

FACTORS AFFECTING FECAL SHEDDING OF *ESCHERICHIA COLI* O157:H7 IN CATTLE

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ABSTRACT

Cattle are reservoirs of *E. coli* O157:H7 and contamination of carcasses during slaughter transfer this potentially deadly pathogen into the human food chain. The goal of this study was to assess: 1) the effect of feeding cattle corn or wheat DDGS, a by-product of the bioethanol industry on fecal shedding or persistence of *E. coli* O157:H7 in cattle; 2) the effect of endemic bacteriophages on fecal shedding of *E. coli* O157:H7 in cattle. Results from this study suggest addition of DDGS in finishing diets of cattle do not affect the fecal shedding or persistence of *E. coli* O157:H7. Three types of endemic phages were identified which may impact levels of shedding of *E. coli* O157:H7 in cattle. Improved understanding of factors which contribute to shedding of *E. coli* O157:H7 and the natural microbiota of individual cattle will improve upon existing systems to reduce *E. coli* O157:H7 in meat.

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LIST OF ABBREVIATIONS

ADF	acid detergent fiber
ADG	average daily gain
AE	attaching and effacing
BIM	bacteria-insensitive mutant
BW	body weight
CDDGS	corn dried distiller grains with solubles
CFU	colony-forming units
CP	crude protein
CTRL	control
CT-SMAC	sorbitol MacConkey agar with potassium tellurite and cefixime
CWB	carcass weight basis
CWDDGS	corn-wheat dried distiller grains with solubles
DDGS	dried distiller grains with solubles
DOF	days on feed
DM	dry matter
DMI	dry matter intake
EHEC	enterohemorrhagic <i>Escherichia coli</i>
EPEC	enteropathogenic <i>Escherichia coli</i>
ETEC	enterotoxigenic <i>Escherichia coli</i>
Gb ₃	globotriaosylceramide
G:F	gain to feed ratio
GI	gastrointestinal tract
HC	hemorrhagic colitis
HCW	hot carcass weight
HUS	hemolytic uremic syndrome
IMS	immunomagnetic separation

LEE	locus of enterocyte effacement
LPS	lipopolysaccharide
LRC	Lethbridge Research Centre
LS	low-shedders
mEC	modified <i>E. coli</i> broth
MOI	multiplicity of infection
NalR	Nalidixic-resistant
NDF	neutral detergent fiber
NEg	net energy gain
NSF	non-sorbitol fermenter
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PFU	plaque-forming units
PHAC	Public Health Agency of Canada
PT	phage type
QG	quality grade
RAJ	recto-anal junction
RFLP	restriction fragment length polymorphism
SRP	siderophore receptor proteins
SS	super-shedders
STEC	Shiga toxin-producing <i>Escherichia coli</i>
Stx	Shiga toxin
tir	translocated intimin receptor
TEM	transmission electron microscopy
TSB	tryptic soy broth
TTP	thrombotic thrombocytopenia purpura
VFA	volatile fatty acids

WDDGS wheat dried distiller grains with solubles
YG yield grade

BACTERIA REFERRED TO IN THIS STUDY

Campylobacter

Cholera

Clostridium spp.

Enterococcus

Escherichia coli

Escherichia coli O157:H7

Histophilus somni

Klebsiella pneumoniae

Lactobacillus acidophilus

Listeria monocytogenes

Mannheimia haemolytica

Pseudomonas aeruginosa

Saccharomyces cerevisiae

Salmonella Enterica

Staphylococcus aureus

1. CHAPTER ONE

1.1. *Escherichia coli* O157:H7 in the feedlot

1.1.1. Introduction

The nature of livestock production yields large numbers of cattle confined to small spaces prior to slaughter. High density production of cattle, nutrient-rich finishing diets to increase animal performance and subsequent stress on the animal in the feedlot have the potential to increase prevalence and/or transmission of pathogens and impact overall contamination of food in the human chain. The frequent bacteria associated with recalls in the food industry are *Salmonella enteritidis*, *Campylobacter jejuni*, *Listeria monocytogenes* and *Escherichia coli* O157:H7 (Soon *et al.*, 2011). *E. coli* O157:H7 has been the source of multiple human disease outbreaks and contaminated beef has been implicated as a source in many *E. coli* O157:H7 outbreaks resulting in massive recalls and substantial economic loss to the beef industry (Vogt and Dippold, 2005). A combination of interventions applied at pre-harvest and throughout processing may be most productive in eliminating this pathogen prior to distribution (Smith *et al.*, 2013). Understanding the ecology of *E. coli* O157:H7 in cattle and identification of methods to eliminate the pathogen are critical to prevent human infections associated with this pathogen in the food industry.

1.1.2. Pathogenicity of *E. coli* O157:H7

E. coli O157:H7 is a zoonotic pathogen responsible for severe gastrointestinal illness including haemorrhagic colitis (HC) and the systemic, sometimes fatal, disease termed haemolytic uremic syndrome (HUS) (Besser, 1999). Although most *E. coli* are harmless commensal organisms found in the gastrointestinal (GI) tract of humans, one subgroup of

pathogenic *E. coli* termed enterohemorrhagic *E. coli* (EHEC) are capable of targeting the intestinal epithelium and releasing toxins that can act both locally or systemically to create damage to other areas and/or organs of the body. Strains in the EHEC pathotype which include *E. coli* O157:H7 have the ability to form attaching and effacing (AE) lesions on the exposed surface of enterocyte cells and release toxins associated with bloody diarrhea, HC and HUS (La Ragione *et al.*, 2009). EHEC strains have a large pathogenicity island termed the locus of enterocyte effacement (LEE) which contains a number of genes (ie. EspA, EspB, EspD, tir, eae) that allow the organism to adhere to intestinal epithelial cells and reorganize the cytoskeletal organization of microvilli resulting in AE lesions (Beier *et al.*, 2004). This process is facilitated by a type III secretion system that transports several crucial proteins needed for intimate adherence between the bacteria and host epithelial cell including a translocated intimin receptor (tir) and the adherence factor, intimin (La Ragione *et al.*, 2009). Polymerized actin accumulates beneath adherent bacteria to produce the AE pedestals which may reduce the absorptive tissue surface and initiate watery diarrhea. The resultant loss of microvilli may allow toxins to gain access to the intestinal lumen of the bloodstream and spread systemically throughout the body (Besser, 1999). The main toxins associated with *E. coli* O157:H7 are the Shiga toxins (Stx) which can be encoded on multiple prophages within the genome of the pathogen and *E. coli* strains releasing this toxin are termed Shiga toxin producing *E. coli* (STEC) (Fogg *et al.*, 2012). These potent toxins bind glycolipid globotriaosylceramide (Gb₃) receptors on colon endothelial cells where they are internalized and effectively block protein synthesis through cleavage of the host 28S (Beier *et al.*, 2004). Damage to the capillaries in the colon may contribute to the onset of bloody diarrhea (Besser, 1999). The cortex of the kidney has abundant Gb₃ receptors and once Stx enter the blood stream they can come in contact with and attach to the glomerular endothelium of the kidney triggering a series of responses which can ultimately

lead to HUS. The Stx stimulate platelet activation and development of microthrombi within the glomeruli narrow blood vessels ultimately leading to the shearing of red blood cells or microangiopathic hemolysis. The development of hemolytic anemia and thrombocytopenia decrease the blood flow which is critical to the kidney, resulting in acute renal failure. These toxins target other Gb₃ receptors found within the central nervous system and organs such as the pancreas resulting in severe vascular damage, strokes, seizures or death (Schmidt *et al.*, 1995). Other important toxins include the EHEC hemolysins which belong to a family of pore-forming cytolysins that disrupt host cell membrane integrity (Schmidt *et al.*, 1995). Certain types of Stx, intimin and haemolysin genes in EHEC have been associated with an increase in severity of disease in humans suggesting virulence factors may account for differences in pathogenicity among strains (Boerlin *et al.*, 1999). Bacterial fimbriae found on the surface of *E. coli* O157:H7 cells have been found to create a physical bridge between bacteria and cultured epithelial cells, and fimbriae mutants exhibit reduced *in vivo* colonization suggesting that fimbriae may play crucial roles in adherence and colonization in the intestinal tract (Rendon *et al.*, 2007; Lloyd *et al.*, 2012). Many *E. coli* O157:H7 strains also possess a pO157 plasmid encoding catalase peroxidases and serine proteases which may protect the bacteria from oxidative stress and iron transport (Beier *et al.*, 2004).

1.1.3. Reservoirs of *E. coli* O157:H7

One of the first recorded outbreaks where *E. coli* O157:H7 was isolated from ground beef occurred in Michigan in 1982 (Riley *et al.*, 1983; Perna *et al.*, 2001). In this outbreak, cattle were recognized as asymptomatic carriers of *E. coli* O157:H7 as they shed the pathogen in their feces. Prevalence of *E. coli* O157:H7 in cattle can range from 0.2% to 48.8% (Snedeker *et al.*, 2012) and long-term colonization may depend on the strain of *E. coli* O157:H7 and/or the

individual animal itself. Fecal shedding in cattle is highly variable with some individuals shedding *E. coli* O157:H7 in feces for a short period of time and others excreting the pathogen for several months suggesting the ability of the organism to persist in the intestinal tract differs for individuals (Grauke *et al.*, 2002). The recto-anal junction (RAJ) has been proposed as the primary site of colonization in the cattle GI tract and occupation of the RAJ with *E. coli* O157:H7 was positively correlated with *E. coli* O157:H7 found on the surface of fecal stools (Naylor *et al.*, 2003). Non- O157 strains of *E. coli* do not persist in the RAJ to the same extent as *E. coli* O157:H7 and the presence of certain types of intimin, tir and virulence factors associated with the pO157 plasmid found in some *E. coli* O157:H7 strains may be more consistent with long term colonization (Sheng *et al.*, 2006a). Most cattle are asymptomatic as they lack the Gb₃ receptors found in humans that are targeted by *E. coli* O157:H7 toxins (La Ragione *et al.*, 2008) making it difficult to differentiate cattle that are long term reservoirs from those that are short-term shedders. In addition to cattle, other mammals such as rabbits, deer, water buffalo, pigs, sheep and goats have been identified as reservoirs for the pathogen (La Ragione *et al.*, 2008). Birds (chickens, seagulls, starlings), rodents (rats) and insects (houseflies) have also been shown to harbour *E. coli* O157:H7 (Soon *et al.*, 2011). With an infectious dose as low as 10 cells and multiple reservoirs for *E. coli* O157:H7, human infection associated with this pathogen will continue to occur until solutions for control are developed. The National Enteric Surveillance program in Canada estimates that gastrointestinal illness associated with STEC including *E. coli* O157:H7 in 2009 was 4.47/100 000 people in Alberta although these figures are only based on a subset of lab isolations and many foodborne illness are still under-reported (Public Health Agency of Canada, 2009).

1.1.4. Sources and transmission of *E. coli* O157:H7

Worldwide, beef remains the most common food source contaminated with *E. coli* O157:H7 (44.2%; Soon *et al.*, 2011), followed by produce (19.5%; Erickson, 2012), multi-ingredient foods (11.8%; Miller *et al.*, 2012), dairy (9.8%; Cagri-Mehmetoglu *et al.*, 2011), other meats (6.9%; Ahn *et al.*, 2009), beverages (4.4%; Besser *et al.*, 1993), bakery (1.0%; Neil *et al.*, 2012), chicken (1.0%; Chinen *et al.*, 2009), seafood (0.5%; Surendraraj *et al.*, 2010), pork (0.5%; Villani *et al.*, 2005) and other poultry (0.3%; Juck *et al.*, 2012). Many pathogenic bacteria that contaminate food products for human consumption can be traced back to farms. Transmission of pathogenic bacteria can occur via air, water, soil, or fomites and as the environment of individual farms can vary greatly, the prevalence and survival of pathogens are equally variable (Beier *et al.*, 2004). Water or feed contaminated with *E. coli* O157:H7 and exposure to pests or wildlife can disseminate the pathogen to multiple food sources (Doyle and Erickson, 2006). Soil properties such as composition of organic material, nutrients, and porosity can influence the survival of *E. coli* O157:H7 and influence the nature of the microbiota in the environment (Dirk van Elsas *et al.*, 2012). Farm surfaces such as ropes, gates, floors, or walls and clothing or shoes of workers can also harbour and spread *E. coli* O157:H7 (Beier *et al.*, 2004). Clean, dry bedding, decreased stocking density and stress, exclusion of wild animals, clean feed and water, and training of farm workers in hygienic practices can result in reduction of *E. coli* O157:H7 in some cases (Soon *et al.*, 2011).

Acquisition of the pathogen at the farm or feedlot can then lead to infected animals entering slaughter plants. Prevalence of *E. coli* O157:H7 on hides and feces is directly correlated with carcass contamination where meat can become contaminated from the environment or hide in the abattoir (Elder *et al.*, 2000). Foods other than meat become contaminated at

multiple stages during growing, packaging or distribution prior to reaching the consumer (Beier *et al.*, 2004). As contaminated food reaches the retail market, many meat-borne outbreaks can be traced back to improper cooking and handling of raw meat (Besser, 1999). *E. coli* O157:H7 is thermal sensitive and thorough cooking of beef to an internal temperature of at least 65°C for at least 7 min has been shown to be a reliable method of eliminating this pathogen (Juneja *et al.*, 1997). Unfortunately, *E. coli* O157:H7 is not inactivated if foods are served raw such as fresh produce and proper hygienic measures must be implemented to prevent cross-contamination of these foods (Russell *et al.*, 2000).

1.1.5. Factors affecting fecal shedding of *E. coli* O157:H7

No single factor has been linked to fecal shedding of *E. coli* O157:H7 in cattle although several factors have been proposed to play a role. Previous studies observed a seasonal trend in shedding of *E. coli* O157:H7 in feces where shedding peaked in the summer months (Bach *et al.*, 2002; Stanford *et al.*, 2005a). Consequently, most human infections associated with *E. coli* O157:H7 are higher in mid-summer, yet clusters of human O157 infections have also coincided with local fecal shedding peaks observed in spring and late summer (Chapman *et al.*, 1997). Several cattle trials have documented atypical shedding patterns in cattle where prevalence of *E. coli* O157:H7 decreased in the summer (Berry *et al.*, 2010) or increased during cold months (Lahti *et al.*, 2003), suggesting that factors other than seasonality may be involved in the etiology of this bacterium in feedlots. Fecal shedding of *E. coli* O157:H7 has been found to persist longer in calves than adults (Cray and Moon, 1995) and calves after weaning shed *E. coli* O157:H7 for longer periods than adult cattle (Ferens and Hovde, 2011).

Transportation has also been found to be a source of stress to cattle (Schwartzkopf-Genswein *et al.*, 2007) and prevalence of *E. coli* O157:H7 was found to increase from 50.3% to

94.4% on hides of cattle transported from the feedlot to the processing plant (Arthur *et al.*, 2007). Many factors associated with transport such as transit time, loading density, temperature, and humidity have previously been linked to increased stress and fecal shedding of *E. coli* O157:H7, although feedlot pen conditions may play a larger role than transport in hide contamination (Stanford *et al.*, 2011a).

Housing including the type of bedding, density and management factors associated with individual farms may contribute to shedding of *E. coli* O157:H7 (Synge *et al.*, 2003; Doyle and Erickson, 2006). Cattle housed at high density as compared to cattle housed in a larger area had higher prevalence of *E. coli* O157:H7 (Vidovic and Korber, 2006) and feedlots associated with larger numbers of cattle had a greater probability of containing shedding cattle (Gunn *et al.*, 2007). Fluctuations in *E. coli* O157:H7 prevalence among pens has also been attributed to the presence of individual high-level shedders termed super-shedders which have been suggested to play an important role in shedding dynamics within pens.

1.1.5.1. Super-shedders

Super-shedders are cattle that are thought to comprise a small proportion of the population and excrete *E. coli* O157:H7 in their feces $> 10^4$ colony-forming units (CFU)/g (Chase-Topping *et al.*, 2008). Previous studies found that only 9% of cattle were considered super-shedders yet these individuals accounted for >96% of the total *E. coli* O157:H7 isolated from cattle feces (Omasakin *et al.*, 2003). A Scottish study reported that 80% of *E. coli* O157:H7 transmission was associated with 20% of cattle that were super-shedders (Matthews *et al.*, 2006). The presence of super-shedders in the commercial feedlot pens was found to increase the prevalence of *E. coli* O157:H7 in low-shedding pen mates (Stephens *et al.*, 2009) and animal-to-animal contact was more important than pen-floor contamination for transmission of *E. coli*

O157:H7 (Stanford *et al.*, 2011b). This suggests that highly colonized individuals may play an important role in dissemination of *E. coli* O157:H7 among cattle. Strong associations have been made between high level shedding and hide contamination suggesting that elimination of high-shedding events could reduce contamination at the abattoir (Arthur *et al.*, 2009). The cause of super-shedding is currently unknown although several factors including the ability to colonize the RAJ (Cobbold *et al.*, 2007) and microbiota within the GI tract may allow *E. coli* O157:H7 to persist in some individuals (Arthur *et al.*, 2010). Microbial populations in the intestinal tract of cattle are influenced by diet, and consequently diet may play a key role in the fecal shedding of *E. coli* O157:H7.

1.1.5.2. Diet and *E. coli* O157:H7

The correlations among diet types and prevalence and intensity of shedding of *E. coli* O157:H7 have been inconsistent. Early studies in the late nineties found that sheep inoculated with *E. coli* O157:H7 and fed hay-based diets shed longer than individuals fed grain (Hovde *et al.*, 1999). The numbers of *E. coli* O157:H7 shed increased after sheep were abruptly switched from corn to hay-based diet yet shedding decreased with the opposite change suggesting the hay-based diet was contributing to shedding of *E. coli* O157:H7 (Kudva *et al.*, 1997). It was proposed that ruminants are induced to shed *E. coli* O157:H7 after abrupt dietary changes and that a switch in nutrient composition can create alterations in the GI environment of ruminants favourable to the proliferation or clearance of *E. coli* O157:H7 (Kudva *et al.*, 1995). The increased fibre and decreased nutrient content found in hay was suggested to decrease the production of volatile fatty acids (VFA) thereby increasing intestinal pH and creating an environment more favourable for the growth of *E. coli* O157:H7 compared to ruminants fed low-fibre high-grain diets (Kudva *et al.*, 1997). Several researchers during this time found an opposite effect where

cattle fed grains shed more generic *E. coli* than cattle fed hay (Russell *et al.*, 2000). Cattle fed grain were found to have a lower colonic pH and it was suggested that feeding cattle grain which is high in starch, increases fermentation and VFA production in the colon, promoting the establishment of acid-resistant *E. coli* (Diez-Gonzalez and Russell, 1998). Early studies regarding acid-resistant *E. coli* have been strongly debated as it was determined that generic *E. coli* behaved differently in bovine hosts than *E. coli* O157:H7, with it also being suggested that *E. coli* O157:H7 acid-resistance is independent of diet (Grauke *et al.*, 2003).

Feed withdrawal or starvation has been considered to affect shedding of *E. coli* O157:H7 as decreased digestion and VFA found during transport may increase proliferation of *E. coli* O157:H7. However, studies showed that fasted cattle had no effect on the shedding of *E. coli* O157:H7 in feces (Callaway *et al.*, 2009). The combination of fasting and type of diet may be correlated as cattle fed barley or forage-based diets did not differ in fecal shedding of *E. coli* O157:H7 yet fasting and re-feeding of forage increased the number of cattle positive for *E. coli* O157:H7 (Buchko *et al.*, 2000a). Cattle are fed high grain diets to maximize growth performance and production efficiency prior to slaughter and studies suggest the type of finishing diet fed to cattle may impact fecal shedding of *E. coli* O157:H7. The number of cattle positive for *E. coli* O157:H7 was higher for cattle fed barley compared to those fed corn or cottonseed and barley (Buchko *et al.*, 2000b). Similarly, the prevalence and number of *E. coli* O157:H7 shed was higher in barley-fed cattle compared to corn-fed cattle (Berg *et al.*, 2004). No difference was observed in survival or rate of decline in the number of *E. coli* O157:H7 in feces from cattle fed barley or corn suggesting persistence in feces did not differ among diet treatments (Bach *et al.*, 2005a). These studies (Buchko *et al.*, 2000b; Berg *et al.*, 2004; Bach *et al.*, 2005a) consistently found that cattle fed barley had an increased fecal pH compared to corn-fed animals, likely as a result of more of the starch in barley being digested in the rumen and more of the starch in corn being

digested in the colon. The type of processing applied to the grain may also have an effect on shedding as cattle fed dry-rolled grains (sorghum or wheat) had less *E. coli* O157:H7 compared to cattle fed steam flaked grains and it was suggested that dry-rolling may allow more substrate to reach the hindgut contributing to an inhospitable environment for *E. coli* O157:H7 (Fox *et al.*, 2007). Other studies determined that prevalence of *E. coli* O157:H7 was higher for cattle fed steam-flaked corn compared to dry-rolled corn yet fecal starch concentration and pH was not related to the prevalence of *E. coli* O157:H7 (Depenbusch *et al.*, 2008). Recent studies examining distiller's grains as a feed source for cattle have contributed to the debate as these by-products have very low starch content, but may still have an effect on fecal shedding of *E. coli* O157:H7.

