces of cattle that meet the definition of the super-shedder state.

CHAPTER 2: Are Super-Shedder Feedlot Cattle Really Super?[2]

2.1 Introduction

Escherichia coli O157:H7 is a major Shiga toxin–producing foodborne pathogen that causes hemorrhagic colitis and hemolytic uremic syndrome among humans worldwide (Karmali, 2004). Healthy cattle transiently host *E. coli* O157:H7 in their gastrointestinal tract and can directly or indirectly transmit this pathogen to humans (Rangel *et al.,* 2005).

The load and frequency of *E. coli* O157:H7 shedding varies greatly among individual cattle (LeJeune *et al*., 2004b; Stanford *et al*., 2005a). Studies related to the prevalence of *E. coli* O157:H7 in cattle typically report shedding of the organism to be sporadic and of short duration (Besser *et al.,* 1997) ranging from 10 to 10^7 CFU/g feces (Chase-Topping *et al.,* 2008). The term "super-shedder" has been applied to cattle that are transiently high ($\geq 10^4$ CFU/g feces) shedders of *E. coli* O157:H7 (Matthews *et al.,* 2006a, b). Super-shedding cattle could potentially have a substantial impact on the on-farm prevalence and transmission of *E. coli* O157:H7 and the risk of adulteration of food products. Matthews *et al*. (2006a) and Omisakin *et al*. (2003) estimated that super-shedders accounted for 80 and 96% respectively, of the total *E. coli* O157:H7 shed into the environment by penned cattle.

Targeting super-shedders for mitigation strategies has been proposed as a means of reducing the incidence and spread of *E. coli* O157:H7 to pen-mates and the environment (Matthews *et al.,* 2006a,b). However, the duration and frequency of release of *E. coli* O157:H7 into the environment by super-shedders is unknown. Shedding profiles of *E.*

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[2] This chapter is an adapted version of the manuscript "Are super-shedder feedlot cattle really super?" 2014. Munns K.D., Selinger L., Stanford K, Selinger L.B. and McAllister T.A. 2014. *Foodborne Pathogens and Disease* **11:**329-331.

coli O157:H7 by cattle have been largely discussed in relation to point estimates within the farm or feedlot environment and often involve clustered sampling with high levels of variation (Robinson *et al*., 2009). More specifically, previous research related to super-shedding has evaluated its incidence at a single time point using a cross-sectional survey from multiple farms (Chase-Topping *et al.,* 2008). More recently, Stephens *et al*. (2009) examined the incidence of super-shedders in two commercial feedlots at two different periods (May-Aug and Sept-Jan) of the year. However, there is currently no research evaluating the extent to which the super-shedding state is maintained in individual animals through the collection of multiple samples over days or weeks.

The main objective of this study was to determine the frequency and duration of super-shedding by cattle through enumerating *E. coli* O157:H7 in feces of cattle identified as super-shedders. These cattle were also subjected to a variety of standard farm management practices including movement through a chute, mixing within pens and a change in diet to determine if these practices altered shedding status. In addition, *E. coli* O157:H7 isolated from super-shedding and low-shedding cattle were genetically characterized using pulsed-field gel electrophoresis (PFGE) and lineage typed to evaluate genetic relationships in an attempt to assess the similarity of *E. coli* O157:H7 from super-shedders and low-shedders.

2.2 Materials and Methods
2.2.1 Identification of super-shedder cattle.
All cattle used in this experiment were handled in accordance with the Canadian Council of Animal Care (1993) and the protocol was reviewed and approved by the Lethbridge Research Centre Animal Care Committee. Crossbred yearling feedlot steers (*n*=400), from a single commercial feedlot

in southern Alberta were sampled in July of 2011 to identify super-shedder cattle. Cattle from 4 pens (with an average of 103 steers per pen) were restrained in a chute, and a fecal grab sample collected from the rectum. All fecal samples were collected in sterile tubes, placed on ice and transported to the laboratory for analysis within 4 h. Fresh gloves were used for fecal collection from each steer. Eleven super-shedders (shedding $\geq 10^4$ CFU/g feces) were identified, purchased and transported (35 km) by truck to the Lethbridge Research Centre (LRC) as soon as enumeration was complete (4 days). Super-shedders were then housed individually in pens (14×15 m) at the LRC experimental feedlot.

2.2.2 Experimental design. The 34-d study involved an initial 6 d sampling period where steers were sampled twice daily by collecting freshly voided fecal pats 2 h before and 6 h after feeding from the pen floor. Five of the 11 steers were shipped for slaughter for detailed analysis of intestinal populations and collection of tissues throughout the digestive tract at days 4, 9 and 11 and the analyses associated with these samples are being undertaken by others in the laboratory. Steers were selected for slaughter based on those with the highest amount of *E. coli* O157:H7 After the first 6 d, three different management practices were implemented and included collection of fecal samples by restraining the remaining 6 steers in a chute once daily, penning the cattle together in a single pen (14×15 m) and finally, a sudden change in diet whereby the forage content of the diet was increased by 20% in a single step.

2.2.3 Salivary cortisol analysis. To determine if increased handling, mixing or a rapid diet change elicited a stress response in cattle, saliva samples were collected for analysis of cortisol as described by Cook *et al.* (1996). Saliva samples were collected using a cotton swab at the same time cattle were restrained in the chute for fecal sampling. After

the swab was saturated with saliva it was placed in a conical-shaped plastic centrifuge tube (15 mL), transported to the laboratory and frozen at -20°C. For analysis, samples were thawed at room temperature for 2 h and saliva was extracted from the cotton swabs by centrifugation at 1930×g for 20 min. Saliva samples (25 µL) were assayed in duplicate using the a high sensitivity salivary cortisol enzyme immunoassay kit (Salimetrics, LLC, State College, PA) according to the manufacturer's directions. The kit had a lower limit of sensitivity of 0.0007–1.8 µg/dL. Cortisol levels were analyzed using the MIXED procedure (SAS Institute, Inc. 2011) with treatment (management practice), day and their interaction as fixed effects in the model and steer as a random effect. Both treatment and day were treated as repeated measures.

2.2.4 Enumeration, enrichment, identification and isolation of *E. coli* O157:H7. *E. coli* O157:H7 was enumerated from thoroughly mixed fecal subsamples (>50 g) by serially diluting 1-g of feces into 9 mL of phosphate buffered saline (PBS) and plating 100 µL (in duplicate) dilutions ranging from 10^{-1} to 10^{-4} onto sorbitol MacConkey agar with 2.5 mg/L potassium tellurite and 0.05 mg/L cefixime (CT-SMAC; Dalynn Biologicals, Calgary, AB) and incubated at 37°C for 18 to 24 h. Colonies were enumerated using a colony counter (Reichert, Depew, NY) and steers identified with $\geq 10^4$ CFU of *E. coli* O157:H7/ g of feces were identified as super-shedders. Three representative nonsorbitol fermenting (NSF) colonies from each sampling point were confirmed to be *E. coli* O157:H7 using the *E. coli* O157:H7 latex test kit (Oxoid Ltd., Basingstoke, Hampshire, UK). Positive agglutination isolates were confirmed by multiplex PCR, whereby template DNA was prepared from a single colony suspended in sterile water and heat lysed. From this, 1 µL of the supernatant was used in PCR with

conditions described by Gannon *et al*. (1997) for the presence of genes specific to the

O157:H7 serotype (*vt*, *eaeA*, *fliC*).

When *E. coli* was not detectable by plating, duplicate 1 g subsamples of feces were

enriched in 9 mL of modified TSB (mTSB) containing novobiocin (20 mg/L; Sigma-

Aldrich Canada Co., Oakville, ON, Canada), bile salts (1.5 g/L; BD – Canada,

Mississauga, ON, Canada), dipotassium phosphate (1.5 g/L; Sigma-Aldrich Canada Co.)

and TSB (30 g/L; BD – Canada) and incubated for 6 h at 37°C. Enriched samples were

then subject to immunomagnetic separation (IMS) using anti- *E. coli* O157:H7

Dynabeads (Invitrogen, Carlsbad, CA) as per manufacturer's instructions. A 50 µL

aliquot of bead-bacteria complex was plated onto CT-SMAC (Dalynn Biologicals) and

incubated at 37°C for 18 to 24 h. Three NSF clear colonies were randomly selected for

latex confirmation and PCR as described above.

Three positive *E. coli* O157:H7 isolates were arbitrarily selected from CT-SMAC

medium from each steer per sampling time point either by direct plating or IMS and

stored in glycerol at -80°C until further analyzed.

2.2.5 Template DNA preparation and lineage typing. A total of 126 isolates (2 from

each positive time point) were recovered over the 34 d trial and lineage typed using

lineage-specific polymorphism (LSPA-6) assay. Lineage I (Sakai and EDL933) and

lineage II (Frik 2000 and ECL1717) strains were used as positive controls. To obtain

DNA template from isolates, 0.5 mL of overnight grown culture was heat lysed, and 1 µL

of the supernatant was used in PCR. The LSPA-6 assay was performed according to

Yang *et al*. (2004), but resolved on a 3% (wt/vol) Agarose A gel (Rose Scientific,

Edmonton, AB) and analyzed using AlphaEaseFC software (version 4.1.0, Alpha

Innotech Corp., San Leandro, CA). As previously described for the LSPA-6 assay, each of the alleles in the order *folD-sfmA, Z5935, yhcG, rtcB, rbsB,* and *arp-iclR* was assigned a number (Yang *et al.,* 2004). Strains showing the LSPA-6 genotype 111111 (lineage I at each of the six loci) were classified as ''lineage I,'' while those with LSPA-6 genotype 2222222 were ''lineage II.'' All deviations from the principle genotypes 111111 and 222222 were designated as intermediate LSPA-6 genotypes.

2.2.6 Pulsed-field gel electrophoresis genotyping. *E. coli* O157:H7 isolates (*n*=126) were PFGE subtyped using a standard protocol (Centers for Disease Control and Prevention, 2000). Genomic DNA was digested in agarose plugs with *Xba*I (Invitrogen, Burlington, ON), and resulting DNA fragments were resolved by electrophoresis using a CHEF DRII PFGE apparatus (BioRad, Mississauga, ON). The *Xba*I-digested *Salmonella enterica* serovar Branderup H9812 was used as marker. Comparison of digested profiles to identify PFGE subtypes and restriction endonuclease digestion pattern clusters (REPCs) was performed using BioNumerics software version 6.0 (Applied Maths, Saint-Martens-Latem, Belgium). Fingerprints were clustered using the Dice coefficient evaluated by the unweighted pair group method (Tenover *et al.,* 1995). A 1% position tolerance and a 0.5% optimization were used to place banding patterns in groups of REPCs, each with 90% or greater similarity. Isolates that were >90% related were considered highly related as described previously (Stanford *et al.,* 2005b).

2.3. Results

2.3.1 Frequency and duration of super-shedding. From sampling 400 cattle at the commercial feedlot, a total of 46 (11.5%) were identified as shedding *E. coli* O157:H7 of

which 11 (23.9%) of these cattle were super-shedders. *E. coli* O157:H7 counts for the super-shedders ranged from 1.5×10^4 to 6.5×10^7 CFU/g feces.

Once transported to the LRC feedlot, only 5 of the steers were identified as super-shedders over the first 5 days of the study and only one animal was a super-shedder after day 2 (**Table 2.1**). After day 5, *E. coli* O157:H7 counts greater than $\geq 10^4$ were not observed in any of the samples collected throughout the remainder of the trial. Even within the same steer, super-shedding was not consistent between fecal samples collected in the morning *vs.* afternoon. For example, on day 4, steer 274 shed 3.5 $\log_{10}$ CFU/g feces in the morning, but shed 8.0 $\log_{10}$ CFU/g feces in the afternoon (**Table 2.1**). Furthermore, steer 651 shed 4.1 $\log_{10}$ CFU/g in the morning on d 4, but was negative for *E. coli* O157:H7 during the afternoon. Steer 236 was identified as a super-shedder at the commercial feedlot, but was negative in all samples collected at LRC.

There was no evidence that the management practices introduced (increased handling, group penning or diet change) affected shedding of *E. coli* O157:H7 by steers. Actually, as the trial progressed less *E. coli* O157:H7 was shed by cattle and after day 14, *E. coli* O157:H7 was only detected after enrichment and IMS (**Table 2.1**). Cortisol analysis revealed no change in salivary cortisol levels as a result of management practices (**Figure 2.1**).

