Impact of Diet on the Abundance and Virulence Properties of *Escherichia coli* in Beef Cattle Overwintering Environments and Dairy Cattle

by

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ABSTRACT

The objective of this study was to determine the effect of nutrient density and housing on *E. coli* populations in beef and dairy production systems. In the first trial, sixty second trimester beef cows were housed in two different overwintering environments and provided dry hay which was either bale grazed or placed in a feed bunk. Selected pens had supplemented with dried distiller's grain with soluble (DDGS; 2.5 kg/cow/every third day) or rolled barley (1 kg/cow/day). In the second trial, six rumen and caecal-cannulated, non-lactating, multiparous Holstein cows received one of the following diets: i) 70% forage ii) grain pellet or iii) alfalfa pellet. The latter two diets were formulated to induce subacute ruminal acidosis (SARA). All animals were randomly sorted to pens and treatments. Fecal samples were collected and cultured on selective media. *E. coli* were enumerated and three isolates were chosen for PCR to detect the presence of 18 selected genes encoding a range of virulence factors. These same isolates were tested for their ability to invade the human adenocarcinoma epithelial cell line HT-29. Diet did not significantly affect *E. coli* abundance but did influence the prevalence of virulence genes involved in adhesion of bacteria to epithelial surfaces. When the diet contained grain, cows shed isolates which were more invasive than those from cows in the other treatments. The data suggest that diet may affect the abundance of *E. coli* shed in the feces and increase the presence of *E. coli* harbouring particular virulence genes that mediate adhesion and invasion of epithelial surfaces.

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ABREVIATIONS

ADF	=	acid detergent fiber
Ca	=	Calcium
cfu	=	colony forming unit
CP	=	crude protein
CTAB	=	cetyl trimethylammonium bromide
DAEC	=	diffusely adherent *E. coli*
DDG	=	dried distiller's grain
DDGS	=	dried distiller's grain with soluble
DG	=	distillers grain
DM	=	dry matter
DNA	=	deoxyribonucleic acid
EAEC	=	enteroaggregative *E. coli*
EHEC	=	enterohaemorrhagic *E. coli*
EIEC	=	enteroinvasive *E. coli*
EMB	=	eosin methylene blue
EPEC	=	enteropathogenic *E. coli*
ETEC	=	enterotoxigenic *E. coli*
ExPEC	=	extraintestinal *E. coli*
HT-29	=	human adenocarcinoma epithelial cell line
LB	=	Luria-Bertani
LPS	=	lipopolysaccharide
$MgCl_2$	=	magnesium chloride
MNEC	=	meningitis associated *E. coli*
NaCl	=	sodium chloride
NDF	=	neutral detergent fiber
P	=	phosphorous
PBS	=	phosphate buffered saline
PCR	=	polymerase chain reaction
S	=	sulfur
SARA	=	subacute ruminal acidosis
SDS	=	sodium dodecyl sulfate
SEM	=	standard error of the mean
UPEC	=	uropathogenic *E. coli*
VFA	=	volatile fatty acid

1.0 INTRODUCTION

Over the last decade, livestock operations have increased in size resulting in a higher density of animals in a single location. As a result, there is a concentration of manure that is primarily used for fertilizing fields dedicated to the production of animal feed as well as produce for human consumption. Since cattle are a known reservoir for pathogenic *Escherichia coli* O157:H7, there is the potential for pathogen dispersal from animal to human via contaminated produce (lettuce, spinach, sprouts), runoff, or contaminated meat from abattoirs (Tarr et al., 2005). Methods to reduce the number of pathogenic bacteria shed from cattle will minimize the likelihood of human diseases resulting from contaminated agricultural products. Evaluation of current livestock management practices is necessary to identify the factors which significantly influence the abundance and shedding of pathogenic bacteria. Such factors may include dietary components (forages, concentrates, and additives), animal housing (intensive or extensive), and animal health (metabolic state, stress). A holistic approach encompassing all of these elements is essential in decoding the complex bacterial interactions that occur in the gut microbiome. Understanding the impact of on-farm management practices on abundance and virulence of pathogenic *E. coli* may serve to reduce public health risk and allow the livestock industry to expand in a sustainable fashion.