1.1.5.3. Distiller grains

Distiller grains are by-products of the bioethanol industry where starch from grains is fermented to produce ethanol and the remaining nutrients are concentrated three-fold (Klopfenstein *et al.*, 2008). Due to availability, grains used for ethanol production are primarily corn in the United States and wheat in Canada, but sorghum, rye, triticale and barley have also been used as substrates for ethanol production with nutrient content of distiller grains varying with grain type (Mustafa *et al.*, 2000). Feeding up to 40% dry distiller grains with solubles (DDGS) or wet distiller grains with solubles (DGS) fermented from corn to cattle has been found to increase both weight gain and efficiency of feed utilization compared to cattle fed traditional grain diets (Ham *et al.*, 1994). Wheat DDGS had a similar average daily gain (ADG) and gain:feed ratio (G:F) compared to barley for cattle fed at 20% of the dry matter (DM) intake (Gibb *et al.*, 2008). Other studies determined that replacing up to 40% barley grain DM with corn or wheat DDGS can increase G:F and decrease days on feed (DOF) with no detrimental effect on meat quality (Walter *et al.*, 2010).

Feeding cattle corn DDGS has been linked to increased fecal shedding of *E. coli* O157:H7. Cattle fed 25% DDGS shed increased numbers of *E. coli* O157:H7 compared to cattle fed traditional corn-based diets and increased numbers of *E. coli* O157:H7 were found in fecal fermentations where DDGS was used as a substrate although no effect on the growth of *E. coli* O157:H7 was found (Jacob *et al.*, 2008a). This group of researchers also documented an increase in prevalence of *E. coli* O157:H7 in feces from cattle fed wet DGS but this association was only significant on one sampling day (Jacob *et al.*, 2008b). Two mechanisms have been suggested as contributing to increased prevalence of *E. coli* O157:H7: 1) that DGS can alter the hindgut ecology providing a more hospitable environment for the pathogen or 2) that a component in DGS can stimulate growth of *E. coli* O157:H7 (Jacob *et al.*, 2009a). Distiller grains have most of the starch removed during the fermentation process so there is less starch and secondary fermentation in the cattle hindgut compared to conditions where complete grains are fed (Speihs *et al.*, 2002). Cattle fed finishing diets containing wet corn DGS had a higher fecal pH and decreased levels of L-lactate and higher prevalence of *E. coli* O157:H7 in feces and on hides (Wells *et al.*, 2009). An increase in pH, decrease in L-lactate concentration and increase in *E. coli* O157:H7 prevalence was also found in manure slurries from cattle fed wet CDGS, an outcome that suggests that the antimicrobial activity associated with L-lactate may decrease *E. coli* O157:H7 concentrations (Varel *et al.*, 2008). The type of diet fed with DGS may also play a role in *E. coli* O157:H7 survival in manure slurries as this bacterium was shed longer in cattle fed dry-rolled corn + 40% wet corn DGS as compared to only dry rolled corn. In contrast, the persistence of *E. coli* O157:H7 in cattle fed high moisture corn+40% wet corn DGS did not differ compared to those fed only high moisture corn (Varel *et al.*, 2010). The form of DG (wet or dry) had no significant effect on *E. coli* O157:H7 shedding, but cattle fed 40% corn DG had higher prevalence with more exhibiting a super-shedder status than those fed 20% or no corn DG, suggesting that

level of corn DG in the diet may also impact shedding (Jacob *et al.*, 2010). Ruminal and fecal bacterial populations were found to differ for animals fed corn DDGS compared to control cattle and DDGS may impact the microbial ecology of individual animals (Callaway *et al.*, 2010). Studies examining the impact of corn DGS on fecal shedding of *E. coli* O157:H7 have been inconsistent as others have found no impact of inclusion of these by-products in the diet. Feeding cattle 20% wet DGS with steam flaked or dry-rolled corn had no effect on fecal prevalence of *E. coli* O157:H7 (Edrington *et al.*, 2010). Other studies reported *E. coli* O157:H7 was not associated with cattle fed 20% corn DDGS although this team lacked a 0% corn DDGS control (Swyers *et al.*, 2011). Another group of researchers found a lack of association between *E. coli* O157:H7 and cattle fed 25% corn DDGS in contrast to their previous studies and suggested that differences among DDGS sources may contribute to inconsistencies among studies (Jacob *et al.*, 2009b). The composition of DDGS has been found to differ among bioethanol plants (Nuez-Ortin and Yu, 2010) due to factors such as type of fermentation, drying temperature and oil extraction (Spiehs *et al.*, 2002). The type of DDGS may affect fecal shedding of *E. coli* O157:H7. In Canada, DDGS are principally made from wheat and contain approximately half the oil content and more protein than corn DDGS (Gibb *et al.*, 2008). There are no studies on the effect of feeding WDDGS and the prevalence or persistence of *E. coli* O157:H7 in cattle. As seen with other types of diets, there is no consistent explanation for variability among studies but unique dietary components may impact the prevalence and shedding of *E. coli* O157:H7 in cattle.

1.1.6. Diet manipulation and *E. coli* O157:H7

Pre-harvest diet interventions may provide a cost-effective means of reducing *E. coli* O157:H7 in cattle prior to slaughter. Plants contain phenolic acids which may have antimicrobial properties and administration of cinnamic, coumaric or ferulic acids common to forage plants

were found to decrease the shedding of *E. coli* O157:H7 in cattle feces (Wells *et al.*, 2005). Tannins which are plant phenolic compounds, reduced shedding of *E. coli* O157:H7 in steers although this reduction was only apparent on certain days (Min *et al.*, 2007) and other studies reported chestnut tannins were not effective in decreasing *E. coli* on cattle hides or in the lower GI tract (Gutierrez-Banuelos *et al.*, 2011). Seaweed extracts, a potential prebiotic have been effective in reducing the intensity and duration of *E. coli* O157:H7 shedding in lambs (Bach *et al.*, 2008) suggesting certain components in plants may play a role in establishment and maintenance of intestinal bacterial populations.

Feeding competitive exclusion or probiotic direct-feed microbials have been found to reduce the shedding of pathogens by cattle. Cattle fed *Lactobacillus acidophilus* over two years were found to shed 35% less *E. coli* O157:H7 than untreated cattle (Peterson *et al.*, 2007a). Yeasts such as *Saccharomyces cerevisiae* have been found to reduce *E. coli* O157:H7 survival in simulated GI conditions (Etienne-Mesmin *et al.*, 2011). A group of colicin-producing *E. coli* strains were found to inhibit *E. coli* O157:H7 as well as other types of non-O157 pathogenic strains (Schamberger and Diez-Gonzalez, 2004). Inclusion of some antibiotics that target gram positive bacteria in the diet, such as ionophores, has also been suggested to potentially increase the proportion of gram-negative organisms such as *E. coli* O157:H7 in the gut but studies have found either no effect (McAllister *et al.*, 2006; Van Baale *et al.*, 2004) or a reduction (Paddock *et al.*, 2011) in *E. coli* O157:H7 after supplementation with monensin, tylosin or ractopamine. In some instances, manipulation of cattle diets shows promise as a means of reducing the shedding of *E. coli* O157:H7 in cattle. However, results are inconsistent and highlight the complexity of the interplay of factors that influence the shedding phenomenon. Consequently, more direct mitigation methods have been sought that are targeted specifically at *E. coli* O157:H7.

1.1.7. Direct methods for reducing *E. coli* O157:H7 in cattle

1.1.7.1. Vaccines

Vaccination as a direct mitigation method to prevent carriage of *E. coli* O157:H7 in cattle has been explored with some success. Two types of vaccines that target type III secretion proteins or siderophore receptor proteins (SRP) have been explored. Type III secretion proteins are involved in attachment of *E. coli* O157:H7 to host cells, so ideally antibodies targeting these proteins would decrease colonization of the pathogen. Cattle administered a vaccine targeting type III secretion proteins were 98.3% less likely to be colonized by *E. coli* O157:H7 at the terminal rectum, although three vaccinations were required for this strategy to be effective (Peterson *et al.*, 2007b). In another study, this group of researchers determined that cattle receiving one, two or three doses increased vaccine efficacy by 68, 66 and 73% respectively, compared to unvaccinated cattle, suggesting vaccine effectiveness was booster dependent (Peterson *et al.*, 2007c). These researchers also suspected herd immunity may play a role as unvaccinated cattle shed less *E. coli* O157:H7 if they were penned with vaccinated cattle. A two-dose regimen of a type III secreted protein vaccination in cattle was found to effectively reduce fecal shedding of *E. coli* O157:H7 by 63% and hide contamination by 55% compared to a placebo (Smith *et al.*, 2009).

Vaccines targeting SRP theoretically generate antibodies that interact with the outer membrane of the *E. coli* O157:H7 and effectively block vital iron-transport into the cell leaving the pathogen at a competitive disadvantage in a mixed microbial environment (Thomson *et al.*, 2009). A SRP vaccine was found to reduce fecal shedding of *E. coli* O157:H7 in cattle by 85.2% by day 98 but required three vaccinations (Thomson *et al.*, 2009). Another group of researchers found SRP-vaccinated cattle had reduced prevalence and duration of shedding of *E. coli* O157:H7

(Fox *et al.*, 2009). This study also found that cattle shedding high concentrations of *E. coli* O157:H7 shed the pathogen for less days when administered the SRP vaccine and suggested that reducing the number of high-shedding animals by vaccination may be an effective pre-harvest strategy. Two vaccine products have been conditionally approved for use in the United States and Canada but these vaccines require multiple doses which is expensive, requiring direct handling of the cattle and forcing cattle to be retained in the same feedlot during the vaccination program (Snedeker *et al.*, 2012). In contrast, bacteriophage therapy may provide a cost-effective natural strategy for reduction of *E. coli* O157:H7 in cattle.

1.2. Phages for control of zoonotic bacteria

1.2.1. Introduction to phages

Bacteriophages (phages) are natural infectious agents of bacteria and represent a significant factor in limiting bacterial populations (Letarov and Kulikov, 2009). Phages can exist in two states, virulent or lysogenic (temperate; Kutter and Sulakvelidze, 2005). Virulent phages adsorb to the surface of a host cell, inject their genome into the host and use the host machinery to create new phage proteins. The phage proteins are assembled within the host and ultimately lyse and kill the bacteria to release new phage progeny. Temperate phages can exist in a lysogenic state where the phage integrates as a prophage within the host genome. The temperate phage can co-exist within the host indefinitely until induced into the lytic cycle. Virulent phages are ideal candidates for phage therapy and bio-control since they do not integrate into the host and can lyse and kill targeted bacterium. The infection process of most phages follows a single-step growth curve and the efficiency and timing of the process is unique for each phage type and may depend on conditions such as host, medium and/or temperature (Kutter and Sulakvelidze, 2005). The number of phages remains constant for an eclipse period

where the phages initiate contact and adsorb to host cells until the latent period commences where the number of phages rise sharply as the cells lyse and liberate completed phages. Bacteria that are infected during exponential growth display shorter latent times where phage replication rates are maximal. Bacteria that are infected past the exponential growth phase display longer latent periods which result in less efficient phage infection (Abedon *et al.*, 2008). The multiplicity of infection (MOI) is the ratio of phage to bacteria that is required for efficient lysis of the targeted bacterium and replication of phages. If the number of phages is too high and lysis occurs too quickly, not enough phages will be produced to effectively carry on the infection cycle and if there are not enough phages, lysis is slow and the explosive replication of phages does not occur. Phages with low MOI's are considered highly lytic and are ideal candidates for phage therapy (Niu *et al.*, 2009a).

1.2.2. *Early human phage therapy*

Since the discovery of phages, their potential for use in antibacterial therapy was immediately recognized. Initially these "invisible microbes" described by Felix d'Herelle from Paris, France, were found in high titers in patients with severe illness and increased in titre during patient recovery. It was suspected that these particles were responsible for recovery of patients afflicted with severe bacterial diseases, leading to the assessment of phages for their therapeutic potential. The success and initial enthusiasm of early experimentation with phage therapy led to phage treatment for bovine septicemia, Cholera, and Staphylococcal wound infections in humans, as well as other bacterial infections (Summers, 2001). Poor knowledge of phage biology in addition to lack of a standardized preparation protocol for phages has led to several clinical failures. Furthermore, the discovery of antibiotics and onset of World War II diverted early phage therapy research in the western world, but research on phage therapy

continued to progress in Eastern Europe and the former Soviet Union. Although several clinical trials in the Soviet Union were deemed a success, the limited research from this era that has been published has been criticised for poor experimental design, lack of detail and the absence of proper controls (Hanlon, 2007). Recently, the surge in antibiotic resistant micro-organisms, advances in microbiology and knowledge of phage ecology has led to re-examination of phage for use in prophylactic and therapeutic applications. In particular, the use of phages targeted at *E. coli* O157:H7 for pre-harvest therapy in animals had been explored.

1.2.3. Early studies on phage therapy

The effectiveness of phage in controlling *E. coli* in calves, piglets and lambs was demonstrated in the 1980's. Treatment with a mixture of two phages reduced enteropathogenic *E. coli* (EPEC) strains introduced in the alimentary tract of calves and lambs and cured diarrhoea in piglets (Williams Smith and Huggins, 1983). Similarly, the use of seven highly active phages against EPEC cured or prevented experimentally induced diarrhoea in calves through a single dose of 10^5 phage or if it was sprayed on calf litter in a 12.0 m² room at a dose of 10^2 phage organisms (Williams Smith *et al.*, 1987a). These researchers encountered the emergence of phage resistant *E. coli* mutants which they suspected were detrimental to the success of phage therapy. To address this problem, they postulated that since the K1 antigen in *E. coli* is required for infection, by using anti-K1 coliphages, any mutant emerging would likely lack the K1 antigen (K-) and therefore be less virulent than the (K+) parent strain (Williams Smith *et al.*, 1987a). Although only K- *E. coli* mutants emerged in calves infected with individual phage, several K+ phage-resistant bacteria which were as virulent as their parent strains emerged in mixed infections. Nonetheless, these K+ resistant bacteria could be controlled by the use of mutant phages derived from the parent strain. Results from subsequent work with oral administration

of phage with CaCO₃ in feed suggested that the buffer reduced the negative effect of the acidic environment of the stomach on phage infectivity. Further determination of optimal temperatures for phage virulence identified the conditions required for the effective therapeutic use of phages targeted at *E. coli* in cattle (Williams Smith *et al.*, 1987b). More recently, advances in molecular biology have allowed researchers to further characterize potential phages for therapeutic and bio-control of pathogens *in vivo*.

1.2.4. *In Vivo phage therapy targeting non-O157 pathogens*

Phage therapy targeted at bacterial pathogens has had some success *in vivo*. Phages have been examined for therapeutic use *in vivo* for *Enterococcus*, *Pseudomonas*, *Staphylococcus*, *Klebsiella*, *Salmonella*, *Campylobacter*, pathogenic *E. coli* and *Listeria*. A single low dosage of phage EF24C was found to efficiently treat mice infected with vancomycin-resistant *Enterococcus* (Uchiyama *et al.*, 2008). Oral administration of phage KPP10 to mice infected with *Pseudomonas aeruginosa* significantly lowered the number of viable cells found in fecal matter shed from the gastrointestinal tract of infected mice compared to saline treated mice (Watanabe *et al.*, 2007). Phage-treated mice also had lower numbers of *P. aeruginosa* in their blood, liver and spleen suggesting that phage administration may be effective in reducing gut-derived sepsis. Prophylactic administration of phage to immunosuppressed mice infected with *Staphylococcus aureus* significantly lowered host cell numbers and stimulated neutrophils, myelocytic and lymphocytic lineages and specific agglutinins which are beneficial to the immune system (Zimecki *et al.*, 2009). Phages targeted at *Klebsiella pneumoniae* have been successfully replicated in mice with high phage titers in the blood, kidney and urinary bladder. These phages may be useful as a prophylactic or therapeutic agent for the treatment of catheter associated urinary tract infections caused by *Klebsiella* (Verma *et al.*, 2009).

The efficacies of phage therapies targeted at *Salmonella* have been mixed. Hurley et al. (2008) found that phage treatment did not reduce *Salmonella* shed by chickens. Borie et al. (2009) found that administration of phage to commercial chickens only reduced *Salmonella* by 80% but that a combination of a competitive exclusion product and phage reduced the incidence of *Salmonella enterica* to 38.7% in the treatment group as compared to all individuals infected within the control group. Compared to controls, levels of *Salmonella enterica* contaminating the cecum of chickens were reduced 24 and 48 h after cloacal or oral administration of phage WT450 (Andreatti et al., 2007).

A single 7-log plaque-forming units (PFU) dose of phage CP220 administered to broiler chickens reduced *Campylobacter jejuni* by 2-log CFU after 48h (El-Shibiny et al., 2009). To achieve a similar reduction in the number of broiler chickens infected with *Campylobacter coli*, a 9-log PFU dose was required. Previous studies of phage CP8 and CP34 administered in an antacid suspension to broiler chickens found a 0.5-5 CFU log reduction in *C. jejuni* compared to controls (Carillo et al., 2005).

A virulent phage isolated from sewage which attaches to the K1 capsular antigen of *E. coli* was administered orally to colostrum-deprived calves and delayed the appearance of *E. coli* K1+ bacterium in the blood (Barrow et al., 1998). This phage has been used to prevent septicemia and meningitis in chickens caused by K1+ strains of *E. coli*. Prophylaxis and therapeutic phage therapy have been examined for their ability to reduce numbers of enterotoxigenic *E. coli* (ETEC) in pigs. Three phages tested as prophylactics significantly reduced the severity of diarrhea in pigs and a mixture of two phages given therapeutically significantly improved composite diarrhea scores in these animals (Jamalludeen et al., 2009). T4-like phages

added to drinking water were able to lyse EPEC cells in both conventional and axenic mice (Chibani-Chennoufi *et al.*, 2004).

Phage P100 was applied to the surface of contaminated cheese and a significant reduction of at least 3.5-log or complete eradication of *Listeria monocytogenes* was achieved (Carlton *et al.*, 2005). Accordingly, on August 18, 2006 the U.S. Food and Drug Administration approved the use of a combination of six phages as antimicrobial agents targeted for *Listeria* in ready-to-eat meat and poultry products (Housby and Mann, 2009). These phages have been deemed safe for the public and can be sprayed onto meat products with activity against over 170 different strains of *Listeria*. The recent commercialization of phages will aid in broadening phage research and applications as phage therapy and bio-control agents can be explored in a new light.

1.2.4.1. *In Vivo* phage therapy targeting *E. coli* O157:H7

Currently, phage targeting *E. coli* O157:H7 in animals have had limited success *in vivo* and face similar challenges to those previously described for therapeutic control of other pathogens. *E. coli* O157:H7 concentrations were reduced in mice after daily administration of a phage cocktail isolated from feces of stock animals and sewage containing SP12-21-22, but differences in *E. coli* O157:H7 concentrations between control and phage-treated mice were less apparent after 9 days (Tanji *et al.*, 2005). Phages SH1 and KH, isolated from bovine feces, eliminated *E. coli* O157:H7 from the feces of mice after 2-6 days and reduced *E. coli* O157:H7 in steers as compared to control steers but did not eliminate it from the majority of steers (Sheng *et al.*, 2006b). Phage therapy studies in sheep have been mixed with some phages reducing *E. coli* O157:H7 shedding (Raya *et al.*, 2006; Bach *et al.*, 2009; Callaway *et al.*, 2008a) while other phages had no effect (Bach *et al.*, 2003; Sheng *et al.*, 2006b). Encapsulated phage administered

to steers did not reduce shedding of experimentally inoculated *E. coli* O157:H7 compared to control steers but did reduce the duration of shedding (Stanford *et al.*, 2010).

1.2.5. Current issues and challenges

Some of the challenges facing recent phage therapy experimentation for success *in vivo* are not unlike those of the past such as specificity of phage to target strains, the optimum phage and host concentrations, a suitable delivery method to the host and the emergence of phage-resistant mutants. Current efforts to improve the efficacy of phage will increase the likelihood of this approach being a successful method for phage therapy *in vivo*.