Table 2.1. *E. coli* O157:H7 detection (log10 CFU/g feces) from cattle throughout 34-day trial. Values $\geq$ 4.0 log10 CFU/g feces were considered super-shedders.

| | | | | | | | | | | | | | | | | | | | | | | Day | | | | | | | | | | | | |
| Steer | 0 | 4 | | 5 | | 6 | | 7 | | 8 | | 9 | | 10 | | 11 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 29 | 30 | 31 | 32 | 33 | 34 |
		AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM																		
																			Cattle handled					Cattle penned together							Cattle diet change			
219	**5.1**	+	-	3.2	-	-	-	-	-	-	-	-	-	2.7	-	-	-	+	+	+	+	-	+	-	-	-	-	-	-	+	-	-	3.2	-
222	**4.2**	-	-	-	3.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
236	**6.0**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
261	**5.1**	-	3.2	-	3.0	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	3.2	-	-	-
274	**6.7**	3.5	**8.0**	3.5	3.0	-	+	3.2	3.0	3.5	+	slaughtered																						
287	**5.4**	2.7	2.7	3.2	2.7	+	-	+	-	+	+	slaughtered																						
294	**5.8**	-	-	2.7	**4.5**	-	-	-	-	-	-	-	-	3.4	3.0	slaughtered																		
299	**7.8**	-	**4.4**	-	-	-	-	+	+	-	-	-	-	+	-	slaughtered																		
310	**7.5**	**5.8**	Slaughtered																															
342	**5.2**	-	-	3.3	-	-	-	-	-	-	-	+	-	+	3.5	3.7	-	+	+	-	-	-	-	-	+	-	-	-	-	+	-	-	3.3	-
651	**6.1**	4.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-	-

AM: 2 h before feeding; **PM:** 6 hrs after feeding; +: Positive for *E. coli* O157:H7 via immunomagnetic separation; -: no *E. coli* O157:H7 detected; **slaughtered**: animal was killed as part of a larger trial; **bold text** identifies super-shedding levels.

2.3.2 Genetic characterization

After lineage typing, 99.6% (125/126) of the isolates were lineage I (111111) with only

one isolate from steer 219 on day 4 (**Figure 2.2**) being classified as lineage type 211111.

There were three distinct PFGE REPC identified (A, B, C) with 97.6% (122/126) of the

isolates being members of one dominant subtype (REPC A; **Figure 2.2**). The dominant

REPC pattern remained constant during the transition from super- to low-shedding status.

Different PFGE subtypes were only briefly observed at d 4 and 22. Steer 219 shed REPC

C on d 4 however, this steer shed PFGE subtype REPC A (**Figure 2.2**) before and after d

4. Similarly, steers 342 and 651 shed REPC B on d 22 but shed REPC A up to d 21 and

again reverted to REPC A after d 22 (**Figure 2.2**). All duplicate isolates were

indistinguishable in terms of lineage type and PFGE profile and as a result only one

isolate per time point is depicted in **Figure 2.2.**

2.4 Discussion

The negative impact on public health, implications for the public's perception of beef and

its ability to restrict exports all make *E. coli* O157:H7 a critical problem for the beef

industry. Contamination of meat from a major southern Alberta meat processing facility

recently resulted in 17 confirmed human cases of *E. coli* O157:H7 infection and the

largest meat recall (>2400 meat items) in Canadian history (Bottemiller, 2012). Although

the cause of the contamination was not confirmed, it has been hypothesized that super-

shedders may have contributed to "high event days" that overwhelmed the critical point

control measures employed in processing plants. Presumably, if the population of

pathogens is kept below the cumulative capacity of in-plant interventions, adulteration
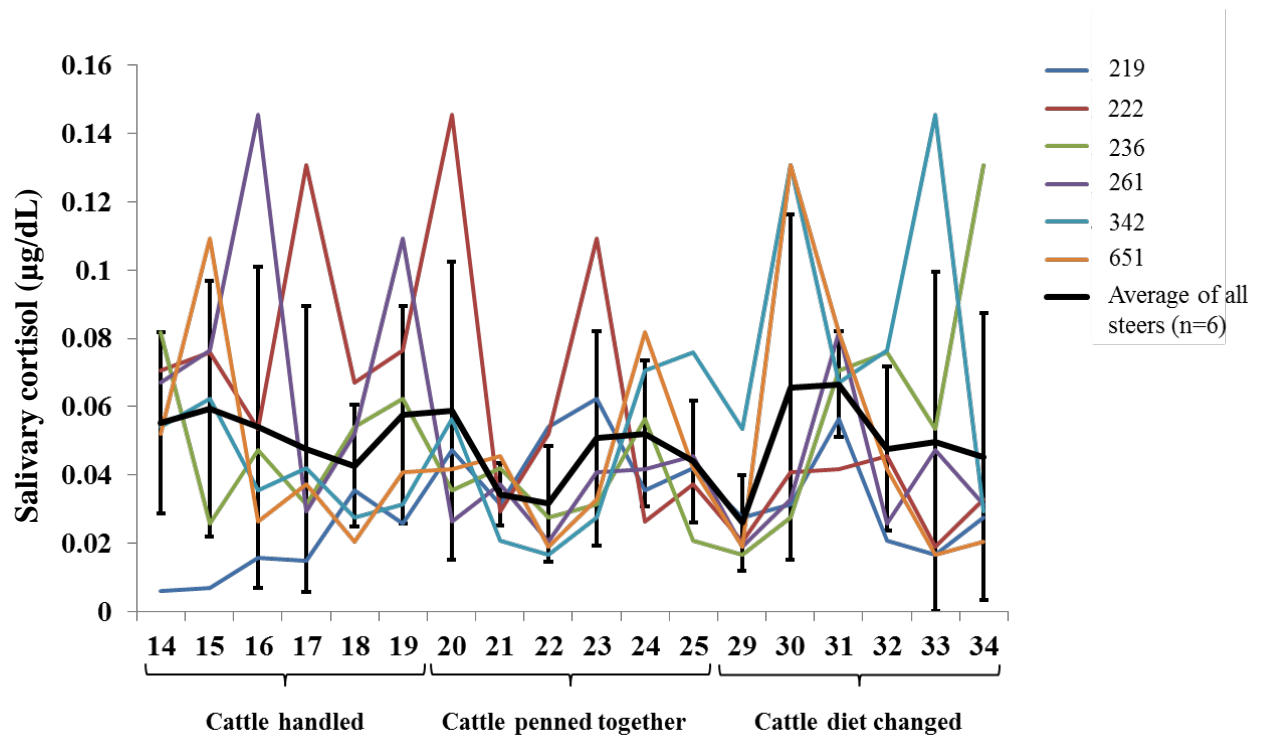
Figure 2.1. Individual and mean (±SEM) salivary cortisol levels from steers (*n*=6). Day 15-20 cattle were run through a chute system and sampled daily, day 21-26 cattle were penned together and run through a chute system and sampled daily, day 29-34 the diet was altered so that it contained 20% more forage, cattle were penned together and run through a chute system daily.
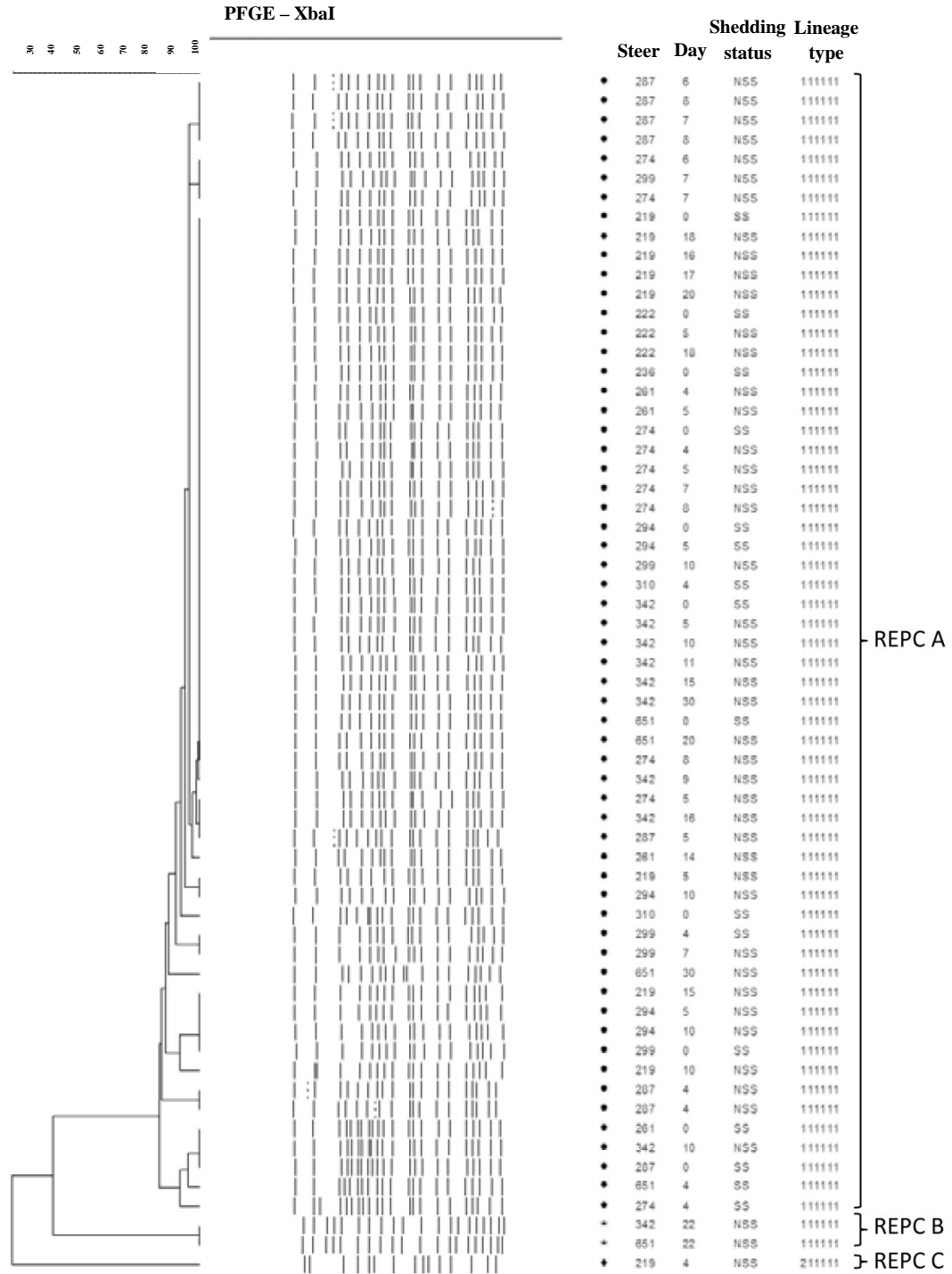
Figure 2.2. Lineage type and dendrogram of pulsed field gel electrophoresis (PFGE) profiles from *E. coli* O157:H7 isolates recovered within feces from steers over time. Like symbols represent strains that are >90% related.

of meat can be avoided (Arthur *et al.,* 2010), but if this threshold is exceeded

contamination can occur (Ahmadi *et al.,* 2007).

To date, researchers have sampled cattle bi-weekly (Cernicchiaro *et al*., 2010;

Jacob *et al*., 2010), weekly (Cristancho *et al*., 2008) and twice weekly (Cobbold *et al*.,

2007) to assess the persistence of *E. coli* O157:H7. More intensive sampling has been

carried out by Robinson *et al.* (2009) whereby levels of *E. coli* O157:H7 in feces of

naturally infected weaned calves (*n*=14) were sampled every three hours over five days.