The focus of the current study was to evaluate the effect of on-farm management practices, including diet (nutrient density) and housing (intensive vs. extensive) on *E. coli* populations in beef and dairy cattle production systems. In each case, the abundance and diversity of *E. coli* were evaluated through culture and molecular techniques.

2.0 LITERATURE REVIEW

2.1. INTRODUCTION

Cattle may shed several species of pathogenic bacteria in their feces, including *Listeria monocytogenes, Campylobacter jejuni, Mycobacterium paratuberculosis, Salmonella spp.,* and *Escherichia coli* (Avery et al., 2004; Pell, 1997). The strains of pathogenic *E. coli* that cause disease in humans and animals are extremely diverse with respect to the mechanisms used for colonization and infection (Donnenberg and Whittam, 2001). *E. coli* pathogenicity can be determined by the virulence factors possessed by the bacterium with only certain combinations of these virulence traits resulting in a highly pathogenic strain (Kaper et al., 2004). Genes encoding adherence factors and toxin production are typically involved in *E. coli* pathogenicity (Kaper et al., 2004; Maurer et al., 1998). *E. coli* colonizing the gastrointestinal tract that are considered to be diarrheagenic can be divided into six main categories: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) (Croxen and Finlay, 2010; Kaper et al., 2004). Included within the EHEC category are Shiga toxin- producing *E. coli* (STEC) which sometimes carry one or both Shiga toxins (also referred to as verocytotoxins) (Vanselow et al., 2005). Extraintestinal *E. coli* (ExPEC) are another category that includes uropathogenic *E. coli* (UPEC), neonatal meningitis *E. coli* (NMEC), and strains associated with septicaemia (Chapman et al., 2006; Croxen and Finlay, 2010; Kaper et al., 2004). Among the GI tract colonizers, ETEC and EHEC can cause disease in animals as well as humans (Kaper et al., 2004).

Members of EHEC, including *E. coli* O157:H7, are a part of the gastrointestinal microbiota of cattle and are routinely shed in their feces (Callaway et al., 2004). *E. coli* O157:H7 is a well known cause of food- and water-borne illness (Callaway et al., 2004; Donnenberg and Whittam, 2001). There is a great deal of diversity among strains of *E. coli*, with respect to their underlying pathogenic mechanisms and degree of virulence.

In livestock production systems, there is a risk for pathogen transfer from animals to people either through direct contact or indirect contact; the latter often resulting from consumption of ground beef or water that has been exposed to feces containing pathogenic organisms (Caprioli et al., 2005; Hrudey et al., 2003; Rhoades et al., 2009). Since there is enteric carriage of pathogenic *E. coli* by cattle when they are brought to slaughter, there is the potential for contamination of meat products though contact with fecal material during processing at the abattoir (Rhoades et al., 2009; Van Donkersgoed et al., 1999). In abattoirs, there are several measures used to reduce the presence of pathogenic organisms, including trimming of contaminated areas, steam vacuuming, and application of antimicrobials to the carcass (Rhoades et al., 2009). Current on-farm efforts are focused on reducing the number of pathogenic organisms including *E. coli* O157:H7 through manipulation of management strategies including diet, housing, handling practices, and waste management. This approach seeks to reduce the pathogen load prior to shipping the animals in an effort to improve food safety (Callaway et al., 2004; Vanselow et al., 2005).

2.2. CHARACTERISTICS ASSOCIATED WITH *E. COLI*

2.2.1. General Growth Characteristics of *E. coli*

E. coli grow optimally at 37°C and a pH between 6 and 7. They can, however, tolerate a pH range of 4.4 to 9 (Prescott et al., 2005). They are facultative anaerobes, gram stain negative, oxidase negative, methyl red positive, and indole positive (Henayl et al., 2000). Simple sugars, including glucose, arabinose, lactose, maltose, sorbitol, trehalose, xylose, and mucate, are metabolized by *E. coli* via the Embden-Myerhoff pathway generating hydrogen and carbon dioxide (Henayl et al., 2000; Prescott et al., 2005). *E. coli* are also capable of utilizing acetate and reducing nitrate (Henayl et al., 2000). *E. coli* can be found throughout the environment including the gastrointestinal tract of animals as a consequence of their ability to grow optimally at temperatures close to the body temperature of most animals (range of 36.5 to 39.5°C) and tolerate a wide range of pH.