1.2.5.1. Specificity of phage to target strains

In order for phage therapy to be successful, selected phages must be highly specific for their targeted bacteria. Commensal bacterial must not be disrupted by therapeutic phages as these bacteria are part of the microbiota in the gastrointestinal tract. In one of the first studies to ensure *E. coli* O157:H7 specificity, phages were screened for their ability to bind O157 antigens, and *E. coli* antigens commonly bound by receptors such as those found on pili, fimbriae, flagella, core lipopolysaccharides (LPS) and other outer membrane proteins (Kudva *et al.*, 1999). The selected O157-specific phages, KH1, KH4 and KH5, successfully lysed all of the *E. coli* O157:H7 strains tested and did not lyse any non-O157 strains or O157-deficient mutant *E. coli* strains. In another study, a potential therapeutic phage, PP01, was isolated from a swine stool sample positive for *E. coli* O157:H7 as it was theorized that phages isolated directly from O157 infected hosts would be more O157-specific (Morita *et al.*, 2002). Phage PP01 successfully infected only O157 strains and not K12 or other O- serotype strains, but a limited range of bacterial strains were examined in this study. Another team of researchers characterized two novel coliphages, MVBS and MVSS, for their specificity for lysis of a large collection of *E. coli*

strains of various serotypes and origins including humans, bovine, buffalo, swine, ovine, deer and rabbit. (Viscardi *et al.*, 2008). MVBS and MVSS displayed broad host ranges by infecting 94.2% of pathogenic isolates which included *E. coli* serotypes O157, O26, O91, O103, O111, O113, O121, O55 and O145. However phages, MVBS and MVSS, also displayed plaque-forming ability in 13.9% and 20.8% respectively, of non-pathogenic *E. coli* isolates. Others studies determined that phenotype of *E. coli* strains may influence susceptibility to phage. The host range of four phages, rV5, wV7, wV8 and wV11, was evaluated against a collection of bovine and human STEC isolates previously characterized by pulsed field electrophoresis (PFGE) and phage typing (PT) (Niu *et al.*, 2009a). Isolates that were sensitive to rV5 or wV11 were genetically different than those that were phage resistant and a high degree of relatedness was found among sub-categories of isolates susceptible to phage wV8. Phage wV7 lysed all isolates irrespective of PFGE subtype or PT. Results from this study demonstrate the unique host range of phages and emphasize the importance of selecting phage cocktails which effectively lyse target strains. The specificity of phages may also differ *in vitro* as compared to *in vivo*. Four T4-like coliphages analyzed for their combined host range targeted 46% of non-pathogenic strains *in vitro* but the normal microbiota was only minimally affected in mice *in vivo* (Chibani-Chennoufi *et al.*, 2004). It was suggested that the resident *E. coli* bacteria may have an altered physiological state or may possess physical factors that prevent or limit phage infection. Understanding the competitive and/or inhibitory factors affecting phage specificity is essential for phage therapy to be applicable at a practical level.

1.2.5.2. Optimum phage and host conditions

The amount of phage and conditions required for pre-harvest control of *E. coli* O157:H7 from the host is unique for each phage and should be considered when selecting phages for

therapy. In addition, doses must promote viral replication and be cost-effective. Studies to determine the most efficacious dose for phage therapy found that a 1:1 ratio of phage to bacteria was more effective at reducing *E. coli* O157:H7 in sheep than either a 10:1 or 100:1 dose, with the authors suggesting that an excess phage to host ratio may interfere with phage replication or attachment (Callaway *et al.*, 2008a). Most *in vitro* phage studies that have been successful at reducing *E. coli* O157:H7 have had a high MOI, required multiple doses and/or aeration or may not be effective at certain temperatures. Phage studies using O157-specific phages were effective in lysing all O157 bovine or ovine strains tested but required aeration and a high MOI (Kudva *et al.*, 1999). Without aeration, complete bacterial lysis occurred at 4°C but not at 37°C and researchers hypothesized that low temperatures resulted in low levels of bacterial growth which increased phage adsorption and infection. Phages that require lower temperatures or aeration for infection of hosts are not ideal candidates for *in vivo* therapy in animals due to higher internal temperature and the anaerobic environment of the ruminal gut, but they may be suitable candidates for environmental bio-control. The O157-specific phage, DC22, was successful in eliminating *E. coli* O157:H7 within 4 h of treatment in clarified rumen fluid, but phage numbers declined following administration and it was suggested that premature lysis of *E. coli* O157:H7 may have been occurring due to a high MOI (Bach *et al.*, 2003). Another phage cocktail consisting of phages PP01, e11/2 and e4/1c isolated from human and bovine feces effectively reduced *E. coli* O157:H7 *in vitro* at 37°C, but once again, required a high MOI of 10⁶ (O’Flynn *et al.*, 2004). A T-Even phage, CEV1, isolated from sheep showed potential for phage therapy as this phage was able to lyse *E. coli* O157:H7 in medium both aerobically and anaerobically and a single dose reduced *E. coli* O157:H7 *in vivo* (Raya *et al.*, 2006). In continuous cultures maintained within a chemostat, repeated administration of phages SP15-21-22 was required to effectively reduce *E. coli* O157:H7 concentrations, but researchers were unable to

eradicate *E. coli* O157:H7 from the culture (Tanji *et al.*, 2005). Each phage has unique physiological properties that present challenges for optimization of their efficacy against target bacteria residing in different environments.

1.2.5.3. Method of delivery

In order for phage therapy to be successful in living systems, phage must be viable when they reach target bacteria. Several methods for administration of phage to *E. coli* O157:H7 have been investigated. Oral administrations of 10^{13} PFU of phage DC22 resulted in a decline of phages to $5.98 \log_{10}$ PFU in feces from sheep after 3 days of treatment and was undetectable after 13 days (Bach *et al.*, 2003). There was no difference in *E. coli* levels between DC22 treated and control groups so it was suspected that the administration of phage did not promote lysis of *E. coli* O157:H7. The lack of phage-mediated lysis was attributed to non-specific binding of phage in the digestive tract, competitive interference or inactivation of phage prior to reaching the lower intestine.

Stability of phages under acidic conditions has been investigated. Phages SP15-21-22 were only slightly active at pH 4, only half were viable at pH 3 and considerably less were viable at pH 2 (Tanji *et al.*, 2005). To protect phage from the acidic nature of the stomach, phage cocktails have been suspended in CaCO_3 to create a cocktail of \sim pH 4 prior to administration to mice. High titers of phage were detected in mice feces but daily administration was required to ensure that viable phage remained in the intestinal tract.

Other studies have investigated the administration of phage to the RAJ, as opposed to oral administration, since this is the primary site of colonization for *E. coli* O157:H7. Application of phage directly to the RAJ mucosa in conjunction with a continuous supply in drinking water reduced the number of *E. coli* O157:H7 in cattle (Sheng *et al.*, 2006b). Oral administration of

phage resulted in higher phage titers than rectally applied phage and it was suggested that oral administration of phage increased contact time of phages with *E. coli* O157:H7 cells and that the phage would have a greater opportunity to replicate and increase in number under these conditions (Rozema *et al.*, 2009). Although the RAJ is the primary site of colonization of *E. coli* O157:H7, rectal application of phages did not introduce phage into the upper regions of the gastrointestinal tract where *E. coli* O157:H7 may also reside (Naylor *et al.*, 2003). Higher mean phage levels were found in feces when phages were both orally and rectally administered as compared to rectal administration alone. Phages were also detected in manure from the pen and isolated in control steers so this study suggested that some steers may acquire phage from the environment and consequently stability of phage in the environment could be exploited as a means of lowering *E. coli* O157:H7 in the feedlot environment.

Further studies on the persistence of phage in the environment and potential administration methods have been investigated. Phages were detected in pooled fecal pats (26.5%), fecal grabs (23.8%), water troughs (21.8%) and pen floor slurry samples (94.6%) demonstrating that phage can be widely distributed within the feedlot environment (Niu *et al.*, 2009b). Prevalence of phage was highest in manure slurry which was composed of a mixture of urine, feces, water, spilled feed and bedding, and suggested that administering phage on pen floors may be an effective means of controlling *E. coli* O157:H7 populations in feedlots. Data also suggested that phage prevalence fluctuates in a manner similar to *E. coli* O157:H7 and that fecal shedding of *E. coli* O157:H7 could be reduced if cattle in the pen harboured phage. Further experimentation in sheep experimentally inoculated with *E. coli* O157:H7 found that orally administered phage numbers declined after 21 days and that a delivery system was needed to protect phages during passage through the intestine in order to allow phage to be administered effectively in feed (Bach *et al.*, 2009). Stanford *et al.*, (2010) formulated a delivery system that

would prevent inactivation of phage in the gastrointestinal tract thereby allowing more phage to reach the RAJ. Since many phages lose viability at low pH, a method for phage encapsulation was proposed that would protect phage from the low pH in the digestive tract and release viable phage at pH > 7 found in the ileum. Cattle received encapsulated phage either orally in gelatin capsules (bolus) or top-dressed in feed, resulting in acceptable levels of viable phage in feces from cattle on each treatment at 1.82 and 1.13×10^9 PFU/g, respectively. However, encapsulated phage did not reduce shedding of experimentally inoculated cattle compared to the control. These researchers suggested that phages may have by-passed regions anterior to the ileum within the upper digestive tract which may have contained populations of *E. coli* O157:H7. More information regarding phage ecology needs to be explored before these methods of administration can be refined to improve the efficacy of phage therapy.

1.2.5.4. Mechanisms of phage resistance

The presence of phage-resistant hosts compromises the success of phage therapy and as a result understanding the mechanisms whereby the host develops phage resistance could be the key to successful phage therapy. The adherence of phage to the outer membrane of bacterial cells is the first step in bacterial attack and therefore its modification is often the first defence of bacterial cells against phage. Since most phage types have receptors unique to that phage, cocktails of phages must be screened and then selected to reduce the likelihood of emerging resistant host cells. In one study, strains that were resistant to phage infection were found to be dependent on the nature of the LPS layer as truncated and abundant mid-range-molecular weight LPS mutants did not support plaque formation (Kudva *et al.*, 1999). Other studies have determined that *E. coli* O157:H7 mutants resistant to phage PP01 had lost their outer membrane protein OmpC (Morita *et al.*, 2002). Restoration of OmpC resulted in *E. coli*

O157:H7 being susceptible to PP01, suggesting that OmpC served as the PP01 receptor. Further studies of phage resistance in *E. coli* O157:H7 have confirmed both LPS and OmpC as possible sites contributing to phage resistance. Deletion of the OmpC outer membrane protein and alteration of the LPS resulted in resistance to the phages, SP21 and SP22, respectively (Tanji *et al.*, 2004). In order to combat phage resistance, a cocktail of both phages SP21 and SP22 collectively were used to infect *E. coli* O157:H7 cells, but new mutants resistant to both phages still emerged. Once again it was suggested that reduced ability of these phages to bind emerging mutant cells was likely a result of interruption in phage adsorption to the cell surface. Phages e11/2, e4/1c, and PP01 were selected to reduce the likelihood of resistant cells forming on meat surfaces but bacteriophage-insensitive mutants (BIM) still emerged at low frequencies (10^{-6}) (O'Flynn *et al.*, 2004). These BIM's exhibited an altered smaller coccoid morphology and commonly reverted to phage sensitivity within 50 generations. The low frequencies of BIM's and their ability to revert to phage sensitivity suggest that phage cocktails should be selected.

1.2.6. Conclusions

Identification of phages that are highly specific to their targeted pathogens is only the first step for successful implementation of these viruses for reduction of bacteria and several challenges or limitations must be addressed. Specific conditions are unique for each phage to survive and replicate in particular, within environments such as the ruminant gut or the farm or feedlot environment. The phages must also be able to interact or compete with other phages and the naturally-occurring microbiota within these environments is currently poorly understood. Future implementation of phage bio-control will require additional knowledge as to how phages thrive in the natural environments in order to be successful.

1.3. Summary

E. coli O157:H7 is a zoonotic pathogen which can result in gastrointestinal illness, severe health complications and even death in humans. The spread or dissemination of this pathogen in the feedlot environment can ultimately lead to contamination of the human food supply. The low infectious dose of this pathogen requires that safe measures be initiated at both the feedlot and abattoir to control this pathogen. Identification of factors that impact the presence of *E. coli* O157:H7 in cattle can aid in the development of precautionary methods to reduce the prevalence of the pathogen in the feedlot environment and during subsequent processing. New methods to eliminate *E. coli* O157:H7 in food prior to distribution to the public will reduce food recalls and improve food safety. In conclusion, development of means to control prevalence of *E. coli* O157:H7 in cattle will lead to a decrease in the number of human infections or outbreaks associated with *E. coli* O157:H7.

The objectives and goals of the thesis were to:

1. Determine the effect of diet on fecal shedding of *E. coli* O157:H7 in naturally colonized cattle
 - I) Determine the effect of wheat DDGS on fecal shedding of *E. coli* O157:H7
 - II) Assess the feed value of an important by-product
 - III) Determine the effect of fecal pH on levels of *E. coli* O157:H7 in naturally colonized cattle
 - IV) Determine the effect of DDGS on persistence of *E. coli* O157:H7 in feces

2. Determine the effect of diet on fecal shedding of *E. coli* O157:H7 in inoculated cattle
 - I) Determine the effect of wheat DDGS on fecal shedding of *E. coli* O157:H7
 - II) Compare experimentally- inoculated animals with naturally- occurring animal studies
 - III) Characterize the longest persisting strains of *E. coli* O157:H7 used in a challenge study

3. Determine the effect of endemic bacteriophages on fecal shedding of *E. coli* O157:H7 in cattle
 - I) Isolate phages specifically targeted to *E. coli* O157:H7
 - II) Identify relationships of endemic phages in cattle with high and low numbers of *E. coli* O157:H7
 - III) Characterize endemic phages

2. CHAPTER TWO

2.1. Effects of wheat or corn distillers' dried grains with solubles on feedlot performance, fecal shedding and persistence of *Escherichia coli* O157:H7

Hallewell, J., T. A. McAllister, J. Thomas, C. W. Booker, S. Hannon, G. K. Jim, L. O. Burciaga-Robles, M. L. May, R. E. Peterson, C. Flaig, E. M. Hussey and K. Stanford. 2012. Effects of wheat or corn distillers dried grains with solubles on feedlot performance, fecal shedding, and persistence of *Escherichia coli* O157:H7. *J. Anim. Sci.* 90:2802-10

2.1.1. Introduction

Expansion of the ethanol industry has led to increased availability of distillers' dried grains with solubles (DDGS) as cattle feed. In Canada, DDGS are principally made from wheat and contain approximately half the oil content and more protein than corn DDGS (Gibb *et al.*, 2008). Feeding corn based distillers grain with soluble (wet or dry) has been shown to improve ADG and G:F in dry-rolled corn based diets (Klopfenstein *et al.*, 2008) with no deleterious effects on carcass quality.

Cattle diets have been investigated for links to fecal shedding and environmental persistence of *E. coli* O157:H7, and it has been postulated that low starch levels in the rumen may raise intestinal pH, reduce concentrations of volatile fatty acids (VFA), and promote *E. coli* growth in the lower digestive tract (Bach *et al.*, 2005b; Jacob *et al.*, 2008b). Conflicting evidence exists related to increased *E. coli* shedding in cattle due to feeding corn DDGS (Jacob *et al.*, 2008a, b; Wells *et al.*, 2009; Edrington *et al.*, 2010) and effects of wheat DDGS on *E. coli* O157:H7 in cattle have been little studied (Yang *et al.*, 2010). The objectives of this study were to determine the effects of feeding wheat or corn DDGS in barley based diets on 1) feedlot performance, carcass characteristics, and animal health in commercial feedlot cattle in western

Canada, 2) *E. coli* O157:H7 shedding in naturally-colonized animals fed WDDGS and 3) fecal pH, and persistence of this organism in the feedlot environment.

2.1.2. Material and Methods

2.1.2.1. *Study facilities*

The study was conducted at a commercial feedlot near Strathmore, Alberta, Canada (113°24'W, 51°C9'N) with a one-time capacity of 30,000 animals. The cattle were housed in standard facilities for western Canada including open-air, dirt-floor pens with central feed alleys and 20% porosity wood-fence windbreaks. All procedures involving live animals were approved by the Feedlot Health Management Services (FHMS) Animal Care and Use Committee with informed consent from the animal owners. Animal handling facilities had a hydraulic chute equipped with an individual animal scale, a chute-side computer with individual animal data collection, management software (*iFHMS*, FHMS, Okotoks, Alberta, Canada) and separation alleys to facilitate the return of animals to designated pens.

2.1.2.2. *Study Cattle*

The cattle utilized in the study were fall-placed, mixed source, auction market-derived male calves. At arrival to the feedlot, animals were subject to standardized animal health management and feedlot production procedures. In brief, each animal received a unique individual animal identification ear tag, a modified-live infectious bovine rhinotracheitis virus, parainfluenza-3 virus, bovine viral diarrhoea virus (types I and II), bovine respiratory syncytial virus and *Mannheimia haemolytica* bacterin-toxoid combination vaccine (Pyramid 5 + Presponse SQ, Boehringer Ingelheim Animal Health, Boehringer Ingelheim Canada Ltd., Burlington, Ontario), a *Clostridium chauvoei*, *septicum*, *novyi*, *sordellii*, *perfringens* Types B, C and D, and

Histophilus somni bacterin-toxoid (Ultrabac 7/Somubac, Pfizer Animal Health), subcutaneous long-acting tulathromycin (Draxxin, Pfizer Animal Health, 0.024 mL/kg. body weight (BW)), and topical doramectin for internal and external parasite control (Dectomax, Pfizer Animal Health, 0.099 mL/kg BW). All intact bulls were banded and animals with retained testicles were surgically castrated. All animals received a hormonal growth implant in the middle third of the ear at an average of 37 and 125 days on feed (DOF), and re-vaccination for infectious bovine rhinotracheitis at 125 DOF.

2.1.2.3. *Experimental design*

Steer calves (n = 6,817) were individually randomly allocated to 3 treatment groups: WDDGS (diets including 22.5% wheat DDGS, DM basis), CDDGS (diets including 22.5% corn DDGS, DM basis) or CTRL (barley substituted for DDGS). Sourcing of cattle for the study required 8 wk and equal numbers of pens for each treatment were filled at each entry of cattle to the feedlot. Standard mixed complete feedlot diets and water were offered *ad libitum* throughout the feeding period and were formulated to meet or exceed the Nutrient Requirements for Beef Cattle (National Research Council, 1996). All cattle were conditioned to high-concentrate finishing diets with the same barley-based step-up rations over a 3 wk period. Subsequently, steers received finishing diets (Table 2.1) as per their treatment group, with feed samples (500 g) collected from bunks on a monthly basis (n = 9) and analyzed by a commercial laboratory (Dairy One, Ithaca NY, USA). Cattle in each treatment group were housed in 10 separate pens with an average of 227 animals/pen (range 152 to 305). Each pen was an experimental unit and the study period was from allocation to slaughter.

Table 2.1. Composition and analyzed nutrient content (DM basis) of diets containing wheat (WDDGS)¹ or corn-based (CDDGS)² dry distillers' grains plus solubles or control (CTRL) in the finishing diet of feedlot steers in western Canada

Item	<i>Treatment Group</i>		
	WDDGS	CDDGS	CTRL
Ingredient, % ³			
Dry-rolled barley	70.58	70.58	93.08
Corn DDGS	0	22.50	0
Wheat DDGS	22.50	0	0
Barley silage	5.00	5.00	5.00
Supplement	1.92	1.92	1.92
Analyzed composition, % DM Basis ⁴			
DM	86.12	86.13	86.28
CP	18.43	17.0	12.50
Ether extract	2.86	4.36	2.52
NDF	18.72	20.51	16.10
ADF	9.30	9.69	7.09
Calcium	0.74	0.70	1.04
Phosphorus	0.59	0.56	0.41
Sulfur	0.38	0.28	0.17

¹WDDGS supplied by Terra-Grain Fuels Inc., Belle Plain, SK.

²CDDGS supplied by Glacial Lakes Ethanol, Mina, SD.

³Monesin (Rumensin[®], Elanco Animal Health, Division Eli Lilly Canada Inc., Guelph, Ontario) and tylosin (Tylan[®], Elanco Animal Health) were added to diets at levels of 25 mg/kg DM and 11 mg/kg DM, respectively.

⁴Bunk samples (500 g) were collected monthly throughout the experiment (n = 9) and the average for those analyses is presented.

2.1.2.4. *Animal health and marketing*

Experienced animal health personnel (blinded to the experimental status of each pen) observed study animals at least once daily. All animal health events including treatment date, presumptive diagnosis, drug(s) administered, and doses were recorded on the chute-side computer system (*iFHMS*). Cattle were sold after pens reached an estimated full weight of 600 kg as per standard feedlot marketing procedures. The animals were shipped for slaughter to local abattoirs and approximately equal numbers of animals from each treatment group were shipped to the same packing plant for slaughter on the same d. At slaughter, the quality grade (QG), yield grade (YG), and hot carcass weight (HCW) were collected by technical staff, with carcass data collected from 2162, 2183 and 2187 cattle from the WDDGS, CDDGS and CTRL treatments, respectively.

2.1.2.5. *Fecal sampling*

Freshly voided fecal pats were collected from the floor of each pen for the first 6 wk and collected on a monthly basis thereafter. Five fecal pats (totaling approximately 400 g) were pooled from the pen floor and placed in a sterile Whirl-Pak[®] (Nasco Canada, Newmarket, Ontario, Canada) bag, with 2 bags of pooled fecal pats collected per pen per sampling. A total of 196 pooled fecal pats were collected per treatment group during the study, with 18 to 20 pooled fecal pats collected per pen depending on slaughter dates of cattle. The samples were placed in a cooler with ice packs and shipped to the laboratory for analysis. Samples were processed at the laboratory within 24 h. To determine fecal pH, 30 g fecal pat sub-samples were weighed into plastic cups with 120 mL distilled water, stirred thoroughly and measured by a portable pH meter (Oakton Acorn, Fisher Scientific, Pittsburg, PA, USA).