In that study, it was found that variation within-animal was greater than between animals

over time. Although, the intensive sampling of Robinson *et al.* (2009) did not take into

consideration whether cattle were super-shedders before or during the time of sampling,

results from their study suggests that all cattle may have the potential to be super-

shedders at certain points in time and that limited samples from the same animal may be

misleading when determining shedding patterns. Consequently, the pattern or duration of

shedding in super-shedders has not previously been characterized with repeated sampling

from the same animal over several consecutive days. It is important to note that levels of

E. coli O157:H7 within feces has been shown to be a stochastic process by which

variability has been observed within a single fecal pat from the same animal (Echeverry

et al., 2005). With this in mind, our ability to define a steer as a super-shedder is a

function of our sampling methodology. In this study, enumeration of *E. coli* O157:H7

occurred from >50-g fecal sample which was thoroughly mixed before a 1-g subsample

was used in subsequent dilutions. Others have identified super-shedding cattle from far

less fecal matter whereby Arthur *et al.* (2013) enumerated *E. coli* O157:H7 using fecal

swabs with <1g fecal mass. The type of methodology may not reflect the most

appropriate representation of *E. coli* O157:H7 as Cobbold *et al.* (2007) has previously

determined that the amount of fecal mass collected per swab to be an average of 0.24 g.

Fecal pats and fecal grab sampling was adopted in our study in order to depict the

magnitude of *E. coli* O157:H7 observed within fecal samples as this level is important to

comment on the potential dissemination of this pathogen into the environment.

In our study super-shedding was a short-lived isolated event and was not

consistent even within the same animal over the course of a day. Furthermore, the

prevalence of super-shedders during their selection from pens of commercial feedlot

cattle was 2.8% as compared to a previous study conducted by our group where it ranged

from 0.45% to 7.1% (Stephens *et al.,* 2009). However, others have found that as many as

22.6% of the cattle examined were super-shedders, with 38/168 steers shedding *E. coli*

O157:H7 at levels of 10^4 CFU/g or higher (Cernicchiaro *et al.,* 2010). A number of

factors other than the intermittent nature of shedding of *E. coli* O157:H7 by feedlot cattle

could contribute to this variability including the feedlot selected, the sampling season or

even year-to-year differences in pathogen prevalence. Seasonality has been shown to play

a role with *E. coli* O157:H7 being more prevalent during summer than winter (Ferens and

Hovde, 2011), an observation that may account for the high numbers of *E. coli* O157:H7

in our samples collected in July and August. Additional studies should be conducted

during the summer and winter months to determine if these super-shedding trends remain

seasonally consistent from year-to-year.

It is imperative to identify how long an animal sheds at the super-shedder level in

order to extrapolate how much these animals are contributing to environmental

contamination. By relating bacterial counts to transmission, and fitting dynamic

epidemiological models to prevalence data from a cross-sectional survey of cattle farms in Scotland, it was proposed that 80% of the transmission of this pathogen arose from 20% of cattle that are shedding at high levels (Matthews *et al.,* 2006a). It is important to note that the conclusions derived from this analysis from a Scottish cattle population may not be relevant to other geographic locations. Chen *et al.* (2013) recently demonstrated that the prevalence of super-shedders is similar to that observed in cross-sectional field data and that both within-host and between-host variability can generate super-shedders. In southern Alberta, Stephens *et al*. (2009) found that super-shedders accounted for as little as 9.9% of the *E. coli* O157:H7 shed in pens of commercial feedlot cattle and that only 1 of 153 steers identified as a super-shedder retained this phenotype in a second sampling at shipment to slaughter (Stanford *et al.,* 2012). The results of the present study further suggest that super-shedding is likely a transient phenomenon in the majority of cattle that achieve this shedding status. However, it is important to note that these observations were observed in only 11 cattle and further research is warranted to determine if super-shedding is sporadic amongst other cattle.

Previous research has examined if stressors such as transport (Cole *et al.,* 1988; Bach *et al.,* 2004), mixing and handling of cattle (Brown-Brandl *et al.,* 2009), and dietary changes (Hovde *et al.,* 1999; Fox *et al.,* 2007; Callaway *et al.,* 2009) increase the number of *E. coli* O157:H7 shed. In the present study, handling, mixing and an increase in dietary forage concentration did not increase the prevalence or levels of *E. coli* O157:H7 shed by steers. Additionally, one time transportation did not increase shedding of *E. coli* O157:H7 as only 2/11 steers were identified as super-shedders immediately upon arrival at the research feedlot. Previous research has demonstrated that transport of cattle increased the

shedding of *E. coli* O157:H7 (Bach *et al.,* 2004); however, other studies have shown that transportation of cattle does not affect levels of *E. coli* O157:H7 (Minihan *et al.,* 2003; Schuehle Pfeiffer *et al.,* 2009; Stanford *et al.,* 2011a). Minihan *et al.* (2003) suggested that increased defecation during transport and lairage may reduce the levels of *E. coli* O157:H7 in feces, but research to support this contention has not been undertaken.

In commercial feedlots, as many as 200 head of cattle are housed within a single pen. In this study, penning the steers together was intended to increase competition for feed resources and promote establishment of a dominance hierarchy, factors that could have induced a physiological response that promoted shedding. Increased transmission of super-shedding strains of *E. coli* O157:H7 among pen mates was also another possibility. Vidovic and Korber (2006) reported a significantly greater prevalence in *E. coli* O157:H7 in feedlot cattle that were housed at high density (13.6-17.3 m^2/head cattle) as compared to those housed at low density (33.2 and 27.8 m^2/head cattle). Previous research reported social grooming of hides as an important source of transmission of the organism among penned cattle (McGee *et al.*, 2004; Stanford *et al.,* 2011b). However, in the present study penning the cattle together did not increase the shedding of *E. coli* O15:H7 even though all individuals had been previously identified as super-shedders. It was originally hypothesized that upon penning the cattle together we would observe increased levels of *E. coli* O157:H7 most likely due to transmission from super-shedder to low-shedder cattle. However, steers were shedding at IMS detectable levels ($<10^2$ CFU/g feces) at the time of penning, therefore potential transfer of this pathogen from super-shedder to low-shedder steers among pen mates as observed by others (Stanford *et al.*, 2011b), was likely reduced. It is also important to note that transmission between cattle may have been

occurring, but would be indistinguishable if steers were shedding the same PFGE type. Another hypothesis may be that the steers in this study have built up "immunity" to this PFGE type during super-shedding and after clearance the establishment of this super-shedding strain type was no longer achievable.

Cortisol analysis revealed no change in salivary cortisol levels as a result of management practices, suggesting that more severe management practices (e.g., weaning, dehorning, and castration) may have been required to induce a significant stress response. Similar to our findings, Kenny and Tarrant (1987) investigated the effects of re-penning, motion, social mixing, and the combination of mixing and motion on behavioral and physiological responses of yearling bulls. The act of moving animals from one pen to another had little effect on cortisol (**Table 2.1**).

There have been conflicting reports in the literature related to whether or not high concentrate diets or forage-rich diets affect *E. coli* O157:H7 shedding patterns in cattle (Diez-Gonzalez *et al*., 1998; Hancock *et al*., 2000). When cattle were fed a forage diet of 70% prairie hay and 30% alfalfa hay, *E. coli* O157:H7 was shed for 60 d compared to 16 d for cattle fed an 80% concentrate based diet (Van Baale *et al*., 2004). Alternatively, other studies have demonstrated that calves consistently shed higher concentrations of *E. coli* O157:H7 when fed high concentrate diets (Tkalcic *et al.,* 2000). In the present study, there was no effect of diet on the level of *E. coli* O157:H7 shed which may be a reflection of the low shedding levels at the time of diet change. Furthermore, it may be a function of a clearing mechanism within the gastrointestinal tract or a "shedding curve" that, by time the diet change was introduced shedding of *E. coli* O157:H7 was complete. Moreover, a

20% forage increase may have been insufficient or imposed for too short of duration (6 d) to elicit a change in the shedding of *E. coli* O157:H7.

2.4.1 Genetic characterization. Octamer-based genome scanning revealed three distinct *E. coli* O157:H7 lineages based on the lineage specific polymorphism assay (Kim *et al.,* 1999; Kim *et al.,* 2001). Typically, two predominant lineages have been described, lineages I and II (Kim *et al.,* 2001; Yang *et al.,* 2004), but intermediate lineages have also been reported (Sharma *et al.,* 2009). While all three lineages have been found in cattle, only lineages I and the intermediate lineage are typically associated with human disease (Sharma *et al.,* 2009). In this study, all isolates were lineage I (99.2%) or lineage I/II (0.8%), suggesting that cattle were shedding *E. coli* O157:H7 that could be associated with human illness. In a broader study, 102 isolates were collected by a single rectal swab of super-shedding cattle in commercial processing plants or commercial feedlots and revealed representatives (36% I/II; 29% I, 35% II) of all three lineages (Arthur *et al.,* 2012). The fact that super-shedders in the present study shed a lineage associated with human disease would make them particularly desirable targets for mitigation strategies that limit the shedding of *E. coli* O157:H7.

Subtyping using PFGE revealed that the majority of the isolates collected in our study were clones. This may reflect the fact that all of our cattle were originally identified as super-shedders from a single feedlot on the same day, albeit from four separate pens. In this study, we examined only three isolates from each steer at each sampling point, but we did not isolate different lineages or PFGE patterns from the same animal at the same time. Others have proposed that super-shedding is a function of a singular strain type

(Arthur *et al.,* 2012) and that "clonal dominance" plays a role in the etiology of *E. coli* O157:H7 within cattle herds (Atwill *et al.,* 2012).

Sequence differences in *E. coli* strains occur as a result of the insertion and deletion of horizontally acquired genomic 'O' islands, as well as the acquisition of plasmids (Schmidt and Hensel, 2004). Pulsed-field gel electrophoresis was used to characterize and identify differences in the genotype of super-shedder strains of *E. coli* O157:H7 as it has been employed to evaluate genetic relationships and the transmission of *E. coli* O157:H7 through the beef supply chain (Avery *et al.,* 2004; Dodd *et al.,* 2010). Results from this study demonstrated that there was a high degree of relatedness among *E. coli* O157:H7 isolated on a single day from super-shedder cattle in separate pens within a feedlot. Previous research examining more than one farm with fecal samples collected over a period of months has shown greater diversity among recovered PFGE strain types from super-shedders. Specifically, Stanford *et al*. (2012) recovered a total of 85 restriction endonuclease digestion clusters (90% or greater similarity) and 86 unique isolates (< 90% similarity) among super-shedders (*n*=153) and low-shedders (*n*=496) sampled at two commercial feedlots at two different times of the year. For 77% of super-shedders, PFGE profiles were distinct from low-shedder isolates collected in the same pen. Furthermore, in this study only 9.5% of super-shedders had fecal isolates in the same genetic cluster at both samplings points, suggesting that a single subtype was not responsible for the super-shedding phenomenon across time. This temporal dynamic suggests that super-shedding may not be an isolated occurrence, but a dynamic event with more than one *E. coli* O157:H7 subtype being shed at high levels by individual cattle

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during the feeding period. However, at a single point in time within the same feedlot a single dominant subtype may be responsible for the super-shedding phenotype.

2.5 Conclusions

In conclusion, this study suggests that super-shedding may not play as great a role in transmission and contamination within the feedlot environment as has been previously proposed (Omisakin *et al*. 2003; Matthews *et al*., 2006a), as the super-shedding state is short lived and lacks continuity. Furthermore, there was a high degree of relatedness among *E. coli* O157:H7 isolated from super-shedder cattle within a single feedlot and this genotype was isolated as cattle transitioned from super- to low-shedders. This may imply that no *E. coli* O157:H7 subtype-specific components are necessary for the development of super-shedding. Alternatively, our results may suggest that there may have been a unique factor that contributed to "subtype dominance" and the super-shedding phenomenon. Super-shedding is simply a measurement of bacterial density within the host and as a result factors within the host may enable the increased level of shedding at any given time. Additional elements such as host genome and the intestinal microbiome should be investigated to gain a better understanding of the factors that contribute to super-shedding in order to develop comprehensive intervention strategies.