2.2.2. Virulence Characteristics Associated with *E. coli*

E. coli produces a number of adhesion factors that allow them to colonize, persist, and cause disease (Kaper et al., 2004; Maurer et al., 1998). The virulence of disease-causing strains of *E. coli* is enhanced by the adherence of the bacteria to host tissues (Van den Broeck et al., 2000). One of the most common morphological adhesion structures present on *E. coli* is fimbriae or fibrillae (Kaper et al., 2004; Maurer et al., 1998). Fimbriae are rod-like, protein filaments that are 5-10 nm in diameter (Kaper et al., 2004; Van den Broeck et al., 2000). The fimbriae formed by *E. coli* can be divided into several classes, enabling differentiation of *E. coli* strains (Kaper et al., 2004; Van den Broeck et

al., 2000). Fibrillae are smaller than fimbriae, having a diameter of 2-4 nm and can be classified as long and wiry or curly and flexible (Kaper et al., 2004). Additionally, UPEC and EHEC express afimbriae or non fimbrial adhesins, which include outer membrane proteins such as intimin and lipopolysaccharide (LPS) (Kaper et al., 2004). The LPS of *E. coli* binds to toll like receptor 4 (TLR 4), which spans the cellular membrane, stimulating a cytokine cascade that can lead to septic shock and death in severe cases (Kaper et al., 2004). Pathogenic *E. coli* often produce greater than one type of adhesion which allows them to be more effective at attaching to host cells (Maurer et al., 1998). Since bacterial attachment plays a crucial role in pathogenesis, adhesins are considered important virulence factors.

Pathogenic *E. coli* can also secrete toxins and other proteins which disrupt numerous fundamental eukaryotic processes (Kaper et al., 2004). EHEC, for example, produces Shiga toxins (Stx) which cleaves 28s ribosomal RNA; this in turn halts protein synthesis and results in apoptosis of infected cells (Donnenberg and Whittam, 2001; Kaper et al., 2004). Strains of EHEC may produce one or both Shiga toxins known as Stx 1 and Stx 2 (Schmitt et al., 1991). Stx 2 exhibits greater lethality to mice while Stx 1 is more lethal to Vero cells (Tesh et al., 1991; Tesh et al., 1993). Several different mechanisms are used to transport toxins from the bacterial cytoplasm to the host cells. Heat labile enterotoxins (LT) produced by EPEC, are transported to the extracellular environment by a type II secretion system (Kaper et al., 2004). Conversely, EHEC-mediated Stx are transported by a type III secretion system (Donnenberg and Whittam,

2001; Kaper et al., 2004). Thus host cell damage results from the combination of a potent toxin and an effective means of introducing the toxin.

As seen in EIEC, invasion of epithelial cells represents another means by which *E. coli* is able to establish itself in the host's gastrointestinal tract (Kim et al., 1998). Invasive *E. coli* have been implicated in Crohn's disease, colon cancer, bovine mastitis, and bovine septicemia (Boudeau et al., 1999; Dopfer et al., 2000; Korth et al., 1994; Martin et al., 2004). The invasiveness of *E. coli* can be tested *in vitro* by culturing cell lines which most closely resemble the target cell type. The HT-29 human colon epithelial cell line and bovine mammary epithelial cell line, MAC-T, have been used in invasion assays (Dopfer et al., 2000; Kim et al., 1998). An established population of EIEC in humans can result in inflammatory colitis and dysentery; however, watery diarrhea is the most common symptom (Kaper et al., 2004). Typically EIEC remains localized at the intestinal epithelia and passage into the submucosa is rare (Kaper et al., 2004).

2.2.3. Detection of *E. coli*

The detection of EHEC in food and fecal samples is achieved through culture and/or molecular techniques. The most commonly used media for the isolation of EHEC O157:H7 is sorbitol-MacConkey agar (SMAC) (Kehl, 2002; Paton and Paton, 1998; Voetsch et al., 2004). Since O157:H7 is sorbitol negative, the colonies appear colorless. Chromogenic agars can also be used since they target the inability of *E. coli* O157:H7 to produce β-glucuronidase (Kehl, 2002).