2.1.2.6. Enumeration and detection of *E. coli* O157:H7

Contents of each bag were mixed manually before weighing. Duplicate 1 g subsamples of feces were enriched in 9 mL modified *E. coli* broth with 20 mg/L novobiocin (mEC) and incubated for 6 h at 37 °C. Enriched samples were then subjected to immunomagnetic separation (IMS) using anti-*E. coli* O157 Dynabeads® (Invitrogen, Carlsbad, CA, USA) as per manufacturer's instructions. A 50 µL aliquot of bead-bacteria complex was plated on sorbitol MacConkey agar with 2.5 mg/L potassium tellurite and 0.05 mg/L cefixime (CT-SMAC; Daylynn Biologicals, Calgary, Alberta, Canada) and incubated at 37 °C for 18-24 h. Three non-sorbitol fermenting (NSF) colonies, visually identified as clear colonies, were randomly selected for latex confirmation and positive colonies stored and frozen in glycerol. For enumeration of naturally occurring *E. coli* O157:H7, 1:10 dilutions from 1 g sub-samples positive by IMS were added to mEC and 100 µL plated in triplicate on CT-SMAC. Serial dilutions were prepared as necessary to achieve plates containing 30-300 colonies for enumeration. Five random NSF colonies from each plate were tested for the O157 antigen using an O157 latex agglutination kit (Oxoid, Nepean, Ontario, Canada). Confirmed O157 colonies were stored and frozen in glycerol.

Perineal hide swabs (n = 367, 12 to 13 per pen) were collected randomly from 4% of the total cattle in each pen before shipment of these animals to slaughter. A sterile sponge (Spongicle® (Med-Ox Diagnostics Inc., Ottawa, Ontario, Canada) was used to scrub a 100 cm² area on the perineum of the animal below the anus, the sponge was then inserted into a bag containing 45 mL mEC, shipped to the laboratory and processed within 24 h. Each sponge was then incubated in the original transport media for 18 h at 37 °C. *E. coli* O157:H7 was detected by IMS and 3 random NSF colonies were tested for the presence of the O157 antigen by latex agglutination as previously described. Isolates were frozen in glycerol.

Polymerase chain reaction (PCR) was used to confirm *E. coli* O157:H7 in feces and hide swab isolates. Colonies used for templates were suspended in 50 µL nuclease-free water and lysed at 95 °C for 10 min before PCR. The O157 PCR included primers specific for Shiga toxin 1 (*Stx1*), Shiga toxin 2 (*Stx2*), intimin (*eaeA*), and flagella (*flicH7*) genes (Gannon *et al.*, 1997; Paton and Paton, 1998). An *E. coli* O157:H7 positive isolate contained *eaeA*, *flicH7* and at least one *Stx* gene.

2.1.2.7. Survival of *E. coli* O157:H7 in feces

Fresh, pooled pen-floor fecal samples were collected from each treatment group at 2 time points (< 14 d on start-up diets or ≥ 14 d on finishing diets). A 967 g sub-sample from each time point was screened for *E. coli* O157:H7 by IMS and negative feces was inoculated with a 5 strain mixture of nalidixic resistant (NalR) *E. coli* O157:H7: C0281-31N, E318N, and R508N (R.P. Johnson, Public Health Agency of Canada, Guelph, Ontario, Canada); E32511N and H4420N (VPJ Gannon, Public Health Agency of Canada, Lethbridge, Alberta, Canada). Each strain was enumerated by direct plating on CT-SMAC including 0.05 mg/L nalidixic acid and a total of 10⁹ CFU was added to feces and mixed by electronic mixer (Kitchen aid, Mississauga, Ontario, Canada) on low speed for 3 min. Inoculated samples (n = 18) from each diet and time point were divided into triplicate 300 g samples, sealed and incubated at 20 °C. One gram aliquots were tested weekly by direct plating and IMS until *E. coli* O157:H7 could no longer be detected for 3 consecutive wk. The pH of each container was determined 1 d after inoculation, 6 wk after inoculation and when *E. coli* O157:H7 was no longer detected.

2.1.2.8. Statistical analysis

The baseline (initial weight, hip height), feedlot performance (DOF, daily dry matter intake, slaughter weight, G:F), and carcass characteristic (dressing %, Canada yield and quality

grades) data were analyzed using the MIXED procedure in SAS (SAS® for Windows, Version 9.2, SAS Institute Inc., Cary, NC, USA) for treatment group effects and corrected for clustering of observations (replicate as random effect). Baseline variables and days on feed were tested as covariates of the feedlot performance variables, and included in the final models for the performance variables when significant ($P < 0.05$) covariate effects were detected (Littell *et al.*, 2006). Overall P -values were derived from the F -test for treatment and comparison and P -values were differences of the least square means. Bacterial counts were log-transformed into CFU/g of feces. Enumerations of *E. coli* O157:H7 were analyzed using the MIXED procedure of SAS. Type III test effects were used to determine significance ($P < 0.05$) of treatments with orthogonal contrasts comparing treatments and least squares means analysis used to evaluate significant differences in treatments over time. Random effects were included for allocation group and repeated effects for sampling periods. Binomially distributed data from IMS detection of *E. coli* O157:H7 were analyzed using sampling period as a repeated measure in the GLIMMIX procedure of SAS. The α level for all analyses was ≤ 0.05 , with P -values between 0.05 and ≤ 0.10 considered tendencies.

2.1.3. Results

2.1.3.1. *Animal performance*

The weight of cattle at allocation to the study was 298.0 ± 11 kg and the average slaughter weight was 615.5 ± 21 kg. Treatment groups did not differ with respect to initial weight and hip height ($P \geq 0.05$, Table 2.2). Morbidity and mortality rates also did not differ across treatment groups ($P \geq 0.05$, data not shown). Initial weight was used as a covariate ($P < 0.05$) for ADG and G:F on a carcass weight basis. Other variables were not adjusted. Compared to CTRL cattle, the WDDGS group had lower dry matter intake (DMI) ($P < 0.001$), ADG on a

Table 2.2. Production data for steers fed wheat (WDDGS) or corn-based (CDDGS) dry distiller's grains plus solubles or control (CTRL) in the finishing period in western Canada

Variable	<u>Treatment Group¹</u>				<u>Treatment Comparisons</u>			
	WDDGS	CDDGS	CTRL	SE	WDDGS	CDDGS	WDDGS	Overall effect of diet
					vs	vs	vs	
					CTRL	CTRL	CDDGS	
Number of cattle	2162	2183	2187	NA ²	NA	NA	NA	NA
Initial weight, kg	297.4	296.7	297.6	3.7	NS ³	NS	NS	0.544
Hip height, cm	122.4	122.2	122.4	1.0	NS	NS	NS	0.656
Slaughter weight, kg	608.6	623.9	617.9	6.5	0.01	0.07	<0.001	<0.001
Average daily gain, kg/steer/d								
Live weight basis	1.24	1.31	1.28	0.01	0.007	0.061	<0.001	<0.001
HCW basis	1.29	1.35	1.32	0.02	0.030	0.024	<0.001	0.078
Gain to feed ratio								
Live weight basis	0.141	0.148	0.149	0.06	<0.001	0.614	<0.001	<0.001
HCW basis	0.145	0.152	0.152	0.08	<0.001	0.955	<0.001	0.020
Dressing percentage, %	61.1	61.1	60.9	0.3	NS	NS	NS	0.106
Days on feed	247.6	248.0	248.0	6.1	NA	NA	NA	NA
Daily dry matter intake, kg/steer/d	8.8	8.9	8.6	0.04	<0.001	<0.001	0.525	<0.001

¹Steers in the WDDGS group were fed dry-rolled barley based diet with 22.5% (on a 100% DM basis), dry wheat distillers' grains with soluble; steers in the CDDGS group were fed dry-rolled barley based diet with 22.5% (on a 100% DM basis), dry corn distillers' grains with soluble; animals in the CTRL group were fed standard dry-rolled barley-based diet.

²NA, not applicable.

³NS, not significant.

carcass weight basis (CWB, $P = 0.030$) and gain to feed ratio (G:F-CWB, $P < 0.001$; Table 2.3). The proportion of Canada Quality Grade (QG) AAA carcasses was reduced ($P = 0.022$) in the WDDGS group compared to the CTRL group (Table 2.3). In contrast, CDDGS cattle had improved weight daily dry matter intake ($P < 0.001$), and ADG-HCW ($P = 0.024$) compared to the CTRL group. The CDDGS group also had a lower proportions of Canada Yield Grade (YG) 1 carcasses ($P < 0.001$), and greater proportions of both Canada YG 2 ($P = 0.003$) and Canada YG 3 carcasses ($P < 0.001$) compared to CTRL.

Comparing WDDGS and CDDGS, the WDDGS-fed animals had lower slaughter weights, weight gains, and HCW ($P < 0.001$). Feedlot performance (ADG and G:F) were poorer for WDDGS fed cattle compared to the CDDGS cohort on a live weight basis ($P < 0.001$). In addition, carcass-adjusted ADG was 5.0% poorer and carcass-adjusted G:F was 4.6% poorer ($P < 0.001$) in the animals fed WDDGS. Cattle in the WDDGS group also had lower proportions of Canada YG 1 carcasses ($P < 0.001$), and greater proportions of Canada YG 2 ($P = 0.02$) and Canada YG 3 carcasses ($P < 0.001$) than cattle fed CDDGS.

2.1.3.2. Fecal shedding of *E. coli* O157:H7 and fecal pH in naturally colonized cattle

The overall mean fecal prevalence of *E. coli* O157:H7 for the duration of the study was 27.7% (163/588; Table 2.4). The frequencies for detection of *E. coli* O157:H7 in feces were 28.57%, 33.16% and 21.43% for cattle in the WDDGS, CDDGS or CTRL groups, respectively. Pens positive for *E. coli* O157:H7 during each month of sample collection ranged from 5.0-61.1% for cattle in the WDDGS group, 5.0-60.0% for the CDDGS group and 0-36.3% for the CTRL group. Dietary treatment did not influence ($P = 0.65$) the overall number of pens positive for *E. coli* O157:H7. Likewise, there were no differences in the overall number of pens positive for *E. coli*

Table 2.3. Carcass characteristics of steer receiving wheat (WDDGS) or corn-based (CDDGS) dry distillers' grains plus solubles or control (CTRL) in the finishing diet

<i>Treatment Group</i> ¹					Treatment Comparisons			
Carcass Characteristic	WDDGS,	CDDGS,	CTRL,	SE	WDDGS	CDDGS	WDDGS	Overall
	%	%	%		vs	vs	vs	
					CTRL	CTRL	CDDGS	
Yield Grade								
Canada 1	74.58	67.33	76.03	2.53	0.434	<0.001	<0.001	<0.001
Canada 2	21.51	25.82	19.98	1.82	0.371	0.003	0.019	0.007
Canada 3	3.91	6.85	3.98	1.01	0.917	<0.001	<0.001	<0.001
Quality Grade								
Canada Prime	0.22	0.56	0.32	0.14	NS ²	NS	NS	0.182
Canada AAA	31.98	39.19	37.48	1.94	0.022	0.446	0.004	0.011
Canada AA	60.10	54.68	56.76	1.53	0.104	0.298	0.012	0.038
Canada A	6.19	4.59	4.09	0.95	NS	NS	NS	0.130
Canada B1	0.18	0.05	0.22	0.09	NS	NS	NS	0.365
Canada B3	0.03	0.00	0.00	0.02	NS	NS	NS	0.387
Canada B4	1.01	0.74	0.54	0.22	NS	NS	NS	0.234
Canada E	0.28	0.20	0.58	0.18	NS	NS	NS	0.133

¹Animals in the WDDGS group were fed dry-rolled barley based diet with 22.5% (on a 100% DM basis), dry wheat distillers' grains with soluble; animals in the CDDGS group were fed dry-rolled barley based diet with 22.5% (on a 100% DM basis), dry corn distillers' grains with solubles; animals in the CTRL group were fed standard dry-rolled barley-based diet.

²NS, not significant.

Table 2.4. Mean pen prevalence, % and log CFU of *E. coli* O157:H7 and pH in fecal pats from pens with cattle fed finishing diets containing 22.5 % wheat (WDDGS) or corn-based (CDDGS) distillers' dried grain with solubles or control (CTRL) in finishing diet from Oct. 2009-Aug. 2010

Period	Date	CDDGS		WDDGS		CTRL	
		% positive (log CFU)	Fecal pH	% positive (log CFU)	Fecal pH	% positive (log CFU)	Fecal pH
1	Oct-Nov	60.0 (0.55)	7.57	35.0 (1.00)	7.69	30.0 (0.62)	7.67
2	Nov-Dec	30.0 (1.88)	6.93	25.0 (2.64)	7.05	20.0 (0.17)	6.91
3	Dec-Jan	55.0 (4.46)	6.63	45.0 (0.00)	6.71	10.0 (0.00)	6.68
4	Jan-Feb	26.3 (3.98)	7.06	36.8 (3.46)	7.17	26.3 (0.00)	7.09
5	Feb-Mar	27.2 (0.00)	7.08	18.1 (0.00)	7.08	36.3 (5.01)	6.90
6	Mar-Apr	35.0 (3.06)	6.75	5.00 (0.00)	6.71	10.0 (0.00)	6.68
7	Apr-May	5.00 (0.00)	7.05 ^b	5.00 (0.00)	7.25 ^c	0.00 (0.00)	6.87 ^a
8	May-Jun	40.0 (0.00)	6.76 ^b	10.0 (0.17)	6.74 ^b	30.0 (4.49)	6.55 ^a
9	Jun-Jul	25.0 (0.00)	6.72	45.0 (2.76)	6.81	25.0 (3.11)	6.67
10	Jul-Aug	33.3 (0.00)	6.61	61.1 (0.00)	6.75	33.3 (0.00)	6.47
11	Aug	12.5 (0.00)	6.54 ^a	25.0 (0.00)	6.33 ^a	25.0 (0.00)	6.01 ^b
Mean		33.2	6.88 ^b	28.6	6.94 ^b	21.4	6.77 ^a

^{a,b,c} Within a row, means without a common superscript differ ($P < 0.05$).

O157:H7 between sampling periods ($P = 0.13$) or among diets for each sampling period ($P = 0.22$). The proportion of fecal pat samples that were enumerable by CT-SMAC plating was 6.46% (38/588) and average concentration of *E. coli* O157:H7 in positive samples was 2.30 log CFU. The magnitude of *E. coli* O157:H7 shed by cattle was not different ($P = 0.87$) among treatment groups. The magnitude of *E. coli* O157:H7 shedding within monthly sampling periods did not differ ($P = 0.07$) as only a small number of animals shed enumerable *E. coli* O157:H7.

The total frequency of positive *E. coli* O157:H7 hide swabs from cattle before slaughter was 23.7% (87/367). There was no difference ($P = 0.20$) in the frequency of positive hide swabs among dietary treatments: WDDGS (18.18%), CDDGS (27.64%), CTRL (25.20%), although positive hide swabs from cattle fed CDDGS tended to be more frequent ($P = 0.08$) than those from WDDGS cattle.

The mean pH of pooled feces was 6.94 for the WDDGS group, 6.88 for CDDGS group and 6.77 for CTRL group. The pH of feces was different ($P < 0.001$) between diets and sample periods. Cattle fed finishing diets containing WDDGS or CDDGS had a higher ($P < 0.001$) mean fecal pH than those fed CTRL. Fecal pH of WDDGS and CDDGS groups tended to differ $P = 0.07$. Fecal pH was highest ($P < 0.05$) for sampling period 1 which occurred at the start of the trial when the highest forage concentrations were fed (Table 2.4).

2.1.3.3. *E. coli* O157:H7 and fecal pH in inoculated feces

The mean concentration of NaIR *E. coli* O157:H7 in feces decreased over time for all diets. There was no difference ($P = 0.58$) in the total number of *E. coli* O157:H7 enumerated among feces from WDDGS, CDDGS or CTRL groups. There was no difference ($P = 0.95$) among dietary treatments for the frequency of *E. coli* O157:H7 isolated. However, *E. coli* O157:H7 was detected more often ($P < 0.01$) in feces collected from cattle adapting to the initial diet

compared to feces from animals receiving finishing diets for at least 2 wk (Figure 2.1). The mean pH of the inoculated feces did not significantly differ ($P = 0.6297$) between diets: WDDGS (7.67), CDDGS (7.56) or CTRL (7.69). The fecal pH increased between the initial inoculation and six weeks ($P < 0.001$) but varied marginally until the final pH was measured ($P = 0.07$).

2.1.4. Discussion

2.1.4.1. *Animal performance*

The dry milling process to make DDGS results in approximately a 3-fold increase in protein, neutral detergent fiber (NDF), acid detergent fiber (ADF), lipid, and mineral concentrations in corn DDGS compared to the original grain (Klopfenstein *et al.*, 2008). Steers fed WDDGS had lower slaughter weight, HCW, and ADG compared to steers fed CDDGS or CTRL. This is in contrast to previous studies where feeding up to 20% WDDGS had no effect on ADG or G:F in steers (Gibb *et al.*, 2008; Walter *et al.*, 2010) and reasons for the reduced performance for the WDDGS diet in the present study are unclear. Possibly, greater numbers of cattle (6817 compared to 120 in the study of Gibb *et al.* 2008) enabled detection of reduced cattle performance for WDDGS diets in the present study. Alternatively, one potential difference between the present study and the aforementioned studies is the proportion of forage included in the diet. The current study utilized 5% barley silage (DM basis) where Gibb *et al.* (2008) utilized 10% barley silage (DM basis) and Walter *et al.* (2010) fed 7.7% barley silage (DM basis). Accordingly, Miller *et al.* (2009) found that ADG was highest for diets containing 9-12% alfalfa hay and DMI highest for 12% alfalfa hay when feeding 25% CDDGS in steam-flaked corn based diets. When feeding DDGS, the concentration of dietary fiber may play an important role in decreasing passage rate and allowing greater ruminal digestion of DDGS (Li *et al.*, 2011).

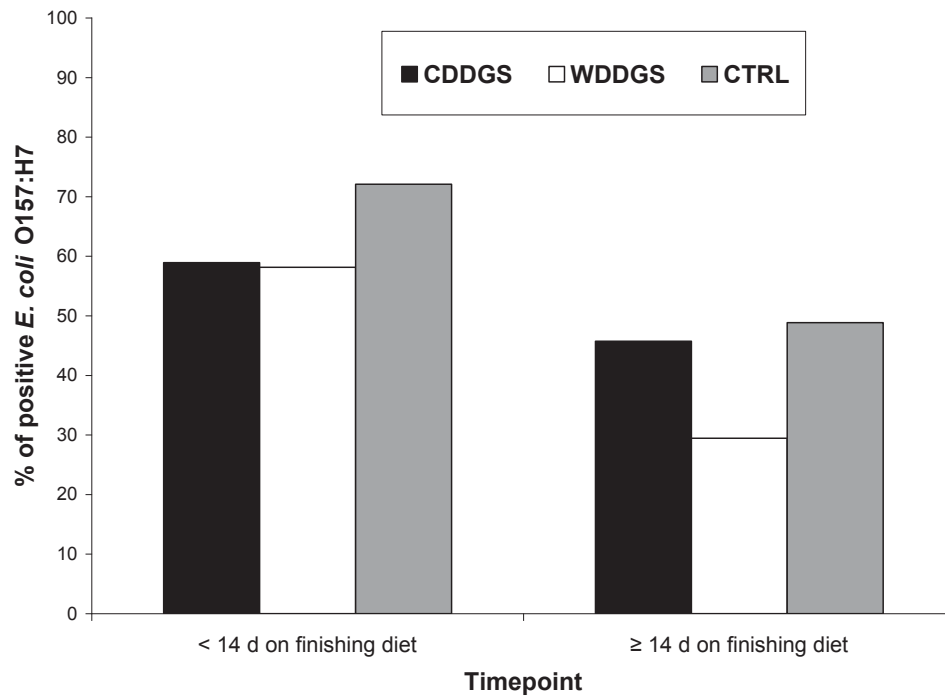


Figure 2.1. Proportion of inoculated feces positive for nalidixic acid -resistant *E. coli* O157:H7 from cattle fed finishing diets containing wheat (WDDGS) or corn-based (CDDGS) distillers' dried grains with solubles or control (CTRL). Feces were collected from cattle on finishing diets for < 14 d or ≥ 14 d.

Steers fed CDDGS had greater HCW and ADG than cattle fed WDDGS or CTRL, which is similar to other studies where steers fed CDDGS gained weight faster and more efficiently than cattle fed corn-based (Ham *et al.*, 1994; Buckner *et al.*, 2007) or barley-based (Walter *et al.*, 2010) diets. Corn grain contains more fat than wheat, suggesting that the corresponding by-product (CDDGS) would have a greater feeding value in feedlot diets compared to WDDGS (Peterson, 2007). At 20% inclusion on a DM basis, energy values of CDDGS have been estimated at 124% the value of dry-rolled corn (Ham *et al.*, 1994), while Gibb *et al.* (2008) estimated WDDGS fed at 20% dietary DM had a similar net energy gain (NEg) to that of barley. However, using NE calculations based on cattle performance (Vasconcelos and Galeyan 2008) the CDDGS and WDDGS included at 22.5% of DM in the present study would be 96.07% and 75.25 % the NE value of dry-rolled barley, respectively. Steers fed WDDGS or CDDGS had increased DMI compared to steers fed the CTRL, in accord with Gibb *et al.* (2008), but contrary to Walter *et al.* (2010) where DMI did not differ for 20% CDDGS or WDDGS diets compared to controls.