CHAPTER 3: What causes super-shedding: Comparative genomic analysis of

Escherichia coli **O157:H7 isolated from super-shedder and low-shedder cattle**

3.1 Introduction

Escherichia coli O157:H7 is a major Shiga toxin–producing foodborne pathogen that poses a serious public health concern and economic problem worldwide. Healthy cattle are a primary reservoir of *E. coli* O157:H7, hosting the pathogen within their gastrointestinal tract (GIT) and shedding the organism into the environment via their feces. The load and frequency of *E. coli* O157:H7 shedding varies greatly among individual cattle (LeJeune *et al.,* 2004b; Stanford *et al.,* 2005a). Previous reports have reported shedding of the organism to be sporadic and of short duration (See Chapter 2; Besser *et al.,* 1997) ranging from 10 to 10^7 CFU/g feces (Chase-Topping *et al.,* 2008). Cattle that shed the organism at levels $\geq 10^4$ CFU/g feces have been termed "super-shedders" and are reported to have a substantial impact on the on-farm prevalence and transmission of *E. coli* O157:H7 and the risk of adulteration of food products. Matthews *et al.* (2006a) and Omisakin *et al.* (2003) estimated that super-shedders accounted for 80 to 96%, respectively of the total *E. coli* O157:H7 shed into the environment by cattle. More recently, Arthur *et al.* (2009) showed that 95% of the feedlot pens that contained at least one super-shedder had cattle with hide prevalence of >80% *E. coli* O157:H7, whereas only 29% of pens without a super-shedder exceeded this level of hide prevalence.

Targeting super-shedders for mitigation strategies has been proposed as a means of reducing the incidence and spread of *E. coli* O157:H7 to pen-mates and the environment (Matthews *et al.,* 2006b). However, the specific factors responsible for

super-shedding are unknown, but are presumably mediated by the characteristics of both the bacterium and the animal host (see Chapter 1). Specifically, Arthur *et al.* (2013) suggested that four components play a role in the super-shedding phenotype: the host genotype, the host phenotype, the intestinal microbiome, and the strain-specific characteristics of the *E. coli* O157:H7. Strain-specific phenotypic and genotypic differences have been noted in *E. coli* O157:H7 recovered from super-shedders as compared to *E. coli* O157:H7 from low shedders cattle. These differences include phage type (see Chapter 2; Chase-topping *et al.,* 2007), degree of clonal relatedness (Stanford *et al.,* 2012), *tir* polymorphisms (Arthur *et al.,* 2013), presence of stx_{2a} and stx_{2c} and antiterminator Q gene alleles (Arthur *et al.,* 2013). These reports have examined differences in specific genomic aspects of *E. coli* O157:H7 strains, but have not undertaken a comparative analysis of whole genomes.

The objective of this study was to determine if there are genetic differences between *E. coli* O157:H7 isolates obtained from super-shedder and low-shedder cattle using a comparative whole genomic approach. Phenotypic differences in carbon utilization and pH sensitivity were also examined in an attempt to identify factors that provide insight into potential mitigation strategies.

3.2 Materials and Methods

3.2.1 *E. coli* O157:H7 isolate collection, enumeration, characterization and selection
E. coli O157:H7 isolates (*n*=10) were selected from two larger studies. The first being the study of Stephens *et al.* (2009) whereby fecal samples were obtained from cattle (*n*=1987) within 11 pens in two commercial feedlots, with an average of 181 steers per pen. The feedlots were both located in southern Alberta and were separated by a distance

of 62 km. The second study was conducted as described in Chapter 2, whereby crossbred yearling feedlot steers (*n*=400), were sampled from a single commercial feedlot in southern Alberta. In both studies a fecal grab sample (50 g) was collected from the rectum. All fecal samples were collected in sterile tubes, placed on ice and transported to the laboratory for analysis within 12 h. Fresh gloves were used for fecal collection from each steer within each study.

Enumeration and confirmation of *E. coli* O157:H7 was carried out by standard methods as described by Stephens *et al.* (2009) and within Chapter 2. Super-shedders were defined as cattle that had $\geq 10^4$ CFU *E. coli* O157:H7/g feces, while low-shedders had $<10^4$ CFU/g feces. A total of 658 *E. coli* O157:H7 isolates were collected during the first study and 126 during the second study. Collected isolates were from both super-shedder and low-shedder cattle.

Isolates were subjected to pulsed field gel electrophoresis (PFGE) as described in Chapter 2 as well as lineage typing using the lineage-specific polymorphism (LSPA-6) assay as outlined by Yang *et al.* (2004) and as described in Chapter 2.

Finally, all isolates (*n*=784) were tested for resistance to antimicrobials using anti-microbial disk susceptibility testing according to the Clinical and Laboratory Standards Institute (CLSI, 2006). Eleven antibiotic discs were applied using a 12-place BBL Sensi-Disc™ disc dispenser (VWR, Intl., Edmonton, AB). The antimicrobials examined were of human and veterinary importance and included enrofloxacin (5 μg), streptomycin (10 μg), amoxicillin/clavulanate (20/10 μg), ceftiofur (30 μg), ampicillin (10 μg), sulphmethoxazole/trimethoprim (23.75/1.25 μg), ceftazadime (30 μg), oxytetracycline (30 μg), neomycin (30 μg), florfenicol (30 μg) and sulfasoxazole (0.25 μg). *E. coli* ATCC

strain 25922 and *Enterococcus faecalis* ATCC 29212 were used as controls in accordance with CLSI guidelines (CLSI, 2006). Zone diameters were measured after 18 h incubation at 37°C using a BIOMIC® V3 digital imaging system and software (Giles Scientific Inc., Santa Barbara, CA).

Upon completion of this genetic and phenotypic characterization a subset of 10 isolates was selected based on degree of relatedness (52.7%-100% based on PFGE), antimicrobial resistance profiles (ranging from susceptible to resistant ≤ 4 antimicrobials), lineage type [lineage I ($n=4$), lineage II ($n=4$) and lineage I/II ($n=2$)] and whether or not they were recovered from super-shedder or low-shedder steers at the time of sampling (**Figure 3.1**). Isolates were further characterized using the Biolog system (Biolog, California), phage typed and subject to whole genome sequence analysis.

3.2.2 Biolog analyses and phage typing

The phenotypic profile of isolates ($n=6$) recovered from super-shedders were compared to those of low-shedders ($n=4$) to determine differences in carbon utilization and pH sensitivity using Omnilog phenotypic microarrays (PM); PM1, PM2 for carbohydrate utilization and PM10 for pH responses (Biolog, Hayward, CA). The Biolog microarrays were carried out according to the manufactures instructions. Briefly, isolates were grown on blood agar overnight at 37°C. Colonies were picked with a sterile cotton swab and re-suspended in 10 mL IF-0a medium (Biolog), and cell density was adjusted to an OD_{600} of 0.035 using a spectrophotometer (Biolog Turbidimeter). An aliquot of 600 μL of this suspension was added to 120 mL of IF-10a medium (Biolog), and 96-well microtiter plates were inoculated with 100 μl/well of this final suspension. The plates were incubated for 48 h in the Omnilog incubator reader, and data were analyzed using the
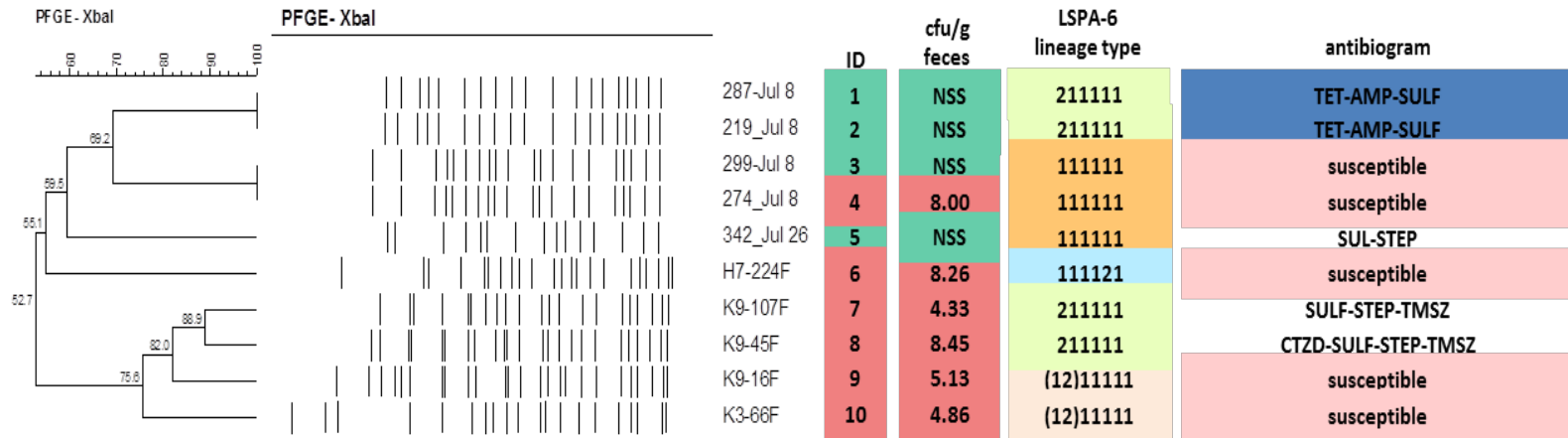
Figure 3.1. Characteristics of isolates selected for whole genome sequencing based on, PFGE relatedness, shedding status, lineage type and antibiogram. NSS: low-shedding; number indicates log CFU g/feces; AMP: ampicillin, 10µg/mL; CTZD: ceftazidime, 30µg/mL; susceptible: no resistance; TMSZ: sulphmethoxazole/trimethoprim (23.75/1.25 µg/mL; STEP: streptomycin, 10µg/mL; SUL: sulfasoxazole, 0.25µg/mL; SULF: sulfisoxazole, 10µg/mL; TET: tetracycline, 30µg/mL

Kinetic Plot and Parametric modules of the Omnilog Phenotype Microarray software suite. Additional statistical analysis was carried out using the "omp" package (Vaas *et al.,* 2013) for R (R Development Core Team, 2014) to identify differences between super-shedder and low-shedder isolates based on the area under the curve (AUC) analysis using ANOVA with significance declared at *P*<0.05.

Isolates (*n*=10) were phage-typed (PT) at the *E. coli* Reference Laboratory of the Laboratory for Foodborne Zoonoses, Guelph, Ontario, using previously described procedures (Ahmed *et al.,* 1987; Khakhria *et al.,* 1990) and 16 phages (numbered 1–16) that differentiated 88 PT.

3.2.3 DNA sequencing, single nucleotide polymorphism (SNP) and clade typing analysis

Genomic DNA was extracted from each *E. coli* O157:H7 isolate (50-ng samples) and prepared for sequencing using the Blood & Cell Culture DNA Maxi Kit (Qiagen, Valencia, CA). Samples were then run on a sequencer (MiSeq, Illumina, San Diego, CA) for paired-end 100-bp sequencing. The FASTX-Toolkit was used to filter low quality reads. Paired-ends Illumina sequencing reads were then assembled into contigs using the Velvet 1.1.06 *de novo* assembler with a kmer length of 49 for all 10 strains. Clean Illumina reads were mapped to the genome of reference strain (EC4115) using Burrows-Wheeler Aligner software packages (Li and Durbin, 2010). Sequence Alignment/Map (SAM) tools (http://samtools.sourceforge.net) were used to split, sort and merge the aligned result, and picard-tools were used to sort the binary sequence alignment data. The Genome Analysis Toolkit (GATK; McKenna *et al.,* 2010) was employed for base quality score recalibration, insertion and deletion (indel) realignment, duplicate removal, and

SNP and INDEL discovery. In-house perl scripts were developed to select SNPs from the GATK output. Single nucleotide polymorphisms loci were annotated by using EC4115 as a reference strain. For comparative purposes, we concatenated all SNPs to create a genotype for each isolate and Circos (2009) was used to visualize SNP distribution within compared genomes.

Clade typing was carried out on the basis of SNPs or combinations of SNPs specific for individual clades according to Manning *et al.* (2008). Additional comparative genomics core and accessory genomic regions analyses were carried out using Panseq (http://lfz.corefacility.ca/panseq/; Laing *et al.,* 2010).

3.3 Results and Discussion

3.3.1 Sequencing and Comparative Genomics

Paired-end 100 bp sequencing yielded 29-30× coverage, and generated 231 to 368 contigs per isolate, representing coverage of 94.1 to 96.6% of the total predicted bases within genomes. An average of 1600 SNPs were identified when the sequenced strains were compared to strain EC4115 which originated from a multistate US outbreak in 2006 (Eppinger *et al.,* 2011) (EC4115; **Figure 3.2**).