The presence of virulence genes such as such as those encoding Stx, intimin, and enterohaemolysin are typically detected using multiplex PCR (Chen and Griffiths, 1998;

Fagan et al., 1999; Kehl, 2002; Paton and Paton, 1998). Overall, PCR is the most sensitive and specific means of screening for the presence of virulence genes (Paton and Paton, 1998). Unfortunately, there are no known culturing techniques to distinguish between non-O157 EHEC and commensal *E. coli* (Paton and Paton, 1998; Tarr, 1995).

2.3. DISEASE ASSOCIATED WITH *E. COLI*

The shedding of EHEC, more specifically *E. coli* O157:H7, from cattle represents a major challenge for food safety at slaughter plants as well as on-farm via waste management to prevent water contamination. The most common route of infection is through the ingestion of meat that has been contaminated with feces or intestinal digesta after slaughter (Rhoades et al., 2009; Van Donkersgoed et al., 1999). Ingested EHEC must pass through the gastric stomach and then attach and colonize the distal small intestine and colon (Paton and Paton, 1998; Vanselow et al., 2005). Once established, EHEC produces Stx that passes through the epithelial layer and enters the blood stream (Paton and Paton, 1998). Infections caused by *E. coli* O157:H7 usually result in bloody diarrhea and severe stomach cramping which can last for as long as seven days. However, if Stx enters the blood stream, a much more severe condition known as haemolytic uremic syndrome (HUS) can result (Kaper et al., 2004; Russell et al., 2000b). In the host, Stx causes apoptosis of intestinal cells, which mediates damage to the colon and renal endothelial cells of the kidneys (Kaper et al., 2004). The low infective dose of O157:H7 (<100 cells) makes this strain of EHEC very dangerous to the public (Kaper et al., 2004; Russell et al., 2000b). The most important virulence factors influencing the pathogenesis of O157:H7 appears to be Stx 2 and intimin (Vanselow et al., 2005).

Approximately 30% of cattle shed *E. coli* O157:H7 and the ones that do typically shed the bacterium sporadically and at low numbers (Callaway et al., 2009; Callaway et al., 2004; Russell et al., 2000a; Russell et al., 2000b). Collectively, these factors make identification of the pathogen difficult. There are also several other serogroups of EHEC (O26, O111, O103, and O145) which are harbored by cattle and are associated with human infections ranging from watery diarrhea to potentially fatal HUS (Caprioli et al., 2005; Pearce et al., 2004; Rhoades et al., 2009). Clearly, a broad spectrum approach to screening for *E. coli* is required to ensure that all pathogens are detected.

2.4. IMPACT OF LIVESTOCK MANAGEMENT ON *E. COLI* SHEDDING

There are several on-farm management practices that can influence shedding of pathogenic *E. coli* by cattle as well as the survival of the shed pathogens. Management practices such as diet selection, feeding schedule and production goals can all affect pathogen shedding by altering the gastrointestinal environment. Since there is the potential for pathogen transfer from animal to animal as well as animal to people, it is essential that we understand the mechanisms underlying transmission, the associated risks, and the role of management practices in pathogen shedding.

2.4.1. Grain feeding

Cattle are ruminants that have developed the ability to consume and ferment fibrous forage with a high cellulose content. In intensive production systems, feedlot cattle are typically fed readily fermentable, energy-dense feed in order to improve feed conversion efficiency and weight gain (Jacob et al., 2009; Russell et al., 2000a; Russell et al., 2000b). Li et al. (2012) observed a starch content of 7.4% DM in caecal digesta when