Steers in the WDDGS group produced fewer AAA and more AA carcasses, whereas cattle in the CDDGS group produced more Canada YG3 and fewer Canada YG1 carcasses; suggesting that feeding CDDGS may increase the amount of fat on the carcass. Walter *et al.* (2010) found increased dressing percentages when feeding either CDDGS or WDDGS while other carcass traits were not affected. These findings are also inconsistent with other studies where feeding WDDGS or CDDGS had no effect on carcass characteristics (Deppenbusch *et al.*, 2009; May *et al.*, 2010) and may be reflective of animal variation in previous studies which utilized 120 to 350 steers.

2.1.4.2. Diet impacts on *E. coli* O157:H7

The fecal prevalence (27.7%) in the current study was higher than other recent studies (Wells *et al.*, 2009; Edrington *et al.*, 2010) which evaluated impacts of feeding CDDGS on

naturally-occurring *E. coli* O157:H7 in the feedlot environment. Two studies by Jacob *et al.* (2008a,b) noted a prevalence of 9.1% (67/738) in fecal samples collected from individual animals and 7.4% (213/2877) from pen floor samples and found a positive association between feeding 25% wet or dried corn DG to cattle and fecal prevalence of *E. coli* O157:H7. More recently this group of researchers evaluated the effect of feeding wet or dried CDDGS to cattle with an initial fecal prevalence of 20.8% (695/3350) for *E. coli* O157:H7 and found no significant differences among cattle fed 0 or 20% CDDGS during the first 12 weeks on finishing diets, although cattle fed 40% DDGS had greater prevalence of *E. coli* O157:H7 (Jacob *et al.*, 2010). Feeding brewer's grains, a fermentation by-product similar to DDGS, was found to significantly increase shedding of *E. coli* O157:H7 in the feedlot environment, although overall pen prevalence for *E. coli* O157:H7 was high (86.7%; Dewell *et al.*, 2005). Feeding corn DDGS or wheat DDGS to cattle did not result in any difference in fecal prevalence of *E. coli* O157:H7 in the present study, but other factors must be evaluated in conjunction with diet.

Other potential risk factors identified for increased fecal shedding have been age, housing, and transmission from other persistent animal reservoirs such as dogs and birds (Cray and Moon, 1995; Synge *et al.*, 2003). Stressors such as weaning, frequent animal turnover, high stocking density and mixing of animals have also been reported to increase fecal shedding (Stanford *et al.*, 2005b). The decreased prevalence of *E. coli* O157:H7 during Apr-May as compared to Oct.-Nov. and July-Aug. is likely attributable to seasonal variations as opposed to dietary factors as all cattle were fed finishing diets over this period.

Cattle are known to be intermittent shedders of *E. coli* O157:H7 with cattle positive for this pathogen one day and negative the next (Lahti *et al.*, 2003). Regardless of the overall *E. coli* prevalence in feedlot studies, ranges of prevalence between pens and over time have been very

high and this may be due to inconsistent shedding among animals and/or within pens (Dewell *et al.*, 2005; Wells *et al.*, 2009; Berry *et al.*, 2010). As well, individual animals may have unique shedding patterns with periods of high prevalence of shedding and longer periods of reduced or undetectable shedding (Synge *et al.*, 2003; Stanford *et al.*, 2010). Inconsistent shedding of *E. coli* O157:H7 by cattle increases the difficulty of determining impacts of factors including diet as animals may be in different stages of shedding.

Cattle within the current study ranged in fecal prevalence from 0-61.1% but it was interesting to note that the shedding rates of *E. coli* O157:H7 for CTRL cattle had a narrower range than those cattle fed DDGS even though overall prevalence was similar for all diets. It has been suggested that periods of high shedding can be attributed to a small group of cattle termed 'super shedders' which excrete $> 10^4$ CFU/g of feces and transfer the organism to the general cattle population (Stephens *et al.*, 2009; Stanford *et al.*, 2010). Jacob *et al.* (2010) found greater numbers of high-shedding cattle fed finishing diets with 40% CDDGS compared to cattle fed 20% CDDGS or grain-based diets. Cattle fed 40% wet CDDGS in the finishing phase before slaughter also had greater prevalence of *E. coli* O157:H7 from rectal grabs of individual steers than did control cattle fed (Wells *et al.*, 2009). Perhaps some cattle are more sensitive to specific components in DDGS or DDGS are causing profound changes in the ecology of the gastrointestinal tract within specific cattle.

Variation in shedding of *E. coli* O157:H7 in cattle may be influenced by other bacteria found in the hindgut. Bacterial tag-encoded FLX amplicon pyrosequencing of cattle fed diets containing 50% DDGS compared to 0% DDGS found DDGS changed relative microbial population proportions in the rumen and lower gut although *E. coli* O157:H7 was not isolated (Callaway *et al.*, 2010). The composition of microorganisms in the gastrointestinal tract can be altered from

competition between organisms for nutrients and attachment sites, host immune response, production of antimicrobial compounds and interaction between organisms (Callaway *et al.*, 2008b). It is possible that feeding DDGS may amplify *E. coli* O157:H7 in some cattle with microbial profiles already supportive of *E. coli* O157:H7 growth.

Cattle hides are the major source of contamination of carcasses by *E. coli* O157:H7 within the abattoir (Keen and Elder, 2000). In the present study, proportions of hide swabs positive for *E. coli* O157:H7 did not differ with the addition of DDGS in finishing diets. Cattle fed WDDGS tended to have a lower prevalence of positive hide swabs and significantly higher fecal pH compared to CTRL cattle suggesting that high fecal pH does not increase persistence of *E. coli* O157:H7 in the environment. Cattle fed CDDGS also had a higher fecal pH than CTRL but did not differ significantly in hide swabs positive for *E. coli* O157:H7 compared to CTRL cattle or those fed WDDGS. Accordingly, contamination of hides with *E. coli* O157:H7 was not affected by fecal pH ($P=0.20$).

The survival and persistence of *E. coli* O157:H7 in inoculated feces was not influenced by diet. *E. coli* O157:H7 decreased gradually over time in feces from cattle regardless of diet, likely due to dehydration and decreasing availability of substrate (Wang *et al.*, 1996; Himathongkham *et al.*, 1999). Feces from cattle adapted to finishing diets for less than 14 d had enhanced persistence of *E. coli* O157:H7 compared to those cattle on the diet for longer than 14 d, in accord with Stanford *et al.* (2005b) where animals on feed for < 30 d were 6 times more likely to shed *E. coli* O157:H7 than animals on feed > 30 d. The fecal pH of inoculated feces from all diets increased after the first six weeks similar to other fecal inoculation studies where decreased concentrations of VFA over time after inoculation was suggested to enable *E. coli* O157:H7 to survive longer (Buchko *et al.*, 2000b; Bach *et al.*, 2005b; Varel *et al.*, 2010).

This study compared the effects of including wheat DDGS and corn DDGS in standard barley-based diets in commercial feedlot cattle in western Canada. Dried distillers grains and solubles composition varies among ethanol processing plants (Spiehs *et al.*, 2002; Nuez-Ortin and Yu, 2010), therefore DDGS inclusion levels (Gibb *et al.*, 2008), grain processing method (Ham *et al.*, 1994), and forage inclusion levels (Peterson, 2007) in diets containing DDGS should be carefully evaluated in conjunction with animal performance and fecal shedding of *E. coli* O157:H7. Based on the results of this study, the inclusion of WDDGS has negative effects whereas including CDDGS has positive effects on feedlot performance and carcass characteristics compared to standard barley-based diets. The cost-effectiveness of using DDGS depends on commodity pricing relative to that of barley and the risk of feedlot performance and carcass grading disadvantages should be considered when feeding WDDGS. Although several studies have indicated an increase in prevalence of cattle fed CDDGS, there have been no studies of the effect of feeding WDDGS on fecal shedding of *E. coli* O157:H7 in cattle. The incorporation of WDDGS in cattle finishing diets had no effect on prevalence or persistence of *E. coli* O157:H7 although DDGS may have increased variability of shedding. The variability of shedding of *E. coli* O157:H7 among studies may be due to other factors and future studies should address some of the factors relating to variable shedding among individual animals.

3. CHAPTER 3

3.1. Fecal shedding in cattle inoculated with *Escherichia coli* O157:H7 and fed corn or wheat distillers dried grain with solubles

Hallewell, J., L. R. Barbieri, J. E. Thomas, K. Stanford, and T. A. McAllister. 2013. Fecal shedding in cattle inoculated with *Escherichia coli* O157:H7 and fed corn or wheat distillers' dried grain with solubles. *J. Food Prot.* 76:114-8

3.1.1. Introduction

Cattle are the primary reservoir for *E. coli* O157:H7, a food and waterborne bacterium that is capable of causing severe illness in humans (Parry and Palmer, 2002; Rasmussen *et al.*, 1993). Cattle are generally asymptomatic and shed *E. coli* O157:H7 in their feces intermittently, a phenomenon that can result in the adulteration of carcasses, ground water, soil, fruits and vegetables (Bach *et al.*, 2002). Multiple factors including diet can influence shedding of *E. coli* O157:H7 by cattle (Synge *et al.*, 2003). Cattle fed barley diets and inoculated with *E. coli* O157:H7 had higher levels of the organism in feces compared to cattle fed diets consisting of corn or a cottonseed/barley mixture (Buchko *et al.*, 2000b). Commercial feedlot cattle fed barley also had a higher prevalence of naturally-occurring *E. coli* O157:H7 in feces than cattle fed corn-based finishing diets, suggesting specific feed ingredients may affect the frequency and magnitude of *E. coli* O157:H7 shedding (Berg *et al.*, 2004). Feed grain processing may also alter fecal shedding as cattle fed dry-rolled corn shed less *E. coli* O157:H7 than cattle fed steam-flaked corn (Deppenbusch *et al.*, 2008; Fox *et al.*, 2007).

Distillers' dried grain with solubles (DDGS) is a by-product of the bioethanol industry and a valuable feed source for cattle due to their concentrated nutrients (Klopfenstein *et al.*, 2008). In the United States, DDGS is mainly derived from corn, but in Canada, wheat is the preferred grain for fermentation in ethanol production plants. Nutrient content of wheat DDGS

(WDDGS) and corn DDGS (CDDGS) differ as WDDGS has approximately half the lipid content and more protein than CDDGS (Gibb *et al.*, 2008; Mustafa *et al.*, 2000), with both by-products containing negligible starch.

Feeding CDDGS has been linked to increased fecal shedding of *E. coli* O157:H7 (Jacob *et al.*, 2008a,b; Wells *et al.*, 2009). It has been proposed that cattle fed DDGS have decreased hindgut fermentation, resulting in increased fecal pH, creating a lower tract intestinal environment favorable for the proliferation of *E. coli* O157:H7 (Jacob *et al.*, 2008b). In contrast, some studies have suggested that fecal pH has no effect on prevalence of *E. coli* O157:H7 (Depenbusch *et al.*, 2008; Varel *et al.*, 2010; Wells *et al.*, 2009) raising the possibility that other unidentified factors may alter the ecology of *E. coli* O157:H7 in the ruminant digestive tract. Recent studies have found that inclusion of up to 20% CDDGS (Edrington *et al.*, 2010; Jacob *et al.*, 2010) or 22.5% CDDGS or WDDGS (Chapter 2) had no effect on fecal shedding of *E. coli* O157:H7 in naturally-colonized cattle. The objective of the current study was to compare the impact of feeding cattle diets containing 40% CDDGS, 40% WDDGS, or a mixture of CDDGS (20%) and WDDGS (20%) (CWDDGS) compared to a traditional barley-based finishing diet (CTRL) on fecal shedding in experimentally inoculated cattle.

3.1.2. Material and Methods

3.1.2.1. Diets and feeding

The study was conducted at the Lethbridge Research Centre feedlot (Lethbridge, AB, Canada) from May to August 2010. Animals were weighed, blocked by weight and randomly assigned to pens. Thirty-two Hereford X Angus steers with an initial weight of 535.0 ± 27.8 kg were housed in eight outdoor pens (four steers per pen). Cattle were adapted to finishing diets for 3 weeks before inoculation, with *ad libitum* intake estimated in the last week. Thereafter,

steers were fed once daily at 95% of the estimated ad libitum intake using one of four total mixed diets (two pens per diet; Table 3.1). Orts were collected and weighed weekly.

3.1.2.2. *Inoculation of steers*

Steers were inoculated with a four-strain mixture of nalidixic acid-resistant (NaIR) *E. coli* O157:H7; E318N and R508N (R. P. Johnson, Public Health Agency of Canada, Population and Public Health Branch, Guelph, ON, Canada), E32511N and H4420N (V. P. J. Gannon, Public Health Agency of Canada, Animal Diseases Research Institute, Lethbridge, AB, Canada). Bacterial cultures were grown in 10 ml of tryptic soy broth (TSB) containing 50 µg/ml nalidixic acid (Sigma, Oakville, ON, Canada) for 6 h at 37 °C, in a shaking incubator at 150 rpm (Innova 4080, New Brunswick Scientific, Edison, N.J). From these cultures, 100 µl was inoculated into 100 ml TSB and grown for 18 h at 37 °C. Cultures of a given strain were pooled and mixed before dispensing (~15 ml/strain) into sterile cups to load into 60 cm³ syringes. Growth of each strain was monitored through turbidity readings at 600 nm and enumerated by direct plating on Sorbitol MacConkey agar with 2.5 mg/l potassium tellurite, 0.05 mg/l cefixime and 50 µg/l nalidixic acid (CT-SMACnal) (Dalynn Biologicals, Calgary, AB, Canada). To inoculate the animals, the strains of *E. coli* O157:H7 were combined in equal concentrations, with a total of 10¹⁰ CFU administered using a sterile syringe and stomach tube followed by the delivery of three 50-ml aliquots of phosphate buffered saline (PBS) pH 7.2 to rinse the stomach tube. Cattle were monitored during feeding and sample collection and treated humanely in accordance with Canadian Council of Animal Care Guidelines (2009).

Table 3.1. Analysis (DM basis) of diets and performance of cattle fed finishing diets containing 40% corn distillers' dried grain with solubles (CDDGS), 40% wheat distillers' dried grain with solubles (WDDGS), 20 % corn + 20% wheat distillers' dried grain with solubles (CWDDGS) or barley-based diets (CTRL). Means in rows with different superscripts differ ($P < 0.05$).

Ingredient	Finishing Diet			
	CTRL	WDDGS	CWDDGS	CDDGS
Corn DDGS	0	0	20	40
Wheat DDGS	0	40	20	0
Barley	80	40	40	40
Barley Silage	15	15	15	15
Supplement	5	5	5	5
Dry Matter (%)	94.1	94.5	94.5	94.6
Analysis (DM basis)				
Crude protein (%)	11.4	18.7	20.3	17.8
Acid Detergent Fiber (%)	8.1	8.5	12.1	12.8
Neutral Detergent Fiber (%)	19.8	22.3	26.8	25.8
Calcium (%)	0.40	0.37	0.54	0.75
Phosphorus (%)	0.29	0.47	0.46	0.39
Magnesium (%)	0.14	0.20	0.20	0.17
Potassium (%)	0.57	0.72	0.72	0.65
Sodium (%)	0.06	0.17	0.18	0.10
Animal performance				
Average Daily Gain kg/day	1.28 ^a	0.925 ^b	1.05 ^c	1.10 ^c
Feed:Gain Ratio	6.87 ^a	9.90 ^b	9.05 ^b	9.07 ^b

3.1.2.3. *Fecal sampling and analyses*

Fecal samples were collected using clean gloves and placed in 90 ml polypropylene containers (Fisher Scientific, Nepean, ON, Canada). Samples were immediately transferred to the laboratory and stored at 4 °C prior to analysis. Fecal subsamples (1 g) were suspended in 9 ml PBS and aliquots (100 µl) of serial dilutions were plated on CT-SMACnal in triplicate and incubated at 37 °C overnight. Plates containing 30 to 300 colonies were enumerated.

Fecal samples (1 g) that were negative by direct plating were enriched in 9 ml modified *E. coli* broth with 20 mg/L novobiocin (EMD Chemicals, Gibbstown, NJ, USA) for 6 h at 37°C and subjected to immunomagnetic separation (IMS) using anti-*E. coli* O157 Dynabeads (Invitrogen, Carlsbad, Calif). A 50 µl aliquot of bead-bacteria complex was plated on CT-SMACnal and incubated at 37 °C for 16 h. Three non-sorbitol-fermenting colonies from all plates were tested for the O157 antigen using an O157 latex agglutination kit (Oxoid, Nepean, ON, Canada). To determine fecal pH, a 5 g subsample from each steer was mixed with 5 ml distilled water prior to measurement with a pH meter (Orion model 310, Fisher Scientific, Ottawa, ON, Canada).

3.1.2.4. *Polymerase chain reaction (PCR) and pulsed field gel electrophoresis (PFGE)*

For each steer, the last two *E. coli* O157:H7 isolates confirmed by latex agglutination (days 23 to 70 of study, depending on steer) were frozen in glycerol and stored at -40 °C. Frozen isolates were resuscitated on LB agar and incubated at 37 °C overnight. Colonies were resuspended in 50 µl nuclease-free water and lysed at 95 °C for 10 min prior to multiplex PCR. The PCR included primers specific for Shiga toxin 1 (*Stx1*), Shiga toxin 2 (*Stx2*), intimin (*eaeA*), and flagella (*fliC*) genes (Gannon *et al.*, 1997; Paton and Paton, 1998). The PCR reaction was performed in a 20 µl reaction volume which contained 1X Master mix solution (Qiagen, Mississauga, ON, Canada), 20 pmol of each primer and 2 µl of colony template using the

following parameters: 15 min denaturation at 95 °C, followed by 35 cycles of 30 s at 94 °C, 45 s at 60 °C and 60 s at 72 °C with a final extension step at 72 °C for 10 min.

Isolates were typed by pulsed field gel electrophoresis (PFGE) of *Xba*I-digested genomic DNA according to the Standardized Protocol for molecular subtyping of *E. coli* O157:H7 (Centers for Disease Control and Prevention, 2004) using a CHEF DR II electrophoresis unit (Bio-Rad Laboratories, Mississauga, ON, Canada). The resulting PFGE patterns were analyzed using Bionumerics software (version 6.5, Applied Math Inc., Austin, TX) with closely related isolates having patterns with at least 95% similarity.

3.1.2.5. *Statistical analysis*

Colony counts were log transformed per gram of feces (CFU/g). Plates confirmed positive by IMS were randomly assigned numbers between 0 and 1 log CFU/g using a Microsoft Excel random number function and analyzed using the mixed model procedure (version 9.1, SAS Institute Inc., Cary, NC, USA). Pen was considered the experimental unit for all analysis. Type III test effects with random effects for pen X diet X day were used to identify differences ($P < 0.05$) among treatments. Orthogonal contrasts and least squares means analysis were used to evaluate differences among treatments and within treatments and days. Binomially distributed data generated from IMS were analyzed using the GLIMMIX procedure of SAS. A three-parameter decay curve model was used within the NLIN procedure of SAS:

$$\text{CFU} = \text{AA} + \text{B} * \exp(-\text{c} * \text{day})$$

with CFU as the log CFU of *E. coli* O157:H7, AA as the curve asymptote, B the intercept of the curve and c as the rate of disappearance of *E. coli* O157:H7. A mixed model was then used to evaluate impacts of dietary treatments among *E. coli* O157:H7 decay curves.

3.1.3. Results

3.1.3.1. *Animal performance*

Average daily gain (ADG) differed among diets ($P < 0.05$). ADG was highest for cattle fed control ($P < 0.05$) and lowest for cattle fed WDDGS ($P < 0.05$) (Table 3.1). Cattle fed control had lower feed/gain ratios than CDDGS, WDDGS, or CWDDGS (Table 3.1).

3.1.3.2. *Fecal shedding of NaIR E. coli O157:H7*

The frequency with which positive *E. coli* O157:H7 isolates were detected was 23.2, 24.6, 24.6, and 27.5% for cattle fed CDDGS, CWDDGS, WDDGS, and barley grain diets, respectively. The prevalence of *E. coli* O157:H7 did not differ when pooled across collection days ($P > 0.05$) among diets. The mean log CFU/g feces of *E. coli* O157:H7 over the duration of the 70 day challenge was 1.7, 2.2, 2.2, and 2.3 (all ± 0.2) for cattle fed CDDGS, CWDDGS, WDDGS, and barley grain diets, respectively. The concentration of *E. coli* O157:H7 in feces did not differ ($P > 0.05$) but a diet X day interaction was observed ($P < 0.05$). The concentration of *E. coli* O157:H7 from cattle fed CDDGS was lower ($P < 0.05$) than WDDGS, CWDDGS or CTRL on days 13, 21, 23, and 35 (Fig. 3.1), but the rate of decline of *E. coli* O157:H7 in feces did not differ among diets ($P > 0.05$; Table 3.2).