The phylogenetic tree based on SNPs differences did not support genetic segregation between isolates from super-shedders *vs.* low-shedders (**Figure 3.3**). The generated tree also contained 26 Genbank reference strains representing isolates obtained from human clinical cases and cattle. Interestingly, strain 342_Jul28, a low-shedder isolate, clustered more closely with isolates of bovine as compared to human origin. The other nine isolates were more closely associated with clinical outbreak strains. This suggests that isolates from super-shedders may be genetically more similar to isolates

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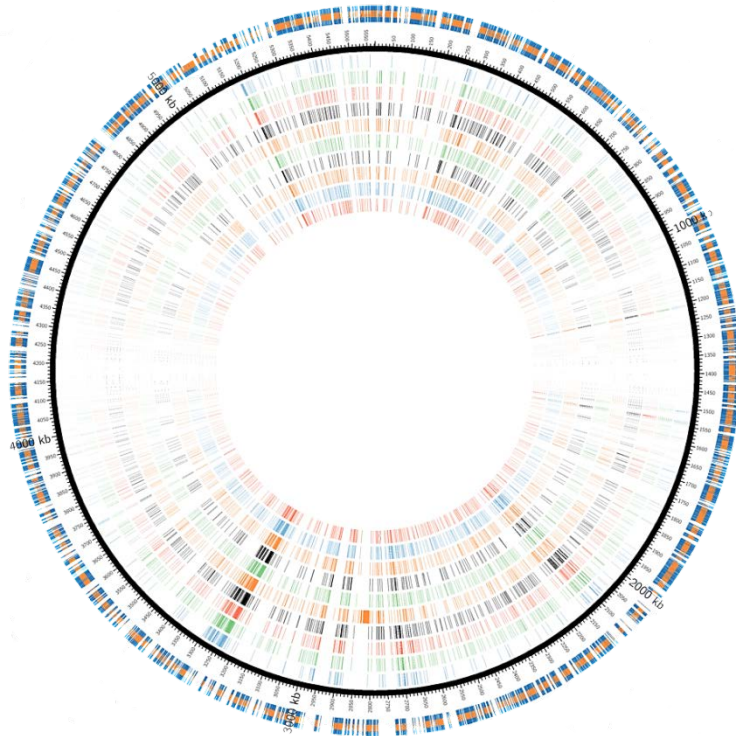
Figure 3.2. Single nucleotide polymorphisms observed within selected isolates (*n*=10) as compared to references stain *E. coli* O157:H7 EC45115 (outer ring). Isolates starting from inner circle are: 287-Jul8 (NSS), 342_Jul28 (NSS), 219_Jul8 (NSS), K9-45F (8.45 log CFU/g), H7-224F (8.26 log CFU/g), 274 (8.00 log CFU/g), K9-16F (5.13 log CFU/g), 299-Jul8 (NSS), K9-107F (4.33 log CFU/g) and K3-66F (4.86 log CFU/g). Intensity of bands depicts frequency of SNPs.
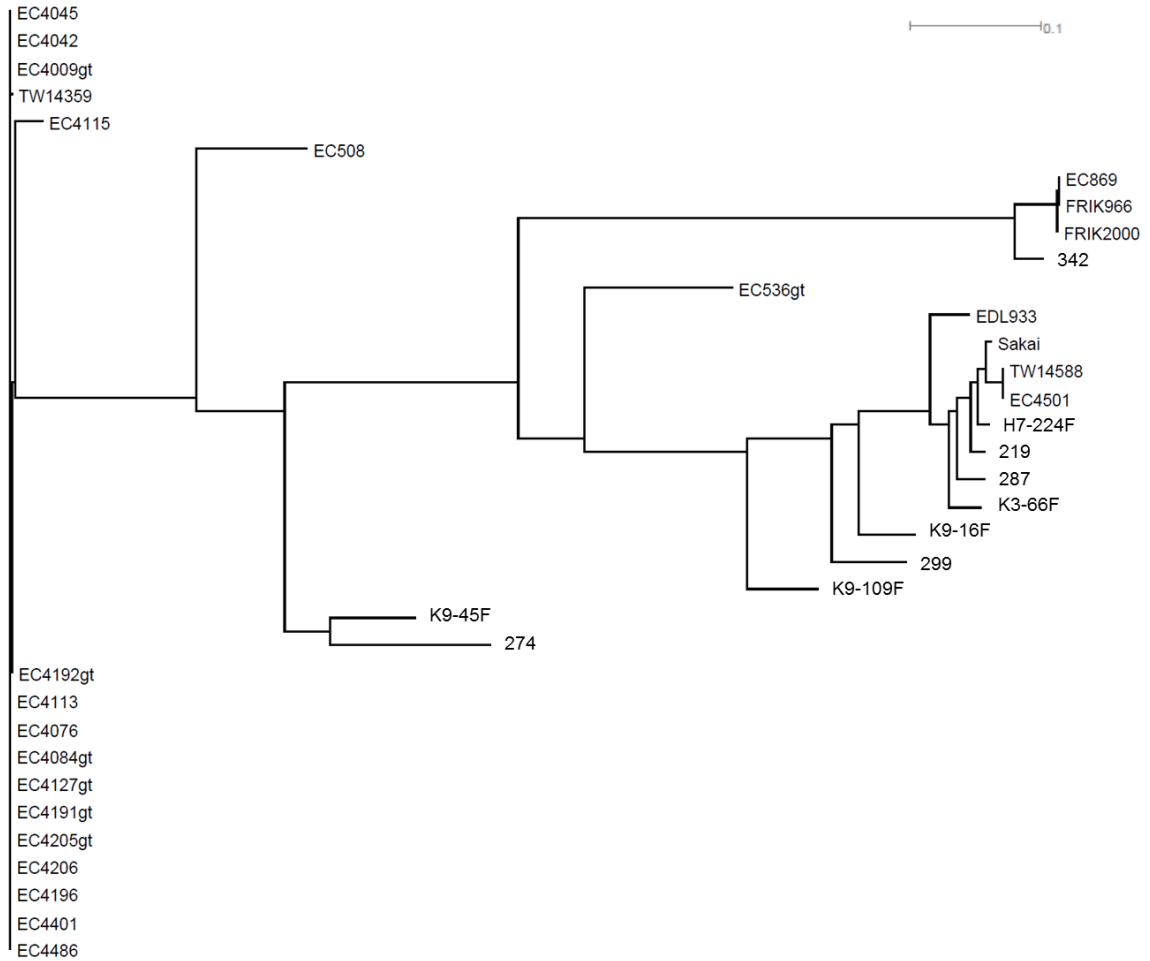
Figure 3.3. Single nucleotide polymorphism based phylogenetic tree of *E. coli* O157:H7. The phylogenetic tree is based on ~1600 intra- and intergenic SNPs identified in *E. coli* O157:H7 isolates from this study (*n*=10) including: 287-Jul8 (NSS), 342_Jul28 (NSS), 219_Jul8 (NSS), K9-45F (8.45 log CFU/g), H7-224F (8.26 log CFU/g), 274 (8.00 log CFU/g), K9-16F (5.13 log CFU/g), 299-Jul8 (NSS), K9-107F (4.33 log CFU/g) and K3-66F (4.86 log CFU/g) and 26 reference *E. coli* O157:H7 GenBank strains

associated with human illness. Arthur *et al.* (2013) recently reported that isolates from super-shedders tended to share more traits in common with isolates collected from humans than isolates collected from cattle with respect to lineage and sequence variation in the *tir* allele. Cote (2013) revealed a clustering of one super-shedder isolate SS17 with the lineage I/II *E. coli* O157:H7 isolates (TW14359 and EC4115) associated with an outbreak caused by adulteration of spinach, after whole genome comparative analysis.

Panseq analysis did not identify differences in the absence or presence of 480 pathogenicity-related alleles between super-shedder and low-shedder isolates (data not shown). Some of these alleles have previously been used to differentiate the pathogenicity of strains of *E. coli* O157:H7 associated with human illness. Bono *et al.,* (2007) evaluated a non-synonymous base change (either an A or T allele) at position 255 and nucleotide repeat polymorphisms at other locations (presence or absence) in the *tir* gene in an attempt to use these differences to delineate isolates from humans vs cattle. This gene is an important virulence factor associated with the type III secretory system and plays a role in adherence of *E. coli* O157:H7 to intestinal epithelial cells (Kaper *et al.,* 2004). Their study revealed that more than 99% of 108 human isolates harboured the *tir* 255 T>A T allele and lacked repeat region 1-repeat unit 3 (RR1-RU3), whereas of 77 bovine isolates, only 55% and 57% of the isolates, respectively, harboured these traits. Interestingly, Arthur *et al.,* (2013) reported that isolates of *E. coli* O157:H7 from super-shedders harbored the T allele more often (71%) than the A allele (29%). Upon examining the genomic sequences from our study, 9/10 sequenced isolates (90%) harboured the T allele and lacked the RR1-RU3 region in their *tir* gene. One isolate (342_Jul28) exhibiting *tir* 255 T>A allele along with RR1-RU3 originated from a low-

shedder. This was the same isolate that clustered more closely with isolates of bovine origin within the phylogenetic tree. In another study that did not differentiate super-shedders from low shedders, isolates from cattle were shown to have equal distribution of *tir* A allele (54.8%) and *tir* T allele (45.2%), while the *tir* T allele was present in 92.9% isolates from humans (Franz *et al.,* 2012). The reason for the specific association of clinical isolates from humans with the T allele is unknown. The major function of Tir protein involves adherence to epithelial cells; allele polymorphisms could affect adherence and consequently the likelihood of these isolates causing infection in humans (Bono *et al.,* 2007). Other important virulence genes associated with the locus of enterocyte effacement including intimin (*eae*), *espA*, *espB*, *espD* did not show SNP differences between super-shedder and low-shedder isolates. Cote (2013) reported a number of non-synonymous single nucleotide polymorphisms (nsSNPs) in virulence and adherence genes including the adhesions *wzzB*, *fimA*, and *csgG*, along with a truncation of *cah* in a single super-shedder isolate. We did not observe nsSNPs among these genes within our isolates.

Shiga toxins are encoded by bacteriophages (Melton-Celsa *et al.,* 1998) with Stx1-, Stx2a-, and Stx2c-associated bacteriophages typically inserted within or adjacent to conserved chromosomal loci *yehV*, *wrbA* or *argW*, and *sbcB*, respectively (Kotewicz *et al.,* 2008; Shaikh and Tarr, 2003; Strauch *et al.,* 2008). Three principal groups of isolates sharing Stx bacteriophage insertion (SBI) site genotypes have been identified: 1) isolates with a Stx2-encoding bacteriophage inserted at a location other than *wrbA* and with *yehV* occupied by a centrally truncated bacteriophage (cluster 1), 2) isolates with a Stx2-encoding bacteriophage inserted into *wrbA* and with *yehV* occupied by a truncated

bacteriophage as in cluster 1 (cluster 2), and 3) isolates with a complete Stx1-encoding bacteriophage inserted into *yehV* and with a Stx2-encoding bacteriophage inserted into *wrbA* (cluster 3). Sequence data from our super-shedder and low-shedder isolates revealed that all but one of the isolates harboured the SBI cluster 3 (**Table 3.1**). One low-shedder isolate, 342_Jul28, belonged to genotype 6, the same isolate that tended to cluster with isolates associated with bovine. Besser *et al.* (2007) observed four different SBI genotypes among human isolates ($n = 282$) from the United States, with 92.5% of all isolates being represented by genotype 1 (30.1%) and 3 (62.4%). Interestingly, Arthur *et al.* (2013) examined differences in SBI sites between *E. coli* O157:H7 isolates ($n=102$) from super-shedders and found more than half (55.9%) belonged to SBI genotypes/clusters 1-3, suggesting that SBI genotypes from super-shedders may be linked to those genotypes that are most often associated with human illness.