3.1.3.3. *PCR and PFGE analyses*

A total of 63 isolates (two per steer) from feces were confirmed *E. coli* O157:H7 positive by multiplex PCR. One isolate collected from a CDDGS-fed steer could not be recovered after storage in glycerol. From these 63 isolates, 28 were R508N; 18 were H4420N; 15 were E318N; 1 was E32511N and 1 exhibited a unique PFGE pattern (Fig. 3.2). The two isolates evaluated

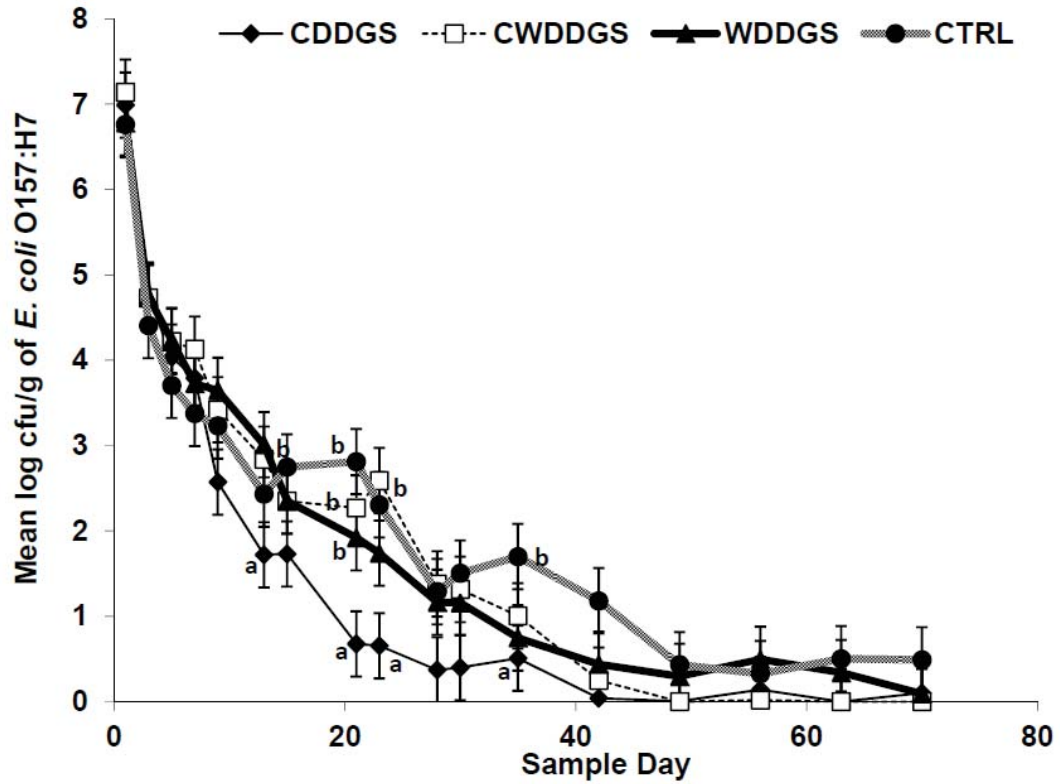


Figure 3.1. Mean log CFU of nalidixic acid-resistant *E. coli* O157:H7 in fecal grabs (n = 544) from cattle fed finishing diets containing 40% corn distillers' dried grain with solubles (CDDGS), 40% wheat distillers' dried grain with solubles (WDDGS), 20% corn + 20% wheat distillers' dried grain with solubles (CWDDGS) or barley-based diets (CTRL) for 70 d post-inoculation with 10^{10} CFU of *E. coli* O157:H7. Samples with differing subscripts differ among diets within sampling day ($P < 0.05$).

Table 3.2. Rate of loss of *E. coli* O157:H7^z 70 days post-inoculation with 10¹⁰ CFU nalidixic resistant *E. coli* O157:H7 in cattle fed diets containing 40% corn distillers' dried grain with solubles (CDDGS), 40% wheat distillers' dried grain with solubles (WDDGS), 20% corn + 20% wheat distillers' dried grain with solubles (CWDDGS) or barley-based diets (CTRL). Means within a column with different superscripts differ ($P < 0.05$).

Diet	Curve Asymptote (AA)	SE	Intercept of curve (B)	SE	Loss (%) CFU/day ⁻¹ (C)	SE
CTRL	1.0289	0.4923	5.8295 ^b	0.3526	7.49	1.37
CWDDGS	0.0711	0.9846	7.2227 ^a	0.4072	7.67	1.58
WDDGS	0.5167	0.5685	6.9814 ^a	0.3770	8.59	1.46
CDDGS	0.1542	0.4923	7.3028 ^a	0.3770	10.87	1.46

^zCFU=AA+B(-C*day) is the three parameter decay model where CFU is the colony forming units of *E. coli* O157:H7 (log₁₀); AA the curve asymptote; B intercept; C rate of disappearance (CFU day⁻¹).

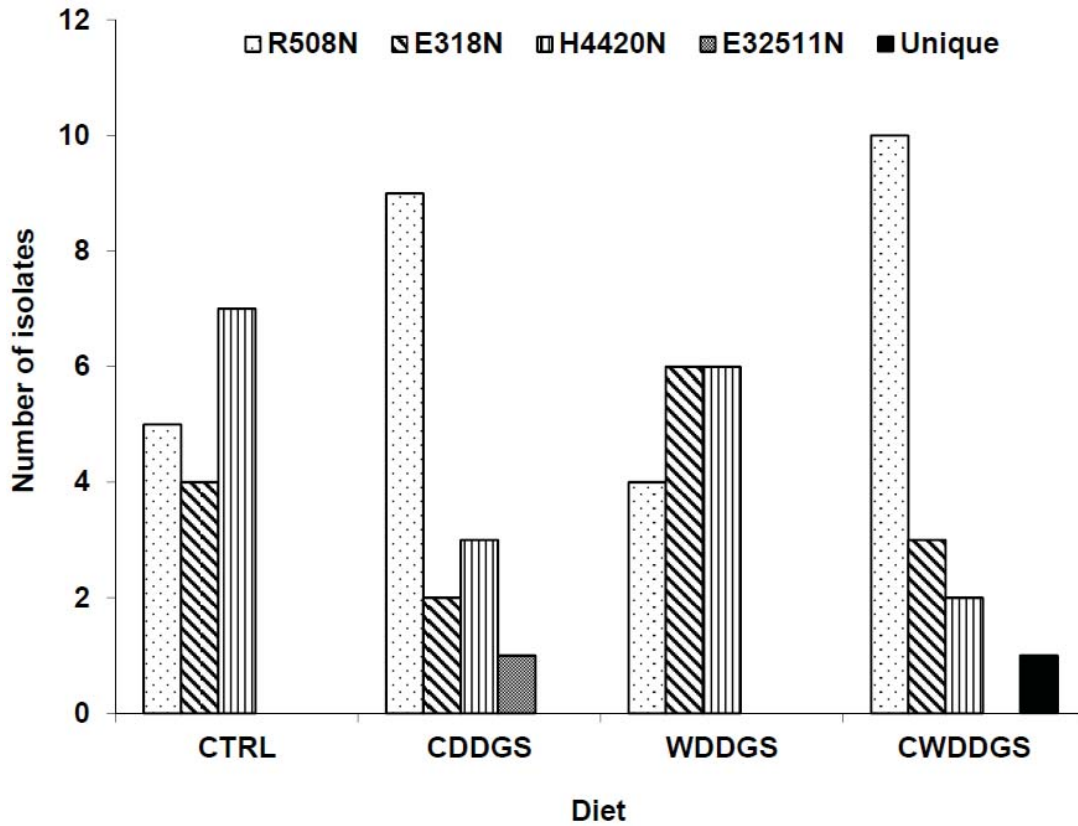


Figure 3.2. Pulsed Field Gel Electrophoresis analyses of the last 2 positive isolates per steer (n = 32) collected between days 23-70 of the 70 day experiment from feces of steers inoculated with nalidixic resistant (NaIR) *E. coli* O157:H7. NaIR strains isolated were R508N, E318N, H4420N, E32511N and one unique strain. The total number of isolates evaluated by PFGE was 63 as one CDDGS isolate was not viable.

for each steer frequently differed, indicating colonization by multiple strains at the end of the 70 day study. Four isolates (three WDDGS and one CTRL) were 91.5% similar to H4420N, and contained PFGE bands identical to H4420N with the exception of a ~410bp insertion and loss of two smaller bands at ~245bp and ~165bp.

3.1.3.4. Fecal pH

The mean fecal pH fluctuated among sampling periods, but did not differ among cattle fed CDDGS, WDDGS, CWDDGS or CTRL ($P > 0.05$). The total mean fecal pH was 6.85, 6.99, 6.91, and 7.02 (all +/- 0.06) for cattle fed CDDGS, CWDDGS, WDDGS, and CTRL diets, respectively.

3.1.4. Discussion

After inoculation, the number of *E. coli* O157:H7 in feces decreased for all diets with some periods of variable shedding similar to other inoculation studies (Bach *et al.*, 2005b; Buchko *et al.*, 2000b; Stanford *et al.*, 2005b). Fluctuations in fecal shedding of *E. coli* O157:H7 after inoculation of cattle have been attributed to oral ingestion of contaminated fecal matter (Stanford *et al.*, 2005b) warmer summer temperatures supportive of *E. coli* O157:H7 growth in feces (Buchko *et al.*, 2000b) and contamination by *E. coli* O157:H7 in feed and water (Bach *et al.*, 2005b). In the current study, fecal shedding of *E. coli* O157:H7 gradually decreased for the first 42 days of the experiment suggesting that growth of the organism in feces and/or re-colonization of cattle within the pens was not occurring. Previous studies monitoring *E. coli* O157:H7 concentrations after inoculation in batch culture fecal microbial fermentations (Jacob *et al.*, 2008a) and fecal pats (Chapter 2) determined that feeding cattle DDGS did not have a positive association on growth or persistence of *E. coli* O157:H7 in the feedlot environment.

The current study found no difference in prevalence or numbers of *E. coli* O157:H7 in feces of cattle fed up to 40% CDDGS, WDDGS, CWDDGS or barley grain-based finishing diet. This agrees with previous studies of naturally colonized cattle, which showed that feeding cattle 22.5% CDDGS or WDDGS had no effect on prevalence or concentration of *E. coli* O157:H7 in feces (Chapter 2). Edrington *et al.* (2012) also found no differences in fecal prevalence after feeding 20% wet corn distiller grains to cattle compared to a corn-based control diet. Similarly, Jacob *et al.* (2010) determined that there was no difference among cattle fed 20% wet or dry corn distiller grains and control, but that cattle fed 40% DDGS had a higher prevalence and shed higher levels at certain stages of feeding. This was our basis for inclusion of 40% DDGS in the present study, but even at this relatively high level of DDGS in the diet we failed to observe any promotion of shedding of *E. coli* O157:H7 in challenged cattle.

Cattle are known to be intermittent shedders of *E. coli* O157:H7 at highly variable levels (Lahti *et al.*, 2003) and it is possible that inclusion of DDGS in cattle diets may increase fecal shedding of *E. coli* O157:H7 in individual cattle that are colonized by *E. coli* O157:H7 (Jacob *et al.*, 2010; Wells *et al.*, 2009). Dietary changes have been linked to shifts in microbial populations including *E. coli* O157:H7 (Callaway *et al.*, 2009). Recent studies (Callaway *et al.*, 2010) have determined that cattle fed 50% CDDGS had a shift in fecal *Firmicutes*:*Bacteroidetes* ratios compared to cattle fed a commercial grain diet without DDGS, potentially opening a niche for other bacteria such as *E. coli* O157:H7 to occupy. The composition of *Bacteroidetes*, *Proteobacteria* and *Firmicutes* communities also varied significantly with fecal starch concentrations and changes in sugars or starch sources have been suggested to alter the digestive habitat and thereby impact pathogen shedding (Shanks *et al.*, 2011).

Feces from cattle fed control diets most frequently contained strain H4420N, similar to results in previous inoculation studies in which the majority of isolates with the longest shedding duration produced banding patterns identical to H4420N in cattle fed corn- or barley-based diets (Bach *et al.*, 2005a, b). PFGE profiles similar to H4420N, but containing an extra band and loss of two smaller bands likely represent a point mutation that resulted in loss of a restriction site (Tenover *et al.*, 1995). Previous inoculation studies have also found the presence of PFGE subtypes resembling initial inoculated strains (Bach *et al.*, 2005b) or different from original inoculated strains (Rozema *et al.*, 2009). In a previous study mutated *E. coli* O157:H7 subtypes were found to be less sensitive to bacteriophages, suggesting that genomic changes conferred greater resistance to these subtypes over the 83 day experiment (Rozema *et al.*, 2009). This illustrates the high plasticity of the *E. coli* O157:H7 genome and its ability to adapt to changing conditions in the intestinal tract and environment. Strain H4420N was less frequently isolated from cattle fed CDDGS or CWDDGS, suggesting that incorporating at least 20% CDDGS into finishing diets may alter microbial persistence of certain *E. coli* O157:H7 strains. Recent studies determined that strain H4420N was sensitive to fatty acids under low pH and the presence of certain oils can exert a bactericidal effect on this strain (Yang *et al.*, 2010). The high oil content of CDDGS may have exerted a bactericidal effect on this strain thereby accounting for its reduced isolation from cattle fed CDDGS. Strains of *E. coli* O157:H7 have demonstrated variability in their ability to colonize the bovine digestive tract (Rasmussen *et al.*, 1993) and this may partially account for variable shedding patterns from cattle fed DDGS. Strains E32511N and E318N are of human origin and have previously been found to persist poorly in cattle as compared to R508N and H4420N (Bach *et al.*, 2005b).

The current study had periods of low-magnitude shedding for cattle fed CDDGS compared to other diets, suggesting that some elements in CDDGS interfere with colonization of

E. coli O157:H7. Although CDDGS and WDDGS are both produced during ethanol production, CDDGS has a higher lipid content and decreased protein content compared to WDDGS (Gibb *et al.*, 2008; Ham *et al.*, 1994; Mustafa *et al.*, 2000). An increase in lipid content and fatty acids could potentially decrease colonization in the gastrointestinal tract, as many unsaturated fatty acids exhibit antibacterial activity towards *E. coli* O157:H7 (Galbraith and Miller, 1973). An increase in protein digestion can also lead to elevated ammonia concentrations in the hindgut providing a more hospitable environment for some *E. coli* O157:H7 strains (Jacob *et al.*, 2009a). No study to date has found reduced shedding of *E. coli* O157:H7 after feeding DDGS, but it is possible that impacts of DDGS on microbial populations may vary at different stages of the feeding period or that feeding DDGS may increase variability in the shedding of the organism. The nutrient composition of DDGS varies with DDGS type and bioethanol plant origin (Nuez-Ortin and Yu, 2010) which may also increase the variability in shedding, leading to contradictory results among studies.

There was no difference observed in fecal pH among cattle fed CDDGS, CWDDGS, WDDGS or CTRL diets. Previous studies found increased fecal pH in cattle fed DDGS compared to barley- or corn-based diets (Chapter 2; Varel *et al.*, 2010; Wells *et al.*, 2009), but fecal pH in the present study was variable among days, which may be due to the smaller number of cattle used in challenge studies. Cattle fed WDDGS had lower ADG than cattle fed CTRL or diets including CDDGS and lower G:F compared to the CTRL diet as reported by previous studies (Chapter 2). Feed efficiency was also notably lower than reported in previous studies, a result that may arise from the fact that the cattle were restrictively fed as well as the low number of replicate pens that were possible with the challenge experimental design.

Cattle fed CDDGS have been linked to fecal shedding of *E. coli* O157:H7 but the effects of feeding WDDGS and fecal shedding of *E. coli* O157:H7 have been little studied. Results from this study suggest that inclusion of up to 40% corn and/or wheat DDGS in finishing diets did not affect numbers of *E. coli* O157:H7 shed in feces or fecal prevalence of this organism compared to a standard barley grain-based finishing diet in cattle challenged with this pathogen. As cattle are known to be intermittent shedders of *E. coli* O157:H7, factors other than diet need to be further explored to understand the complexities of fecal shedding of *E. coli* O157:H7 among cattle. The results of this study suggest that incorporation of corn or wheat DDGS into the finishing diets of cattle does not promote the shedding of *E. coli* O157:H7.

4. CHAPTER 4

4.1. Isolation and identification of endemic bacteriophages from cattle shedding high and low numbers of *Escherichia coli* O157:H7 in feces

J. Hallewell, Y. D. Niu, K. Munns, T. A. McAllister, R. P. Johnson, H-W. Ackermann, J. E. Thomas and K. Stanford. Isolation and identification of endemic bacteriophages from cattle shedding high and low numbers of *Escherichia coli* O157:H7 in feces. *Appl. Environ. Microbiol.* Submitted

4.1.1. Introduction

Escherichia coli (*E. coli*) O157:H7 is a foodborne organism responsible for severe illness and death in humans (Besser, 1999). Cattle are asymptomatic carriers of *E. coli* O157:H7 and contamination of foods through production and distribution chains has resulted in massive recalls and substantial economic loss to the food industry (Rangel et al., 2005; Vogt and Dippold, 2005). Shedding of *E. coli* O157:H7 is inconsistent, with some cattle shedding the organism for a few days while others may shed *E. coli* O157:H7 for weeks or months (Chase-Topping et al., 2008) suggesting that the nature of the host plays a role in etiology of this bacterium. Super-shedders are cattle that excrete the pathogen at concentrations $\geq 10^4$ CFU·g⁻¹ of feces (Matthews et al., 2006) and are thought to account for a small proportion of the cattle population yet may contribute 80-96% of the total load of *E. coli* O157:H7 in the herd (Jeon et al., 2013; Omisakin et al., 2003; Matthews et al., 2006). Multiple factors contribute to shedding of *E. coli* O157:H7 in cattle including diet (Callaway et al., 2009), housing (Vidovic and Korber, 2006), season (Stanford et al., 2005), stress (Schwartzkopf-Genswein et al., 2007) and age (Cray and Moon, 1995). However, knowledge of the microbial ecology of the gastrointestinal tract of cattle is limited and relationships among microbiota may impact the ability of *E. coli* O157:H7 to persist in some cattle (Arthur et al., 2010).

Endemic bacteriophages (phages) are a factor which could directly impact shedding of *E. coli* O157:H7 by cattle. Phages are natural predators of bacteria and represent a significant factor in limiting bacterial populations (Letarov and Kulikov, 2009). Endemic phages have been isolated from cattle and their environment and these phages may be highly specific and effective for therapy of *E. coli* O157:H7 in cattle (Callaway et al., 2008; Niu et al., 2012; Viscardi et al., 2008). Knowledge of *E. coli*/phage ecology *in vivo* is relatively limited and the effects of endemic phages on the efficacy of phage cocktails used in therapy are unknown, making interpretation of these studies difficult (Kropinski et al., 2012a). A wider knowledge of the natural microbiota in the cattle environment and the relationship between naturally-occurring phages and *E. coli* O157:H7 would improve current mitigation methods. Accordingly, the objectives of this study were to identify endemic phages in the feedlot environment and determine the relationship of these phages with naturally-occurring *E. coli* O157:H7 in cattle identified as shedding high and low numbers of *E. coli* O157:H7.

4.1.2. Material and Methods

4.1.2.1. *Sample collection*

The study was conducted at the Lethbridge Research Center (LRC) containment feedlot (Lethbridge, Alberta, Canada) from July 8 to Aug. 7, 2011. All cattle were handled in accordance with the Canadian Council of Animal Care (2009) with procedures approved by the LRC Animal Care Committee. Fecal grab samples from crossbred yearling steers (N = 400) at a commercial feedlot in southern Alberta were sampled once to identify super-shedders (Munns et al., 2014). Six cattle excreting $\geq 10^4$ CFU·g⁻¹ of feces were identified as super-shedders (SS) and five cattle excreting $< 10^4$ CFU·g⁻¹ of feces were identified as low-shedders (LS). Cattle were then transported to LRC and feces sampled in conjunction with a companion study (Munns et al.,

2014). Cattle were penned individually for 2 wk and grouped together for the final 3 wk of the experiment. Approximately 50 g of freshly voided fecal pats were collected from each steer daily for the first 6 d of the trial using sterile gloves and placed in 90 ml polypropylene containers (Fisher Scientific, Nepean, ON, Canada). Fecal grabs (50 g) were collected from cattle for the remainder of the trial with the exception of 5 d when no samples were collected. Samples were immediately transported to the laboratory and stored at 4°C for further analysis.