The determination of SBI for a particular strain is not only dependent on where the *stx* are inserted, but also which *stx* subtypes (Scheutz *et al.,* 2012) are present. Previous research has reported that strains with stx_2 are more often associated with hemolytic-uremic syndrome (HUS) than strains harboring stx_1 (Boerlin *et al.,* 1999; Friedrich *et al.,* 2002). These two genes have been further divided into several subtypes: stx_{1a}, stx_{1c}, and stx_{1d} for stx_1, and subtypes stx_{2a-g} for stx_2 (Scheutz *et al.,* 2012). Furthermore, the subtypes stx_{2a} and/or stx_{2c} are more often found to be associated with HUS (Orth *et al.,* 2007) than other *stx* types. Our data revealed that all isolates except one low-shedder isolate (342_Jul28) carried both stx_{1a} and stx_{2a} toxin genes whereas 342_Jul28 carried stx_{1a} and stx_{2c} (**Table 3.1**). Arthur *et al.* (2013) examined the presence of these genes in isolates from super-shedders and found that they carried stx_{2a} or stx_{2c}

separately more frequently than they carried these toxin genes together. Gene stx_1 was carried by half (51%) of the super-shedder strains while all isolates from low-shedders were found to possess the collective set of stx_{2a}, stx_{2c}, and stx_1. Cote (2013) reported that a fully sequenced super-shedder isolate had a Shiga toxin gene profile of stx_1- stx_2+ stx_{2c}+ and contained two plasmids, pO157 and pSS17. Interestingly, isolates within our study carried stx subtypes (stx_{2a} and stx_{2c}) that are more often associated with increased Shiga toxin production and HUS regardless of shedding status.

The antiterminator Q gene alleles have been found to be distributed differently between human and bovine *E. coli* O157:H7 isolates (LeJeune *et al.,* 2004a) and may serve as a genetic marker for super-shedder isolates. The Q_{21}/ Q_{933} PCR assay targets two different alleles of the antiterminator gene Q upstream of the prophage stx_2 region (LeJune *et al.,* 2004a). Allele Q_{933} is responsible for a strong anti-terminator activity resulting in relatively high expression levels of stx$_2$, while allele Q_{21} generates weak anti-terminator activity resulting in lower stx$_2$ expression (Wagner *et al.,* 2001). The Q gene may indirectly affect the colonization of bovine GIT through differences in Shiga toxin expression. *E. coli* O157:H7 strains harboring the Q_{933} variant of the anti-terminator gene produced significantly higher levels of Stx2 toxin than strains with the Q_{21} variant or strains harboring both Q_{933} and Q_{21} (LeJeune *et al.,* 2004a; Ahmad and Zurek, 2006). Previous research from our laboratory has shown increased levels of Stx2 enhances adherence of *E. coli* O157:H7 to the intestinal epithelium in cattle (Baines *et al.,* 2008). In our study, all but one isolate harboured the Q_{933} allele with one low-shedder isolate (342_Jul28) containing the Q_{21} allele (**Table 3.1**). Interestingly, 59.8% of super-shedding isolates examined by Arthur *et al.,* (2013) harboured the Q_{933} either alone (41.2%), in

Table 3.1. Genetic characteristics among isolates examined within this study and reference strains.

Isolate ID	Characteristic	*tir* 255 T>A polymorphism and RR1-RU3	SBI Cluster/ Genotype	*Stx* presence	Anti-terminator Q gene alleles
287-Jul8	low-shedder	T – lacking RR1-RU3	Cluster 3	stx_{1a}, stx_{2a}	Q_{933}
342_Jul28	low-shedder	A – RR1-RU3 present	6	stx_{1a}, stx_{2c}	Q_{21}
219_Jul8	low-shedder	T – lacking RR1-RU3	Cluster 3	stx_{1a}, stx_{2a}	Q_{933}
299-Jul8	low-shedder	T – lacking RR1-RU3	Cluster 3	stx_{1a}, stx_{2a}	Q_{933}
K9-45F	super-shedder	T – lacking RR1-RU3	Cluster 3	stx_{1a}, stx_{2a}	Q_{933}
H7-224F	super-shedder	T – lacking RR1-RU3	Cluster 3	stx_{1a}, stx_{2a}	Q_{933}
274	super-shedder	T – lacking RR1-RU3	Cluster 3	stx_{1a}, stx_{2a}	Q_{933}
K9-16F	super-shedder	T – lacking RR1-RU3	Cluster 3	stx_{1a}, stx_{2a}	Q_{933}
K9-107F	super-shedder	T – lacking RR1-RU3	Cluster 3	stx_{1a}, stx_{2a}	Q_{933}
K3-66F	super-shedder	T – lacking RR1-RU3	Cluster 3	stx_{1a}, stx_{2a}	Q_{933}
Sakai	clinical strain	T – lacking RR1-RU3	Cluster 3	stx_{1a}, stx_{2a}	Q_{933}
EDL933	clinical strain	T – lacking RR1-RU3	Cluster 3	stx_{1a}, stx_{2a}	Q_{933}
EC4115	clinical strain	T – lacking RR1-RU3	Cluster 1	$stx_{2a + 2c}$	Q_{21}/Q_{933}
FRIK2000	bovine strain	A – RR1-RU3 present	15	stx_{1a}, stx_{2c}	Q_{21}

combination with Q_{21} (18.6%) suggesting that Q_{933} may be play a role in enabling *E. coli* O157:H7 to be shed at super-shedder levels through increased Stx2 expression.

3.3.2 Carbon utilization profile and phage type (PT) of *E. coli* O157:H7 from super-shedders and low-shedders

There is very little research examining carbon utilization within *E. coli* O157:H7 isolates from different sources. *E. coli* O157:H7 possess multiple metabolic pathways to oxidize a range of carbon sources making it difficult to equate differences in metabolism to a single metabolic pathway. If observed differences in carbohydrate metabolism could be linked to increased fitness, it may provide insight into the factors that promote the survival and proliferation of super-shedder strains within the GIT. Franz *et al.* (2011) described a significantly higher oxidization rate of propionic acid, α-ketobutyric acid, and α-hydroxybutyric acid among *E. coli* O157:H7 strains that survived for a longer (mean 211 days) as compared to a shorter duration (mean 70 days) in manure-amended soil. No significant differences in the oxidation of these substrates were observed between super-shedders and low shedder isolates. However, galactitol, 3-O-β-D-galactopyranosyl-D-arabinose and thymidine, displayed increased oxidation in super-shedder as compared to low-shedder isolates (**Table 3.2**).

Galactitol/tagatose transport involves the *gatYZABC* operon consisting of *gatY*, *gatZ*, *gatA*, *gatB*, and *gatC* as well as two genes, *gatD* and *gatR*, located immediately downstream of *gatYZABC* operon. This operon encodes for tagatose bisphosphophate aldolase, tagatose-6-phosphate kinase, phosphotransferase system (PTS) as well as galactitol-specific transporter subunits IIA, IIB and IIC. Gene *gatD* encodes for galactitol-1-phosphate dehydrogenase and the *gatR* is the repressor for the galactitol

Table 3.2. Differences in average *Escherichia coli* O157:H7 oxidative activity on various carbon sources between super-shedder (*n=6*) and low-shedder (*n=4*) isolates as measured by Biolog phenotypic microarrays. Bold values represent higher oxidation rates.

Carbon Source	Key genes involved in metabolic pathways	Area under the curve analysis using kinetic data (Omnilog units)		*P*-value
		Super-shedder isolates (mean±SEM; n=6)	Low-shedder isolates (mean±SEM; *n=4*)	
Galactitol	*gatY,gatZ, gatA, gatB, gatC, gatD, gatR*	**4416** ± 141	1292 ± 24	0.00068
Thymidine	*deoA, deoB, deoC, deoD, deoR, yegT*	**5688** ± 134	3415 ± 78	0.032
3-O-β-D-Galactopyranosyl -D-Arabinose	*hns, ebgA, ebgB, ebgC, ebgR*	**4305** ± 168	2178 ± 79	0.014
D-Raffinose	*rafY2, lacY*	2593 ± 98	**5649** ± 179	0.0012
L-Methionine	*metN, metI, metQ, metK*	229 ± 8	**657** ± 11	0.051
N-Acetyl-D-Galactosamine	*aga/gam regulon (agaR, kbaZ, agaV, agaW, agaE, agaF, agaA, agaS, kbaY, agaB, agaC, agaD, agaf)*	2801 ± 134	**5150** ± 201	0.013

utilization operon. Upon examining our sequence results, no sequence differences were observed between super-shedders and three of four low-shedder isolates. The outlier, low-shedder strain (342_Jul28) exhibited an amino acid substitution Ile95Val in the *gatC* gene. This substitution may not contribute to a difference in galactitol utilization as both isoleucine and valine are non-polar amino acids and as a result this substitution may not affect the secondary or tertiary structure of the protein.

A cytoplasmic protein, dH-NS, plays a role in the expression of many genes either directly or indirectly by modifying the condensation of chromosomal DNA. Previous research has demonstrated that when the *hns* structural gene and upstream regulatory regions were replaced with a mutant cassette, O-β-d-galactopyranosyl-d-arabinose was no longer metabolized. Genetic differences within *hns* between super-shedder and low-shedder isolates within the study were not observed. The *ebg* gene cluster of *E. coli* O157:H7 consists of *ebgA*, *ebgB*, and *ebgC*, which encodes for the subunits of the ebg ß-galactosidase (evolved- ß-galactosidase; EbgA), and *ebgR* which encodes for the operon repressor. The natural substrate of this operon is unknown (Hall, 1999), however its involvement in catabolizing galactopyranosyl-D-arabinose has been reported (Hall and Zuzel, 1980). Three of the four low-shedder isolates and one super-shedder isolate exhibited an amino acid substitution Arg871His in EbgA. The remaining super-shedder isolates and one low-shedder isolate (342_Jul28) harbored arginine at this position, a substitution also observed in two strains associated with infection in humans, EDL933 and Sakai . Arginine and histidine are both positively charged and whether this mutation has an impact on the utilization of O-β-d-galactopyranosyl-d-arabinose requires further investigation.

Thymidine catabolism in *E. coli* involves a number of enzymes including thymidine phosphorylase (*deoA*), 1,5 phosphodeoxyribomutase (*deoB*), deoxyribose-5-phosphate aldolase (*deoC*) and nucleoside phosphorylase (*doeD*). The expression of the operon associated with these genes is regulated by the *deoR* repressor and a nucleoside transporter protein (*yegT*) that may play a role in thymidine uptake in *E. coli*. The sequence of these genes and their promoter regions were identical between super-shedder and low-shedder isolates.

In contrast, N-acetyl-D-galactosamine (Aga), D-raffinose and L-methionine displayed significantly higher rates of oxidation in low-shedder than super-shedder isolates (**Table 3.2**). The phosphoenolpyruvate:carbohydrate PTS is an enzyme complex responsible for the transport of a large number of different types of carbohydrates in bacteria (Postma *et al.,* 1993). A gene cluster encoding the N-Acetyl-d-galactosamine PTS and other catabolic enzymes are responsible for transport and catabolism of Aga. *E. coli* O157:H7 strains typically display an Aga+ phenotype, however, isolates recovered from a 2006 spinach-associated *E. coli* O157:H7 outbreak displayed an Aga⁻ phenotype (Mukherjee *et al.,* 2008). Sequence alignment of the 11,745-bp *aga/gam* cluster of genes in EDL933 with those in the Sakai strain and isolates from the 2006 spinach outbreak revealed a single nucleotide difference (G:C→A:T) in the *agaF* coding for EIIA$^{Aga/Gam}$, changing a conserved glycine residue to serine (Gly91Ser). Upon analyzing these genes in our sequenced isolates we found no differences in the *aga/gam* cluster between super-shedder and low-shedder isolates.

Raffinose, is a trisaccharide composed of galactose, glucose, and fructose and was oxidized in all the isolates in this study, but to a lesser extent in super-shedder isolates

(**Table 3.2**). The plasmid born Raf operon in generic *E. coli* has been well characterized (Aslanidis *et al.,* 1989) and contains genes coding for proteins involved in the uptake and utilization of raffinose including an α-galactosidase (*rafA*), Raf permease (*rafB*), and sucrose hydrolase (*rafD*) as well as the repressor (*rafR*). Downstream of *rafRABD* operon, *rafY* encodes a glycoporin involved in the uptake of maltose, sucrose, and raffinose (Ulmke *et al.,* 1997). The product of *rafY* impacts the transport of raffinose through LacY. No homologs of the *rafRABD* gene cluster are present in *E. coli* O157:H7, however a *rafY* homolog annotated as *rafY2* were present in the genomes of both super-shedder and low-shedder isolates and displayed no sequence differences.

Methionine is one of two sulfur-containing amino acids and plays a role in a variety of methyltransferase reactions as a precursor of S-adenosyl-Methionine (SAM). S-adenosylmethionine is synthesized from methionine and ATP by SAM synthetase (*metK*). As with raffinose, L-methionine was oxidized to a lesser extent in super-shedder than low-shedder isolates (**Table 3.2**). The methionine transport system includes *metN*, *metI* and *metQ* encoding for ATPase, permease and substrate binding protein, respectively. No genetic differences could be found for methionine-related genes among super-shedder and low-shedder isolates.

A critical element in the emergence of *E. coli* O157:H7 was the evolution of acid resistance under positive selective pressure within the GIT of ruminants, an attribute that promotes survival in acidic environments (Lin *et al.,* 1995) and may result in increased infection and proliferation within the GIT of cattle fed high grain diets (Diez-Gonzalez *et al.,* 1998). When super-shedder and low-shedder isolates were subjected to a range of pH

from 3.5 to 10, 67.7% of super-shedder isolates (4/6 isolates) displayed increased growth between pH 3.5 to 7 than other isolates tested in this study (**Figure 3.4**).

If super-shedders isolates have a greater ability to survive in a more acidic GIT environment, then perhaps these strains are more likely to proliferate and achieve super-shedder levels, especially in cattle fed grain-based diets. *E. coli* O157:H7 achieves acid resistance using three systems, of which the glutamate decarboxylase (gad) system is the most efficient and has been shown to be essential for the organism's ability to colonize the intestinal epithelium in cattle and to play an important role in the protection against oxidative stress (Bearson *et al.,* 2009). No genetic differences were observed among genes involved in the glutamate decarboxylase acid-resistance system including *gadA*, *gadB*, *gadBC*, *gadX*. Additional genes that play a role in the maintenance of cell wall integrity (*tolQ* and *tolR*), oxidative stress resistance (*soxS)*, osmoregulation (*proP* and *proB*), as well as several genes encoding proteins involved in DNA repair and protein turnover (*uvrA, uvrB, uvrC, uvrY, ruvB* and *mfd*) and molecular chaperones (*msrB/yeaA, grpE, ybbN, ybiY, hslO, clpS* and *clpA)* were examined and no SNP differences were identified between super-shedder and low-shedder isolates.

It is important to note that genes that exhibit identical sequences may still be differentially expressed. *E. coli* O157:H7 genomes contain several open reading frames (ORFs) encoding for proteins of unknown function. Further tests looking at those ORFs of unknown function could elucidate their role, if any, in the differential utilization of compounds examined within this study along with their possible role in the development of the super-shedder phenotype. In addition, several unidentified/hypothetical genes associated with regions corresponding to O-islands and S-loops (Perna *et al.,* 2001;
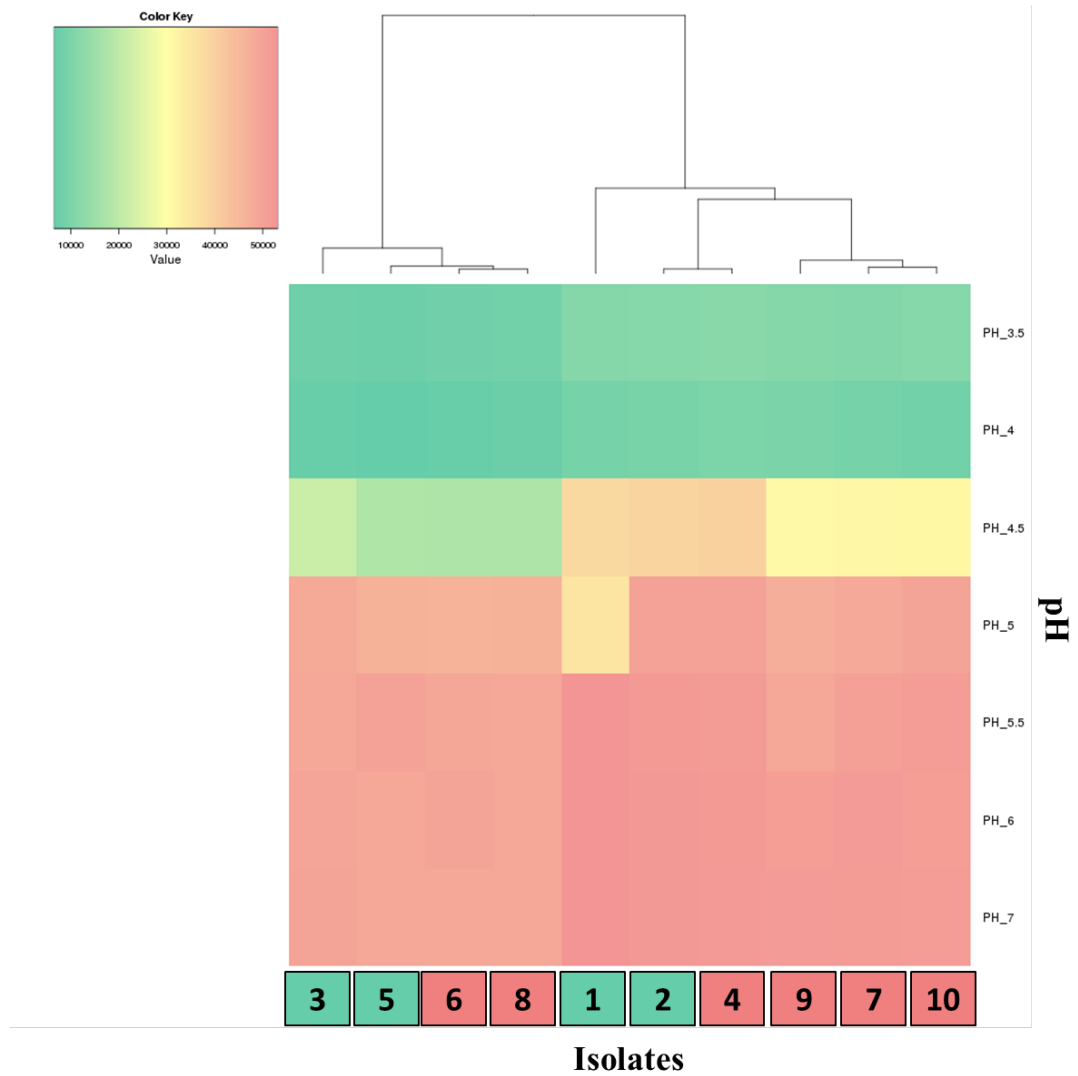
Figure 3.4. Growth of isolates over a pH range from 3.5 – 10. Isolates were grown for 48 h at various pH ranging from 3.5 to 10. The colour key represents relative growth based on oxidative activity using area under the curve (Omnilog units). Isolates are identified in pink (super-shedders) and teal (low-shedders) boxes as in Figure 3.1.

Hayashi *et al.,* 2001) in *E. coli* O157:H7 genomes may be involved in the uptake and utilization of the substrates found to be differentially oxidized within this study. Therefore, along with genetic sequencing, comparative expression microarray or RNAseq-based gene expression assays from isolates grown in identical conditions may aide in in deciphering differences in overall gene expression profiles between super-shedder and low-shedder isolates. However, it would be challenging to perform these comparative studies in real-time with samples collected from the GIT considering the transient nature of super-shedding and the challenges with detecting the low levels of *E. coli* O157:H7 in low-shedders. Simulating the environmental conditions within the GIT to differentiate expression levels between super-shedder and low-shedder isolates also presents challenges.

All the isolates recovered from super-shedders were PT14a, whereas those from low-shedders included phage type PT91 (219 Jul_8), PT43 (342_Jul28) and PT14a (*n*=2). Previous research has identified five different PTs (PT8, PT14a, PT21, PT33, and PT34) among the *E. coli* O157:H7 isolates collected within Canada with PT14a and PT8 being most common (Jokinen *et al.,* 2011). In contrast, Arthur *et al.* (2013) examined super-shedder isolates (*n*=102) from the United States and identified 19 different phage types with PT4 accounting for 30% of isolates. Another study found an association between PT21/28 and super-shedders within a population of cattle in Scotland (Chase-Topping *et al.,* 2007; Halliday *et al.,* 2006). Interestingly, PT4 is also the most common phage type associated with *E. coli* O157:H7 infections in humans in the United States, and similarly PT21/28 is the most common phage type isolated from clinical cases in Scotland (Pearce *et al.,* 2009) as well as PT14a isolated from clinical cases in Canada (Jokinen *et al.,*

2011). suggesting that super-shedders may harbour those strains that are most frequently associated with human illness.

3.3.3 Clade typing

Manning *et al.* (2008) genotyped more than 500 clinical strains of *E. coli* O157:H7 based on 96 SNPs that separated strains into genetically distinct clades and identified a 'hyper-virulent' clade (clade 8) of *E. coli* O157:H7 among isolates obtained from a 2006 outbreak associated with raw spinach consumption in the United States. Clade 8 strains have been shown to have 2-fold greater adherence to bovine epithelial cells (MAC-T) and increased expression of virulence genes, including those that are LEE-encoded (*espAB*, *tir*, *eae*, *stx₂*) and plasmid encoded (*hlyA*, *toxB*, *tagA*) (Abu-Ali *et al.,* 2010)*.* One study reported that a super-shedding isolate genotyped as clade 8 (Katani *et al.,* 2014). Due to the fact that only a single isolate was examined this finding may not be associated with the super-shedder phenotype. Our study is the first to examine six super-shedding isolates all of which were clade 2 (**Table 3.3**). This suggests that super-shedding isolates from our study are not associated with a hyper-virulent strain.

3.3.4 Biofilm formation

In Chapter 1, I hypothesized that super-shedder isolates have a superior ability to form biofilms on the intestinal epithelium. Biofilm sloughing in the intestine could be responsible for the fecal densities of *E. coli* O157:H7 that are required for the host to be designated as a super-shedder. Intermittent sloughing of the intestinal biofilm could also account for the sporadic nature of super-shedding in cattle. Previous research, using DNA microarrays, discovered that 79 genes, representing 1.84% of the *E. coli* genome, were differentially expressed during biofilm formation as compared to planktonic growth

Table 3.3. Clade typing results from isolates collected in this study compared to clinical and bovine *E. coli* O157:H7 strains.

Gene (ORF)	ECs0517	ECs0654	ECs2357	ECs2521	ECs3881	ECs3942	ECs4130	ECs4380	
SNP target (nt position)	247	281	539	1060	438	348	630	776	
Amino acid position	83	94	180	354	146	116	210	259	
Codon polymorphism	a/g GC	A t/c C	T c/a C	g/t CC	A C t/c	G C a/c	G C t/c	G g/a G	
Nonsynonymous or synonymous	NON	NON	NON	NON	SYN	SYN	SYN	NON	**Clade**
Function	AcrR family transcriptional regulator CDS	citrate lyase subunit alpha	Hypothetical protein	p-Aminobenzoate synthetase component I	Hydrogenase-2 small subunit	glycerol-3-phosphate acyltransferase PlsY	Sodium/pantothenate symporter	heme utilization/ transport protein	
EC4115 strain (clinical)	**G**	T	**A**	**G**	**C**	A	**C**	G	8
EDL933 strain (bovine)	A	T	C	**G**	**C**	A	T	G	3
Sakai strain (clinical)	A	T	C	T	T	A	T	G	1
219_Jul 8 (low-shedder)	A	T	C	**G**	T	A	T	G	2
287-Jul 8 (low-shedder)	A	T	C	**G**	T	A	T	G	2
299-Jul 8 (low-shedder)	A	T	C	**G**	T	A	T	G	2
342_Jul 26 (low-shedder)	**G**	T	C	**G**	**C**	A	**C**	**A**	2
274-Jul 8 (super-shedder)	A	T	C	**G**	T	A	T	G	2
H7-224F (super-shedder)	A	T	C	**G**	T	A	T	G	2
K3-66F (super-shedder)	A	T	C	**G**	T	A	T	G	2
K9-16F (super-shedder)	A	T	C	**G**	T	A	T	G	2
K9-45 (super-shedder)	A	T	C	**G**	T	A	T	G	2
K9-107F (super-shedder)	A	T	C	**G**	T	A	T	**A**	2
Amino acid change	S → G	I → T	S → Y	S → A	–	-	–	G → E	

(Schembri *et al.,* 2003). Among the genes that showed increased expression in biofilms were three involved in adhesion and auto aggregation, several encoding structural proteins such as OmpC, OmpF and OmpT, and *slp* (encoding an outer-membrane lipoprotein induced after carbon starvation). Some of these genes (*slp* and *ompC*) have been associated with the initial steps of *E. coli* biofilm formation on abiotic surfaces (Prigent-Combaret *et al.,* 1999; Otto *et al.,* 2001). Upon identifying these genes within our sequence, no differences were observed in these genes between super-shedder and low-shedder isolates further emphasizing that gene expression and adherence assays would be important in deciphering differences between super-shedder and low-shedder isolates. To date, most bacterial adherence assays have been standardized using cell lines derived from human cancers such as the HeLa cells, HEp-2 cells and Caco-2 cells (Cookson and Woodward 2003; Halpin *et al.,* 2010). These cell lines may not accurately reflect the mechanistic processes involved in the adherence of bacteria to cattle GIT epithelial cells. Kudva and Dean-Nystom et al. (2011) standardized a protocol for a RAJ squamous epithelial (RSE) cell–bacterial adherence assay. One study reported *in vitro* analysis of adherence profiles of one super-shedder isolates' ability to bind and adhere to this cell line (Cote, 2013). Results showed that all of the RSE cells exposed to the super-shedder isolate had a significantly higher number of bacteria/cells as compared to RSE cells exposed to a low-shedder strain, EDL933 (Cote, 2013). This suggests that super-shedder isolates may have a superior capacity to adhere to bovine rectal epithelial cells, although I could find no evidence to support this through comparative genomics of super- vs low shedder isolates.