4.1.2.2. Isolation and enumeration of *E. coli* O157:H7

E. coli O157:H7 in fecal pats and fecal grab samples were enumerated by direct plating on sorbitol MacConkey agar with 2.5mg·L⁻¹ potassium tellurite and 0.05 mg·L⁻¹ cefixime (Daylynn Biologicals, Calgary, Alberta, Canada) and plates with 30-300 colonies were used for enumeration. Duplicate 1 g subsamples of feces were enriched in 9 ml of modified *E. coli* broth with 20 mg·L⁻¹ novobiocin and incubated 6 h at 37°C. For detection of *E. coli* O157:H7, enriched samples were subjected to immunomagnetic separation using anti-*E. coli* O157 Dynabeads® (Invitrogen, Carlsbad, CA, USA) as per manufacturer's instructions. Colonies were confirmed positive by latex agglutination and PCR (Chapter 2). Positive *E. coli* O157:H7 isolates were frozen in glycerol and stored at -40°C.

4.1.2.3. Pulsed field gel electrophoresis of *E. coli* O157:H7 isolates

Frozen isolates were resuscitated on Luria-Bertani agar (EMD Chemicals, Gibbstown, NJ, USA) and incubated at 37°C overnight. Isolates were typed by pulsed field gel electrophoresis (PFGE) of *Xba*I (New England Biolabs, Pickering, Ontario, Canada) digested genomic DNA according to the standardized protocol for molecular subtyping of *E. coli* O157:H7 (Centers for Disease Control and Prevention, 2004) using a CHEF DR II electrophoresis unit (Bio-Rad Laboratories, Mississauga, ON, Canada). Resulting patterns were analyzed using BioNumerics

Version 6.6 software (Applied Maths Inc., Austin, TX, USA) with closely related isolates having at least 90% similarity.

4.1.2.4. *Isolation of E. coli O157:H7-infecting phages*

E. coli O157:H7 R508, a bovine phage type (PT) 14 strain supplied by the Laboratory for Foodborne Zoonoses, Public Health Agency of Canada (PHAC), Guelph, ON, Canada was used as the host for isolation of phages. To isolate phages from fecal samples (grabs or pats), 2 g of feces was suspended in 8 ml lambda diluent (10 mM Tris Cl [pH 7.5], 8 mM MgSO₄·7H₂O), vortexed thoroughly and left to stand at room temp for 30-60 min. Samples were then centrifuged at 5250 × g for 10 min (X-15R, Beckman Coulter, Mississauga, ON, Canada) to sediment feces. A subsample (1.8 ml) was extracted from the top layer, centrifuged at 11,000 × g for 10 min and filtered through a 0.22 μm pore syringe filter (Pall Life Science, Mississauga, ON, Canada). Filtrates were subjected to short-(1 h) and/or long (overnight; Niu et al., 2009) enrichment for detection of phages and evaluation of phage populations. Phages were purified 3 times by single-plaque isolation and stock filtrates were prepared using *E. coli* O157:H7 R508 as a host (Niu et al., 2012). Titers of phages (~1×10⁸ PFU·ml⁻¹) in stock filtrates were determined using the soft agar overlay technique (Sambrook and Russell, 2001).

4.1.2.5. *Genome size estimation, restriction fragment length polymorphism, transmission electron microscopy of phage isolates*

Phage stocks (50 ml) were treated with DNase (10 μg·ml⁻¹) (Sigma-Aldrich, Oakville, ON, Canada) and RNase (20 μg·ml⁻¹) (Sigma-Aldrich) for 1 h at room temperature on a magnetic stirrer. Phage suspensions were then treated with 1 M NaCl and 10% PEG 8000 and slowly mixed overnight at 4°C. Overnight samples were centrifuged at 14,000 × g for 30 min (236 HK Hermle, Edison, NJ, USA) at 4°C and supernatants were removed. The pellet was re-suspended in 2 ml

lambda diluent (pH 7.2). Genome size of concentrated phage lysates was determined by PFGE (Niu et al., 2012) using the using a CHEF DR II electrophoresis unit (Bio-Rad Laboratories, Mississauga, ON, Canada). Resulting band sizes were analyzed using BioNumerics. Determination of restriction fragment length polymorphism (RFLP) was performed on pre-treated phage stocks using PFGE (Niu et al., 2012). Filtered phage lysates were examined by transmission electron microscopy (TEM) was completed by H-W Ackermann at the Department of Microbiology (Quebec City, QUE, Canada) according to methods described by Niu et al. (2012).

4.1.2.6. *Phage typing*

E. coli O157:H7 isolates were serotyped and phage typed at the *E. coli* Reference Laboratory for Foodborne Zoonoses, Guelph, Ontario. Phage typing was performed as originally described by Ahmed et al. (1987) and Khakhria et al. (1990), with 16 phages that differentiate 89 phage types.

4.1.2.7. *Microplate phage virulence assay*

The susceptibility of *E. coli* O157:H7 to isolated phages was determined by microplate phage virulence assay (Niu et al., 2012). A collection of commonly isolated PT of *E. coli* O157:H7 (N = 30) supplied by PHAC were used in the assay. Six *E. coli* O157:H7 isolated in the current study (from each PFGE subtype) were also included in the phage assays. The multiplicity of infection (MOI) was estimated by the minimum concentration of phage required for complete lysis of the culture. *E. coli* O157:H7 were defined as extremely sensitive: MOI < 0.01; highly sensitive: $0.01 \leq \text{MOI} < 1$; moderately sensitive: $1 \leq \text{MOI} < 10$; and resistant: no lysis upon exposure to phage isolates.

4.1.2.8. Statistical analyses

Bacterial enumerations were log-transformed into CFU per gram of feces. Phage and *E. coli* O157:H7 prevalence data was converted to binary data where the presence of plaques or colonies was considered positive and the absence of plaques or colonies was considered negative. Binomial data was analyzed using GLIMMIX procedure of SAS (SAS® for windows, Version 9.2, SAS Institute Inc., Cary, NC, USA) with type III test effects used to determine significance ($P < 0.05$) of groups and least square means analysis used to evaluate differences among groups.

4.1.3. Results

4.1.3.1. Prevalence of *E. coli* O157:H7

The total prevalence of *E. coli* O157:H7 isolated from feces of 11 steers was 41/285 (14.4%). Incidence of fecal samples positive for *E. coli* O157:H7 did not differ ($P = 0.972$) between SS (22/156, 14.1%) and LS groups (19/129, 14.7%; Figure 4.1). Although there was no difference in the prevalence of *E. coli* O157:H7 among sampling days ($P = 0.989$) across all cattle, the incidence of *E. coli* O157:H7 tended to vary by week. Prevalence of the pathogen (18.4–21.2%) was higher in the first two weeks of the study than in the following two weeks (7.6–9.1%; $P < 0.05$). The average concentration of *E. coli* O157:H7 was 3.38 log CFU·g⁻¹ feces for SS steers and 2.50 CFU·g⁻¹ feces for LS steers. Only two SS steers and no LS had a fecal sample with ≥ 4.00 log₁₀ CFU·g⁻¹ of *E. coli* O157:H7 during the trial.

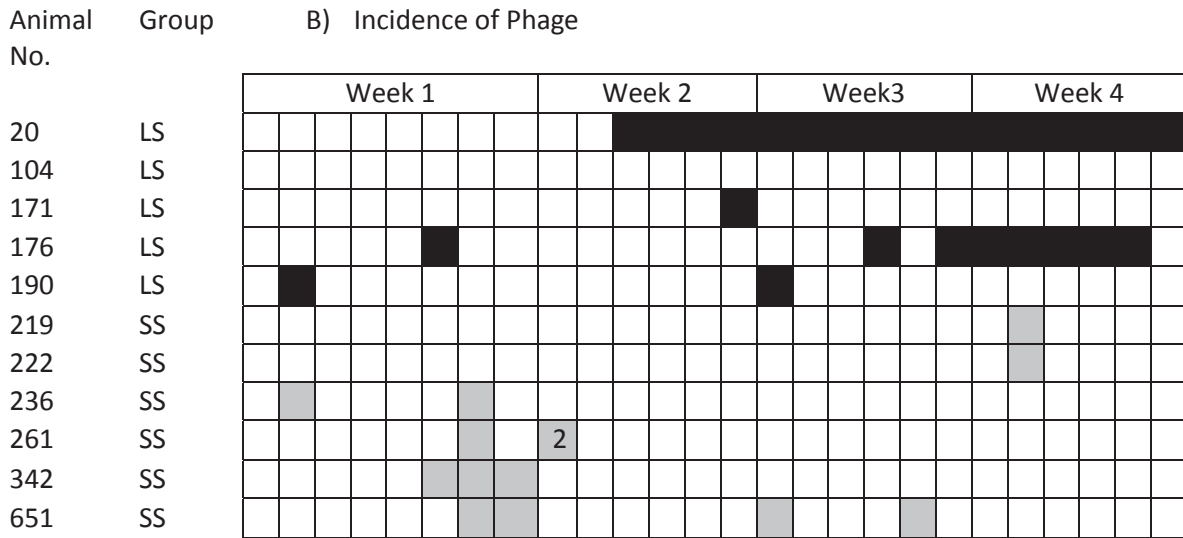
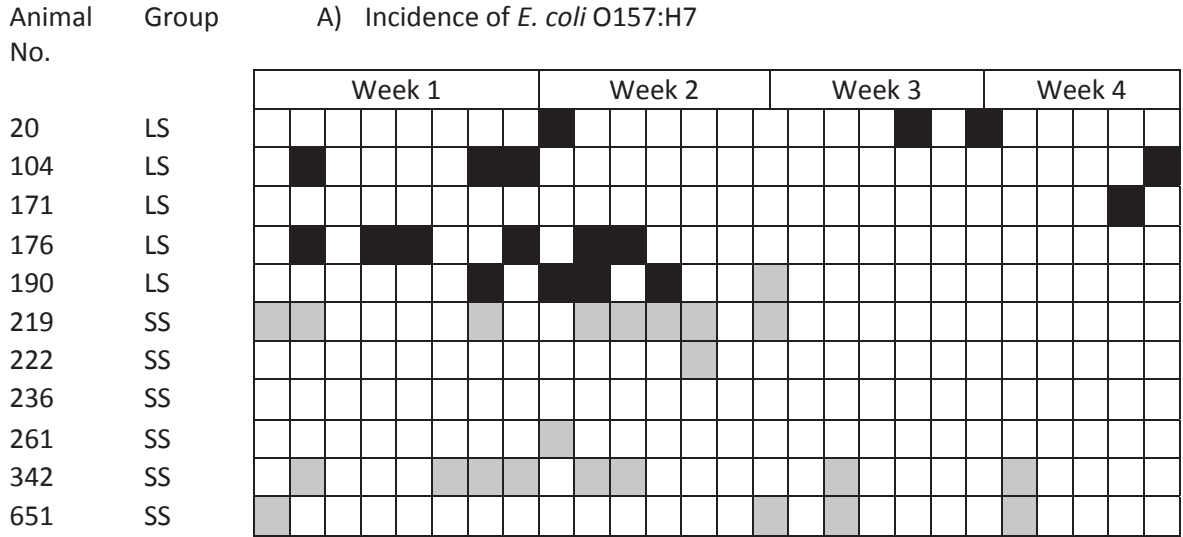


Figure 4.1. Fecal samples (pats or grabs) from steers (n= 11) positive for *E. coli* O157:H7 and phages from July 8 to August 7, 2011. Black filled boxes represent low-shedding (LS) steers and grey filled boxes represent super-shedding (SS) steers. The number 2 represents steers from which two types of phages were isolated from and individual steer on the designated sampling day.

4.1.3.2. *E. coli* O157:H7 genotypes

A total of 41 isolates were confirmed as *E. coli* O157:H7 by multiplex PCR. Based on PFGE patterns, positive isolates were classified into 6 subtypes with 90% similarity (Table 4.1). *E. coli* O157:H7 subtype 3 was isolated from both SS (86.4%) and LS (63.2%) steers. *E. coli* O157:H7 subtypes 1 and 6 were only isolated from SS, while subtypes 2, 4, and 5 were only isolated from LS.

4.1.3.3. Prevalence of phages

Fecal samples positive for phages were found in both SS and LS cattle (Fig. 1B). Overall, the prevalence of phages in the SS group (13/156, 8.3%) was considerably lower ($P = 0.01$) than in the LS group (27/129, 20.9%). In SS steers, phages were detected more frequently during the first week (8/40, 16.7%) than in subsequent weeks (2.8–5.6%), although this difference was not significant ($P = 0.078$). By contrast, phages were more frequently isolated from LS steers during the last sampling week (36.7%), at levels higher ($P < 0.05$) than that obtained in the first two weeks (5.1–13.3%). Moreover, the prevalence of phages in the third and fourth weeks from LS animals was substantially higher ($P \leq 0.01$) than in the corresponding weeks from SS steers. All phages isolated from short-(1 h) enrichments were from LS cattle, suggesting high phage populations in (2/5) of these steers.

4.1.3.4. Phage genome sizing and phage groups

A total of 41 isolated phages were divided into 3 groups (α , β , γ) based on PFGE genome sizes (Table 4.1). The genome size was 42 kb for phage group α , 92 kb for phage group β and 183 kb for phage group γ . Two types of plaque morphology were observed: small (< 1 mm diameter) or bulls-eye (~ 1-2 mm diameter clearing zone with translucent halos). All isolates in groups β

Table 4.1. Number of isolates of *E. coli* O157:H7 PFGE subtypes and phage groups from feces of each steer (n = 11). *E. coli* O157:H7 subtypes (1-6; n = 41) are based on ~90% similarity among isolates. Phage groups (α , β , γ ; n = 41) are based on genome size. SS represents super-shedder steers and LS represents low-shedder steers.

Steer	Type	<i>E. coli</i> O157:H7 PFGE Subtype						Phage Group		
		1	2	3	4	5	6	α	β	γ
20	LS			3					1	15
104	LS		1	3						
171	LS				1				1	
176	LS			1		5		1		7
190	LS			5				1	1	
219	SS			7			1			1
222	SS			1					1	
236	SS							1	1	
261	SS			1				2	1	
342	SS	1		7				3		
651	SS	1		3				3	1	

and γ displayed small plaques, while isolates in group α displayed the bulls-eye morphology. On one occasion, phages isolated from a single fecal sample from one SS steer exhibited both morphologies. Phage group α was most frequently isolated from SS (64.3%), while group γ was most frequently isolated from LS (81.5%). One phage was selected for further characterization from each phage group based on having high titer against strain R508 with vB_EcoS_ALC35 selected from Group α with a MOI of 1, vB_EcoM_ALS20 selected from Group β with a MOI of 0.001 and vB_EcoM_ALC54 selected from Group γ and with a MOI of 0.0001.

4.1.3.5. RFLP of phage groups

RFLP patterns showed multiple restriction sites for phage ALS20 (Fig 4.1B) and ALC35 (Fig 4.1C) by *HindIII*. In contrast, the ALC54 phage genome was left intact using enzymes *HindIII*, *EcoRI*, *PstI*, *XbaI*, *KpnI*, *XhoI*, *SacI*, *Acc651*, *Sall* and *AluI* although partial restriction sites were found for this phage using enzyme *EcoRV* (Fig 4.1D).

4.1.3.6. TEM and characterization of phages

Phages from each group displayed morphologically distinct structures with TEM. Structurally, phage ALC35 had a head diameter of 58 nm and a long tail of 156×8 nm with tail fibres (Fig 4.3A). Based on morphology and genome size ALC35 was identified as a T1-like phage of *Siphoviridae*. ALS20 had an isometric head of 72 nm in diameter and a tail of 107×18 nm and was identified as an O1-like phage of *Myoviridae* (Fig 4.3B). ALC54 had a large head of 105 nm and a striated contractile tail of 133×16 nm and was characterized as a T4-like phage of *Myoviridae* family (Fig 4.3C).

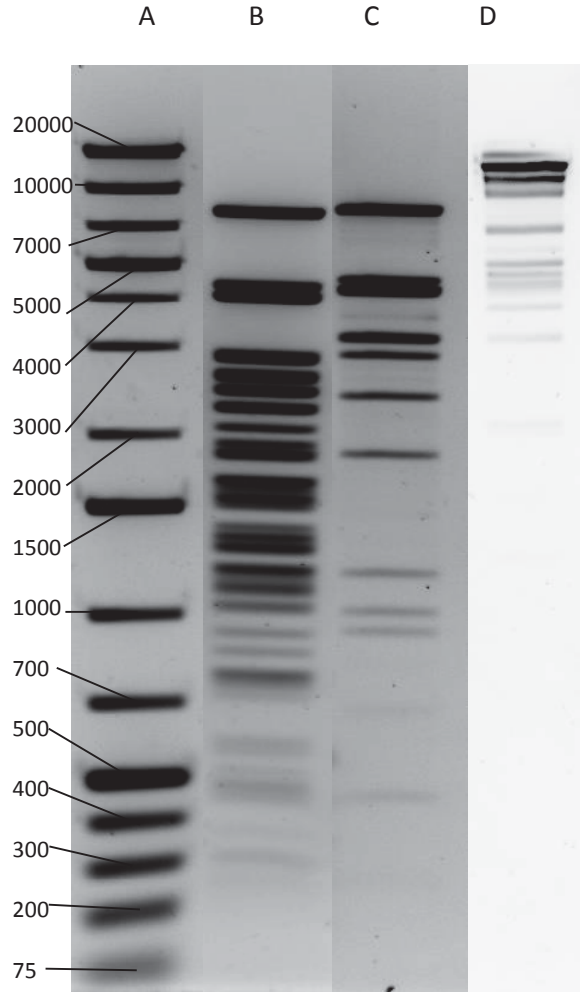


Figure 4.2. Restriction fragment length polymorphism (RFLP) of phage genomes. A: 1kb plus DNA ladder (Fermentas, Carlsbad, CA); B: ALS20 digested by *Hind*III; C: ALC35 digested by *Hind*III D: ALC54 digested by *Eco*RV

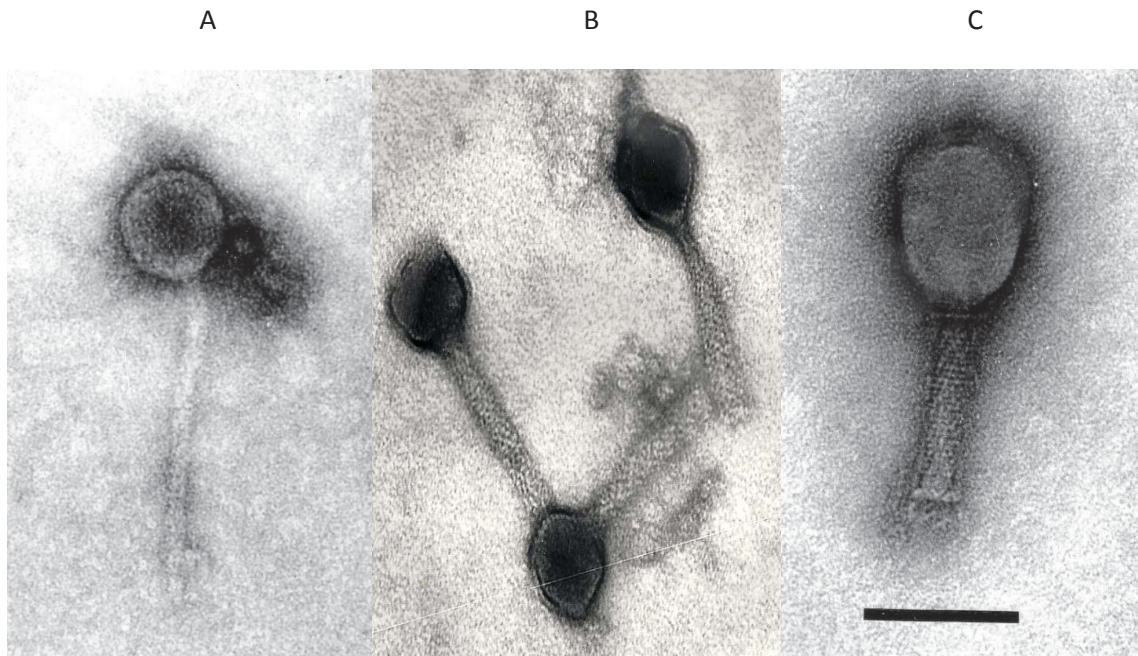


Figure 4.3. Transmission electron microscopy of A) ALC35, T1-like phage; B) ALS20, O1-like phages; C) ALC54, T4-like phage, bar represents 100nm; negatively stained with uranyl acetate

Table 4.2. Susceptibility of *Escherichia coli* O157:H7 phage types to phages ALC35, ALS20 and ALC54. +++ (MOI < 0.01); ++ (0.01 ≤ MOI < 1); + (1 ≤ MOI < 10); R (no lysis observed)

Phage	Phage group ^a	Extremely sensitive (+++)	Highly sensitive (++)	Moderately sensitive (+)	Resistant (R)
ALC35	α	PT ^b :1,10,14,14a,21,23,28,32,34,46,47,48,68,80, 88; LPT ^c :4,14a	PT:4,24,49,67,74 ; LPT43	PT:2,8,31,33,38,45,50,51,54,63	LPT91
ALS20	β	PT:1,4,24,28,45,46,47,49,50,67,68,74,88; LPT:14a,43	PT:2,8,10,14,14a,21,23,31,32,33,34,38,48,54,63,80; LPT4		PT51; LPT91
ALC54	γ	PT1,2,4,8,10,14,14a,21,23,24,28,31,32,33,34,38,45,46,47,48,49,50,51,54,63,67,68,74,80,88; LPT: 4,14a,43,91			

^aPhage groups are based on genome sizes: 42.4 kb for group α, 91.6kb for group β, 182.7 kb for group γ.

^bPT are phage types from *E. coli* O157:H7 collection of the Laboratory for Foodborne Zoonoses, (Public Health Agency of Canada (PHAC), Guelph, ON, Canada).

^cLPT are phage types of *E. coli* O157:H7 isolated during the present study.

4.1.3.7. Sensitivity of *E. coli* O157:H7 to endemic phages

E. coli O157:H7 host strain R508 was confirmed as highly sensitive to the phages selected from each group (ALC35, ALC54 and ALS20; Table 4.2). Six strains of *E. coli* O157:H7, isolated with the phages (3 from SS and 3 from LS) and representing the six different PFGE subtypes, were also tested against phages to determine *E. coli* O157:H7 sensitivity. The 6 'local' *E. coli* O157:H7, were phage-typed as PT4, PT14a (N = 3), PT43 and PT91. All 30 PT in the strain collection and all 6 locally-isolated *E. coli* O157:H7 were extremely sensitive to phage ALC54. For phage ALS20, resistance was noted for *E. coli* O157:H7 PT51 and PT91, although all other strains were extremely or highly sensitive. Lysis by phage ALC35, was variable, ranging from extremely sensitive 52.7% (19/36), highly sensitive 16.7% (6/36), moderately sensitive 27.7% (10/36) or resistant 2.78% (1/36).

4.1.4. Discussion

4.1.4.1. *E. coli* O157:H7 prevalence

The overall prevalence of fecal shedding of *E. coli* O157:H7 was 14.4%, similar to other studies of feedlot cattle in summer months (Chapman et al., 1997; Stanford et al., 2005). The prevalence of *E. coli* O157:H7 in the study in SS and LS groups was similar suggesting that most SS steers did not remain persistent shedders after transport to the research facility. Classifying cattle with relation to shedding level was difficult as one SS did not shed *E. coli* O157:H7 for the duration of the study after relocation. Individual cattle may only be super-shedders for a short period of time (Arthur et al., 2009; Stephens et al., 2009) and *E. coli* O157:H7 prevalence gradually decreased over the course of the study. Although most SS did not continue to shed *E. coli* O157:H7 at SS levels, the numbers of *E. coli* O157:H7 excreted were higher in SS comparatively to LS, possibly due to enhanced colonization of the gastrointestinal tract by *E. coli*

O157:H7 in SS. Accordingly, cattle persistently shedding *E. coli* O157:H7 have been shown to have 10-100 times greater colonization of intestinal sites compared to non-persistent shedding cattle (Baines et al., 2008).

4.1.4.2. *E. coli* O157:H7 subtypes

The majority (75.6%) of *E. coli* O157:H7 isolated from both SS and LS cattle was from one dominant PFGE subtype as has been previously reported (Lahti et al., 2003; Stanford et al., 2005) and suggests that certain dominant subtypes persist or readily disseminate among cattle. Some studies indicate that SS cattle may carry unique subtypes of *E. coli* O157:H7 (Stanford et al., 2012; Chase-Topping et al., 2008) whereas other studies do not identify common subtypes exclusive to high-shedding animals (Jeon et al., 2013; Cobbold et al., 2007; Arthur et al., 2013). In the current study, distinct PFGE subtypes were found exclusively in SS or LS cattle suggesting a relationship exists between subtypes of *E. coli* O157:H7 and number of *E. coli* O157:H7 shed by cattle.

Phage typing revealed PFGE subtypes 2, 3, and 5 as PT14a, which has emerged as the predominant PT of *E. coli* O157:H7 isolated from human disease outbreaks in Canada (National Microbiology Laboratory, 2010) and the presence of PT14a in both groups suggested SS and LS cattle harbored a highly pathogenic strain capable of causing disease in humans. The heterogeneity of excreted *E. coli* O157:H7 by individual cattle involves colonization by the bacteria of the gastrointestinal tract. Persistently shedding cattle may be influenced by unknown factors that favor colonization by specific strains of *E. coli* O157:H7 (Baines et al., 2008). SS and LS cattle are affected by seasonality (Stephens et al., 2009), but relationships within intestinal microbiota are still poorly known and endemic phages may influence clearance, proliferation or possibly super-shedding of *E. coli* O157:H7 by individual cattle.

4.1.4.3. Prevalence of phages

The overall prevalence of phages from fecal samples with SS (8.3%) and LS (20.9%) was similar to the range (11.7-23.3%) reported by Callaway et al. (2006). Phages may fluctuate in concert with populations of *E. coli* O157:H7 (Niu et al., 2009) and low populations of *E. coli* O157:H7 may have been due to presence of O157-specific phages in the present study. Low-shedding cattle had higher concentrations of phages and lower numbers of *E. coli* O157:H7 compared to SS suggesting that the presence of phages may influence shedding.

4.1.4.4. Characterization of endemic phages

Based on genome size and TEM morphology, the phage isolates were classified into T1-like phage of *Siphoviridae*, O1-like and T4-like phages of *Myoviridae*.

4.1.4.5. ALC35: T1-like phage

Other endemic T1 phages isolated from the feedlot environment have had genome sizes of 44 kb, plaques with wide opalescent zones while restriction digestion analysis indicated 8-12 fragments after digestion with *Hind*III similar to phage ALC35 (Niu et al., 2012; Kropinski et al., 2012a). Transmission electron microscopy revealed a general resemblance to phage T1 (Niu et al., 2012). ALC35 displayed highly variable lysis patterns on *E. coli* O157:H7 isolates indicating that lytic capability of this phage probably varied with the host. *Escherichia coli* PTs 2 and 8, which are commonly isolated from outbreaks in nursing homes (Woodward et al., 2002) and elsewhere in Canada (National Microbiology Laboratory, 2010) were only moderately sensitive to ALC35.

4.1.4.6. ALS20: O1-like phage

The genome size of ALS20 from phage group β was 91.6 kb and very close to that of Felix O1 (88.9kb) and the O1-like wV8 virus (86.1kb; Villegas et al., 2009). Restriction analysis of ALS20 showed multiple cleavage sites by *Hind*III but restriction sites differed with those of the O1 virus, FO1, where two cleavage sites were found after *Hind*III digestion (Kuhn et al., 2002). Most *E. coli* O157:H7 PTs were extremely or highly sensitive to ALS20 although resistance to PT51 and the locally-isolated PT91 was observed. PT51 and PT91 are not commonly isolated from humans in Canada (National Microbiology Laboratory, 2009). In addition to *E. coli* O157:H7, the Felix O1-like virus and phage wV8 virus are able to lyse many common *Salmonella* strains (Villegas et al., 2009), suggesting that O1-like phages may make good candidates for phage therapy by targeting multiple pathogenic bacterial species capable of causing disease in humans.

4.1.4.7. ALC54: T4-like phage

The genome size at ~182.7 kb of ALC54 was similar to that of several T4 phages specific for *E. coli* O157:H7 that have been isolated showing genome sizes ranging from 166-180 kb (Abuladze et al. 2008; Kropinski et al., 2012b; Raya et al., 2006). Restriction endonuclease digestion of ALC54 determined most enzymes were unable to cut the genome similar to other previously isolated *E. coli* O157-specific T4 phages (Liao et al., 2011; Raya et al., 2006). Electron microscopy of ALC54 revealed myovirus morphology and a large head like other T4-like phages (Kropinski et al., 2012b; Raya et al., 2006). Most endonucleases do not digest T4-DNA due to the presence of glycosylated hydroxymethyl cytosine instead of cytosine (O'Flynn et al., 2004). All *E. coli* O157:H7 PT and locally isolated- *E. coli* O157:H7 were extremely sensitive to lysis by ALC54, suggesting this T4-like phage is an efficient predator of *E. coli* O157:H7. T4-like phages isolated from dairy and beef feedlot manure lysed 94-98% of *E. coli* O157:H7 as determined by Viazis et

al. (2011), and although their lytic capabilities varied among isolates, an 8-phage cocktail was capable of reducing *E. coli* O157:H7 populations by $> 5 \log \text{CFU}\cdot\text{ml}^{-1}$ *in vitro* at 37°C. The broad host range of T4-like phages stems from their ability to recognize several host lipopolysaccharides, outer membrane proteins and C-termini specific for adsorption of *E. coli* O157:H7, making T4 phages excellent candidates for phage therapy (Liao et al., 2011; Raya et al., 2006). Phages are widespread in livestock (Niu et al., 2009) and the current study indicates T4-like endemic phages are persistent in some cattle.

4.1.5. Conclusions

Most steers harbored *E. coli* O157:H7 PT 14a, which is capable of causing severe disease in humans. Compared to T1- and O1- like phages, T4-like phages exhibited broader host range and stronger lytic capability when targeting *E. coli* O157:H7. Moreover, the T4-like phages were more frequently isolated from feces of LS than that of SS, suggesting endemic phages may play an important role in mitigating *E. coli* O157:H7 in cattle and the different shedding level of the pathogen in each animal might be partially dependent on phages. Some of the challenges facing recent phage therapy attempts are successful replication of phages and selection of phages. The impact of endemic phages on optimum phage: host concentrations and/or competition or synergy among phages should be considered when assessing phages for therapy. A better understanding of the microbial ecology of the gastro-intestinal tract of cattle is critical for successful management of *E. coli* O157:H7.

5. CHAPTER 5

5.1. Implications and Conclusions

E. coli O157:H7 was first identified in 1982 from a Michigan ground beef outbreak (Wells *et al.*, 1983; Riley *et al.*, 1983) and has been the source of multiple human disease outbreaks worldwide (Greig and Ravel, 2009). Infection with *E. coli* O157:H7 in humans can result in hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), or thrombotic thrombocytopenia purpura (TTP) prompting health complications including renal impairment, seizures, diabetes, hypertension or even death (Thomas and Elliot, 2013). Mortality rates after development of HUS are 2-10% and children, elderly and people with underdeveloped immunity are particularly at high risk (Kiranmayi *et al.*, 2010). The low infectious dose (~10 cells) and association with severe human disease or death makes *E. coli* O157:H7 a significant threat to public health. The National Enteric surveillance program in Canada estimated that gastrointestinal illness associated with Shiga toxin-producing *E. coli* including *E. coli* O157:H7 was 4.47/100 000 people in Alberta and may be under-reported (Public Health Agency of Canada, 2009). Prevention of *E. coli* O157:H7 related illness would notably reduce healthcare costs and improve public health.

Cattle are natural reservoirs of *E. coli* O157:H7 and contamination of carcasses during slaughter can transfer pathogens into the human food chain. Worldwide, beef remains the most common food-source contaminated with *E. coli* O157:H7 (Juck *et al.*, 2012) and outbreaks due to improper handling and consumption of undercooked meat remain a vehicle for transmission of the bacteria to humans. Contamination of foods in production and distribution chains has resulted in massive recalls and substantial economic loss to the beef industry (Vogt and Dippold, 2005). In Sept. 2012, *E. coli* O157:H7 detected in beef resulted in 4000 tonnes of beef and beef products recalled from Canadian, American and international markets with losses to the beef

industry estimated at \$16-27 million (Lewis *et al.*, 2013). *E. coli* O157 is estimated to be the fourth-most costly foodborne disease in Canada and the USA and federal inspection agencies have enforced a “zero-tolerance” policy towards *E. coli* O157:H7 in beef (Kiranmayi *et al.*, 2010). In processing, cross contamination exists when infected carcasses are in direct contact with each other, personnel and/or equipment (Elder *et al.*, 2000) and the magnitude of *E. coli* O157:H7 in cattle prior to slaughter may impact the likelihood of pathogen transmission occurring in processing facilities. Sequential interventions at the farm level have been found to reduce both prevalence and concentrations of *E. coli* O157:H7 in cattle feces and on cattle carcasses (Smith *et al.*, 2013). Many outbreaks of *E. coli* O157:H7 can be traced back to farms and pre-harvest strategies reduce the risk of carcass contamination at slaughter and processing plants (Soon *et al.*, 2011).

Several factors have been linked to fecal shedding of *E. coli* O157:H7 in cattle including season (Stanford *et al.*, 2005b), age (Cray and Moon, 1995), stress (Schwartzkopf-Genswein *et al.*, 2007), housing (Synge *et al.*, 2003) and diet (Callaway *et al.*, 2009). The correlation among diet types and prevalence and intensity of shedding of *E. coli* O157:H7 has been inconsistent and clarification of the impact of specific dietary components on microbial ecology of the cattle gastrointestinal tract of cattle could aid in reducing prevalence of the organism. Distillers’ dried grains with solubles (DDGS) are by-products of the bioethanol industry and are a valuable feed source for cattle. Starch from grains is converted to ethanol through fermentation leaving a residue which has higher protein, fat and fiber content than complete grains (Klopfenstein *et al.* 2008). Cattle fed diets containing up to 40% DDGS on a dry matter basis gain faster and more efficiently than cattle fed traditional grains (Ham *et al.* 1994). World ethanol production is steadily increasing as more countries are motivated both economically and environmentally towards sustainable renewable fuel production. On September 1, 2010 the government of

Canada finalized the Federal Renewable Fuel Regulations which require an average of 5% renewable content in gasoline and 2% in diesel and heating distillate oil (Canadian Environmental Protection Act, 2010). New policies in Canada have seen biofuel production increase to ~3 billion litres from grain, oilseeds and biomass. The expansion of the bioethanol industry has led to increased availability of by-products and depending on market forces, increased costs for traditional feed grains are making DDGS an attractive alternative to traditional grains. Several factors need to be considered in terms of environmental and economic sustainability of DDGS as a feed source for cattle. Feeding of corn-based DDGS (CDDGS) in the United States has been linked to increased fecal shedding of *E. coli* O157:H7 (Jacob *et al.* 2008a,b). Fecal shedding of *E. coli* O157:H7 in cattle contribute to contamination of the environment and subsequently increased potential for transmission into the human food chain. Corn is the primary source of DDGS in the United States but in Canada wheat is the principal grain used due to its high starch content and ability to be grown in northern climates (Gibb *et al.* 2008). Canada's trading partners are certain to inquire if feeding wheat-based DDGS (WDDGS) causes similar impacts on the shedding of *E. coli* O157:H7. Results from my current research suggest that adding WDDGS in the finishing diets of cattle does not affect fecal shedding or persistence of *E. coli* O157:H7. Feeding CDDGS or WDDGS to cattle failed to produce any difference in fecal prevalence of *E. coli* O157:H7 in the present study, but suggests other factors must be evaluated in conjunction with diet. Cattle are intermittent shedders and overall *E. coli* prevalence in feedlot studies, range of prevalence between pens and over time have been very high and this may be due to inconsistent shedding among animals and/ or within pens (Dewell *et al.*, 2005; Wells *et al.*, 2009; Berry *et al.*, 2010). As well, individual animals may have unique shedding patterns with periods of high prevalence of shedding followed by longer periods of reduced or undetectable shedding (Synge *et al.*, 2003; Stanford *et al.*, 2010). Previous

studies assessing inclusion of DDGS and fecal shedding have collected limited fecal samples, inoculated small numbers of cattle or had short sampling periods with limited O157 prevalence. Inconsistent shedding of *E. coli* O157:H7 by cattle increases the difficulty of determining impacts of factors including diet as animals may be in different stages of shedding. In the present study it is possible a barley-based finishing diet may have increased *E. coli* O157:H7 populations since cattle fed barley diets have had higher prevalence of *E. coli* O157:H7 compared to cattle fed corn and corn/cottonseed mixed diets (Buchko *et al.*, 2000b; Berg *et al.*, 2004). The composition of DDGS has also been found to differ among bioethanol plants (Nuez-Ortin and Yu, 2010) due to factors such as type of fermentation, drying temperature and duration (Spiels *et al.*, 2002). Recent studies regarding type of finishing diet fed to cattle and the impact on fecal shedding of *E. coli* O157:H7 have been mixed and illustrate the importance of these studies in clarifying inconsistencies among studies. The type of diet (starch source, processing, inclusion level, basal diet) season, sampling and/or individual cattle microbiota are all factors which may have contributed to the variation among studies which emphasizes the importance of evaluating these factors. Even though DDGS may have considerable value as a feed, it would be false economy if the use of this feed resulted in elevated environmental contamination and prevalence of *E. coli* O157:H7 in processing facilities thereby increasing risk of passage into the human food chain. As feeding DDGS in cattle feed has not been previously studied, results from this study are novel and will aid in identifying factors which may contribute to fecal shedding of *E. coli* O157:H7. As shedding was not increased after feeding of DDGS, other factors such as the individual microbiota should be explored.

Multiple combinations of interventions prior to slaughter have the greatest potential to reduce both prevalence and concentration of *E. coli* O157:H7 on cattle carcasses (Smith *et al.*, 2013). Clean, dry bedding, decreased stocking density and stress, exclusion of wild animals,

clean feed and water and training of farm workers in hygienic practices can result in a significant reduction of *E. coli* O157:H7 in the environment (Soon *et al.*, 2011). Improved managerial practices at the feedlot may reduce likelihood of *E. coli* O157:H7 fecal shedding in cattle but interventions targeted at eradicating *E. coli* O157:H7 will aid in reducing *E. coli* O157:H7 prior to slaughter.

Bacteriophages (phages) are natural predators of bacteria, ubiquitous in the environment including foods and are prime candidates for reduction of pathogens since have the ability to replicate in host cells, self-limiting and specific to their targets (Letarov and Kulikov, 2009). The use of phage cocktails increases the host range that phages can infect and the large pool of candidates (10^{30} - 10^{32} virions in biomass) provide immeasurable alternate preparations for eradication of *E. coli* O157:H7 (O'Flynn *et al.*, 2004). Recently, the Food and Drug Administration in the United States approved a phage preparation for elimination of *Listeria monocytogenes* in raw and ready-to-eat meats predating an era of new strategies for reduction of pathogens in the meat industry thereby improving food safety for the public and reducing meat recalls (Guenther *et al.*, 2009). Humans are naturally exposed to phages in food and phages are consumed regularly with no apparent ill effects. The effectiveness of phage in controlling *E. coli* has been demonstrated *in vivo* for mice, cattle, sheep and pigs and environmental application for reduction of pathogens in the feedlot has also been studied (Summers, 2001). Unfortunately, long term control of *E. coli* O157:H7 using phages has not yet been successful and knowledge of the natural microbiota including endemic phage, is relatively limited making interpretation of these studies difficult (Kropinski *et al.*, 2012a). Knowledge of *E. coli*/phage ecology in the natural environment would aid in improving current mitigation methods. Characterization of endemic phages will be valuable in understanding the diverse nature of these agents and contribute to a pre-existing database of phage genomics.

Results from my research identified three species of endemic phages which may impact levels of shedding of *E. coli* O157:H7 in cattle and be valuable for reduction of *E. coli* O157:H7. Some challenges facing recent phage therapy experimentation for success *in vivo* are specificity of phage to target strains, the optimum phage and host concentrations, a suitable delivery method to the host and emergence of phage-resistant mutants. Characterization of endemic phages is relevant as interaction of these phages may alter phage/host concentrations or create competition among phages. Sequencing of endemic phage genomes will improve our understanding of phage genes and function which can then be used to produce superior phages for phage therapy. It may be possible to broaden host range, reduce occurrence of mutants by altering phage genomes for host specificity or extract phage genes coding for proteins such as lysins which are effective against bacterial pathogens (Coffey *et al.*, 2010). Although suitable delivery methods have been explored to ensure effective delivery of phage in cattle gastrointestinal tracts (Bach *et al.*, 2003; Stanford *et al.*, 2010), knowledge of phages is limited and a greater understanding of phage mechanisms of infection and interaction will ensure improved survival and targeting of phages for effective phage therapy. As the natural microbiota in the animal is complex, endemic phages specific to *E. coli* O157:H7 in cattle can also effectively be developed for bio-control in processing plants or within retail stores to reduce risk of contamination of foods with *E. coli* O157:H7. Preliminary studies have demonstrated reductions in *E. coli* O157:H7 on hard surfaces, meats and vegetables using phage cocktails (Abuladze *et al.*, 2008; O'Flynn *et al.*, 2004). Improved understanding of the natural microbiota from individual cattle will improve current phage bio-control methods and aid in understanding the complex factors which affect fecal shedding of *E. coli* O157:H7 in cattle.

Hazard analysis critical control points decrease the incidence of carcass contamination in processing facilities but cross contamination in processing plants still occurs (Elder *et al.*, 2000).

In addition to preventative measures for reducing *E. coli* O157:H7 at the abattoir, new technologies such as phage bio-control can improve upon existing systems to reduce *E. coli* O157:H7 in meat. Despite preventative measures after production such as health education campaigns, proper food handling practices and testing to prevent dissemination of *E. coli* O157:H7, outbreaks due to *E. coli* O157:H7 still occur and costs to public health and industry can be high. Multiple strategies to reduce fecal shedding of *E. coli* O157:H7 in cattle prior to slaughter will reduce risk of this pathogen entering the human food chain thereby improving public health and safety. By identifying factors that are associated with fecal shedding of *E. coli* O157:H7 we can design or improve our existing mitigation methods to reduce transmission of this pathogen to humans.

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