RNA sequencing (RNA-seq) is a relatively new technology enabling a wider dynamic range for transcriptome characterization (Wang *et al.,* 2009), while quantifying transcript expression levels. Furthermore, this information is more useful in interpreting data generated through proteomic analysis (Fu *et al.,* 2009) allowing complete annotation and quantification of all genes and their isoforms across samples (Garber *et al.,* 2011). In order to fully understand the nature of *E. coli* O157:H7 during super-shedding, transcriptomic studies examining *E. coli* O157:H7 specific RNA isolated from the feces of super-shedder and low-shedder cattle should be assessed.

3.4 Conclusions

The focus of this study was to conduct comparative genomics on *E. coli* O157:H7 isolates collected from super-shedders and low-shedders in an effort to identify specific genes associated with super-shedders in the hope that it would provide information that could be deployed in future mitigation strategies. Interestingly, one low-shedder isolate (342_Jul 28) was an anomaly and displayed characteristics that were more closely related to bovine lineages than clinical or outbreak lineages. This 'bovine-like isolate (342_Jul28), typed as clade 5, SBI genotype 6, displayed differences in carbon utilization, exhibited *tir* 255 T>A allele with RR1-RU3 repeating unit, carried stx_{1a} and stx_{2c} and the weak anti-terminator Q_{21} variant of the antiterminator Q gene alleles. Whereas the remaining three low-shedders as well as all super-shedder isolates were more 'clinical-like'; typing as clade 2, SBI cluster 3, lacking the *tir* polymorphism and RR1-RRU, possessing stx_{1a} and stx_{2a} and the Q_{933} variant. It can be reasoned that although the 'clinical-like' strains of *E. coli* O157:H7 may be present in a host at any given time in low numbers, host factors including the microbial community within the

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GIT as well as the composition of the diet may play a role in their colonization and likelihood of achieving sufficient density for super-shedding to occur. The time of sampling could also have an impact on the likelihood of finding differences in the genomes of super-shedder isolates. For example, if cattle are sampled before they increase to super-shedding levels then super-shedder and low-shedder isolates could be identical.

It is important to note that there may be several other factors, in addition to the pathogen itself, which could be contributing to the super-shedding phenomenon. Previous studies have provided evidence that the microbiomes of a super-shedder host are unique from low-shedding hosts (Xu *et al.,* 2014; Dugat-Bony *et al.,* 2013). Furthermore, transcriptomic profiles of rectal tissue collected from super-shedding and low-shedding cattle using RNA-seq exhibited 38 differentially expressed genes, of which 15 genes were associated with innate and adaptive immune functions (Wang *et al.,* unpublished data). Future studies examining super-shedder cattle need to take into account additional factors that are playing a role in this phenomenon if effective mitigation strategies are to be identified.

CHAPTER 4: Summary and Conclusion

Findings from this study shed light on some important unknown questions related to super-shedding of *E. coli* O157:H7 in cattle. Firstly; how long do cattle super-shed? It is important to identify how long cattle shed at the super-shedder level in order to estimate the degree to which these individuals contribute to environmental contamination. In the past, studies have sampled cattle bi-weekly, weekly, twice weekly and every three hours to assess the persistence of super-shedding *E. coli* O157:H7 in the feces of cattle. However, the pattern or duration of shedding in super-shedders has not previously been characterized with repeated sampling from the same animal over several consecutive days. This study implemented twice daily sampling of super-shedding cattle over 34 days. Results found super-shedding to be a short-lived, isolated event and was not consistent even within the same animal over the course of a day. Subjection of the animals to management practices including transportation, handling and mixing of steers, and an increase in forage concentration did not increase the prevalence or levels of *E. coli* O157:H7 shed by steers. These results suggest that super-shedding may not play as great a role in transmission and contamination within the feedlot environment as previously proposed.

A limitation associated with this observation is that only 11 super-shedders were monitored over time. I could, of course, always include more animals; however, the cost and time associated with identifying super-shedding steers within a feedlot setting makes it difficult to include more. Ideally it would be best to sample more cattle, daily, at the commercial feedlot. However, this is highly disruptive to the everyday production practices at a commercial feedlot with the daily running of cattle through a chute for

sampling unlikely to be permitted by the feedlot owners. Another suggestion for sampling is to identify super-shedders and segregate them into a separate pen and sample them daily. This also would not be representative of a normal feedlot practices and animal to animal transmission may not be the same as when these individuals are housed within a larger herd of cattle within a pen. It has also been proposed that individual cattle could be tracked in the feedlot and feces collected when they defecate. This would be difficult as commercial feedlot pens can have over 400 cattle in one pen making following individual cattle difficult and entering pens potentially dangerous for workers. Given sampling challenges, this study provides novel information related to the nature of super-shedding as it is the first of its kind to repeatedly sample super-shedders over time.

Enumeration of *E. coli* O157:H7 within feces has been shown to be a stochastic process because of the variability in the distribution of the bacterium within a single fecal sample. *Escherichia coli* O157:H7 may be present in the bovine GIT, but may not necessarily be excreted with every bowel evacuation and as a result is unevenly distributed in feces. A limitation associated with detecting super-shedders is that CFU/g feces is commonly determined via direct plate counts, a process that involves serial dilution of sampled feces. This technique is time consuming, making it difficult to process large numbers of samples rapidly. Other methods of enumeration include the most probable number technique, which also requires a high degree of replication at the appropriate dilution to narrow confidence intervals. Quantitative PCR (or real-time PCR) can also be employed to obtain accurate counts of *E. coli* O157:H7; however, this method requires the extraction of high-quality DNA. This step can be time limiting and expensive, especially if the fecal sample is found to be negative for *E. coli* O157:H7 at

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the time of sampling. Furthermore, real-time PCR lacks sensitivity ($\geq 3.5 \times 10^4$ CFU/g) within soil, manure, feces and wastewater (Ibekwe et al., 2002).

The second question examined was what causes super-shedding? There are several possible factors that may influence the ability of an individual to become a super-shedder including complex interactions among the microbe, host and environment. This study focused on the role that the pathogen may have on the super-shedding phenomenon. This included attempting to identify factors within strains of *E. coli* O157:H7 that contribute to their survival in the environment which in turn could influence their likelihood of being acquired by the host. In addition, factors that enable the bacterium to survive within the digestive tract, out compete other microbiota, proliferate within digesta, colonize the epithelium at the RAJ and undergo mass release into the rectal contents were explored. Upon describing the sporadic nature of super-shedding, it was hypothesized that biofilm sloughing into the rectum could be responsible for the fecal densities of *E. coli* O157:H7 associated with super-shedders (i.e., 10^4 CFU/g feces). Intermittent sloughing of this intestinal biofilm would account for the sporadic nature of shedding and super-shedding in cattle.

Upon examining the whole genome of ten representative isolates recovered from super-shedding (*n*=6) and low-shedding (*n*=4) cattle, an average of 1600 single nucleotide polymorphisms (SNPs) were identified when compared to the outbreak reference strain EC4115. A phylogenetic tree based on SNPs did not support the genetic segregation between isolates from super-shedders *vs.* low-shedders, but it was noted that super-shedder isolates were more closely related to clinical than environmental *E. coli* O157:H7 strains. All super-shedder isolates and three of four low-shedder isolates were

typed as phage type 14a, SBI cluster 3 and clade 2. Super-shedder isolates also displayed increased utilization of galactitol, thymidine and 3-O-β-D-Galactopyranosyl-D-Arabinose as compared to low-shedder isolates although no SNPs could specifically be mapped to genes that code for proteins known to play a role in the metabolism of these substrates.

Interestingly one low-shedding isolate (342_Jul 28) was an anomaly and displayed characteristics that were more closely related to bovine lineages than clinical or outbreak lineages. This 'bovine-like isolate (342_Jul28), typed as clade 5, SBI genotype 6, displayed differences in carbon utilization, exhibited tir 255 T>A allele with RR1-RU3 repeating unit, carried stx_{1a} and stx_{2c} and the weak anti-terminator Q_{21} variant of the antiterminator Q gene alleles. Whereas the remaining three low-shedders as well as all super-shedder isolates were more 'clinical-like' typing as clade 2, SBI cluster 3, lacking the *tir* polymorphism and RR1-RRU, possessing stx_{1a} and stx_{2a} and Q_{933} variant. It can be reasoned that although the 'clinical-like' strains of *E. col*i O157:H7 may be present in a host at any given time in low numbers, host factors including the microbial community within the GIT as well as the diet/feed may be playing a role in their colonization to reach super-shedding status.

Results from this study were unable to successfully identify specific marker traits that could be used to differentiate super-shedder from low-shedder isolates. It is important to note that despite genes being genetically identical they may not always expressed at the same level due to genetic variations in regulatory regions elsewhere in the genome. Therefore, along with genetic sequencing, comparative expression microarray or RNAseq-based gene expression assays from isolates grown in identical conditions could be an approach to decipher differences in overall gene expression

profiles between super-shedder and low-shedder isolates. However, it would be challenging to perform this comparative study using real-time gastro-intestinal tract (GIT) samples considering the transient nature of super-shedding along with the need to simulate growth substrate/environmental conditions within the GIT in a manner that resulted in analogous gene expression. Simulating conditions that are identical to those in the host is a major obstacle to developing approaches to advance the understanding of the etiology of super-shedder *E. coli* O157:H7 within the host. An additional limitation identified within this study pertains to the isolation and characterization of single isolates from a single super-shedding event. It is unknown if the isolate I characterized was in fact a clonal representation of all *E. coli* O157:H7 present in the sample. However, in the past studies from our laboratories have characterized more than three isolates from a single culture plate and identified them as being 100% clonally related based on PFGE (Sharma *et al.,* 2008).

In conclusion, results from our study do not directly contribute to the ability of us to mitigate or control super- shedders within a herd as no specific biomarkers were identified within *E. coli* O157:H7 associated with super-shedder cattle. However, insight into the intermittent nature of super-shedding suggests that mitigation of these individuals may be ineffective unless *E. coli* O157:H7 reductions strategies are directed towards the herd as a whole. As discussed, super-shedding is a result of a combination of interactions between the pathogen, host and the environment and, as a result, more research on the nature of microbial communities in the host is required to clarify the roles of bacterial richness and how the predator-prey relationship among bacteriophage and *E. coli* O157:H7 influence super-shedding. As well, a better understanding is required of the

innate immune response specifically within the super-shedder host. Once these factors are

better understood, improved methods for identifying and mitigating super-shedders

within cattle populations may be developed.

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