cows were fed a high starch diet (33.7% DM) in comparison to a low starch (14.2% DM), forage diet which had starch levels of 2.8% of DM in caecal digesta. As a consequence of the large influx of starch in these diets, a portion of it passes through the rumen and small intestine and is degraded into volatile fatty acids (VFA), maltose, and maltodextrins, by bacteria in the colon. These substrates can then be utilized by *E. coli* (Callaway et al., 2003; Jacob et al., 2009; Khafipour et al., 2009b; Russell et al., 2000a). Cattle receiving high grain diets also tend to shed greater numbers of all *E. coli*, including O157:H7, in comparison to cattle receiving a forage-based diet (Callaway et al., 2009; Russell et al., 2000a; Su et al., 2011). Diets containing high proportions of barley have been linked to an increase of O157:H7 shedding by cattle (Dargatz et al., 1997). The observed difference in fecal shedding between these two diets has been attributed to the fact that high-grain diets result in reduced pH and higher VFA concentrations in the gastrointestinal tract in comparison to a forage-based diet (Cobbold and Desmarchelier, 2004). The combination of low pH (6.4) and increased VFAs (120 mM; Cobbold and Desmarchelier, 2004) may reduce the adherence of EHEC to the gut wall therefore resulting in more *E. coli* being shed into the feces (Cobbold and Desmarchelier, 2004). Therefore, the combination of increased substrate, low pH and increased VFAs likely leads to increased shedding of pathogenic *E. coli* (Cobbold and Desmarchelier, 2004; Russell et al., 2000a).

The inclusion of a grain supplement or a primarily grain-based diet may increase the acid resistance of pathogenic *E. coli* colonizing the hind gut. This is due to the passage of undigested starch to the colon resulting in fermentation in the hindgut, causing

increased VFA production coupled with a decrease in pH (Diez-Gonzalez et al., 1998; Russell et al., 2000a). The lower pH may promote the proliferation of acid resistant bacteria in the hindgut; in fact an increase in acid resistant *E. coli* has been noted when feeding high grain diets (Diez-Gonzalez et al., 1998; Krause et al., 2003). Conversely, feeding a hay diet following a high grain diet has been shown to decrease the number of acid-resistant *E. coli* shed in the feces (Diez-Gonzalez et al., 1998). This group of *E. coli* may be particularly dangerous to humans since they are more likely to survive passage through the host's gastric stomach (Hovde et al., 1999; Russell et al., 2000a). Formulating diets to decrease the abundance of acid-resistant pathogenic bacteria may represent a potential means of decreasing human infections.

2.4.2. Grain processing

The main goal in grain processing is to improve the starch digestibility but excessive availability can result in rapid fermentation of starch to organic acids and cause disorders such as bloat, acidosis, and abscessed livers (Huntington, 1997). Processed grain has been shown to increase fecal *E. coli* numbers in comparison to unprocessed grains (Gilbert et al., 2005). Grain processing influences whether the feedstuff is fermented rapidly in the rumen or passes through the rumen and undergoes fermentation in the hindgut (Huntington, 1997). It has been demonstrated that grain processing such as rolling and steam-flaking increases digestibility and availability of substrates for microbial growth in the hindgut (Jacob et al., 2009). Steam-flaking of grain has been shown to increase O157:H7 shedding compared to grain which has been dry rolled (Fox et al., 2007). It is believed that a greater proportion of starch passes to the hind gut when

feeding dry rolled grain resulting in increased production of organic acids and reduced O157:H7 viability (Fox et al., 2007). Since the method of grain processing shifts the primary site of starch digestion from the rumen to the small and large intestines (Russell et al., 1992), it represents one means by which microbial populations in cattle can be manipulated.

2.4.3. Addition of DDGS

Ethanol plants produce DDGS as a by-product from the fermentation of corn and/or wheat (Ortin and Yu, 2009; Stein and Shurson, 2009). Through the fermentation process, the starch content of the grain is removed leaving a condensed by-product with a more concentrated nutrient content than the original grain (Ortin and Yu, 2009). The quality of DDGS produced depends upon the type of grain, the fermentation conditions, the drying methods, and the grinding procedure (Ortin and Yu, 2009).

2.4.3.1. Nutritional composition of corn and wheat DDGS

The nutrient profile of DDGS depends upon whether corn, wheat, or a mixture of the two was used to produce the ethanol (Ortin and Yu, 2009). The levels of crude fat (49.8 g/kg of DM vs. 165.3 g/kg of DM), neutral detergent fiber (NDF; 480.7 g/kg of DM vs. 494.6 g/kg of DM), acid detergent fiber (ADF; 109.9 g/kg of DM vs. 146.8 g/kg of DM), and sulfur (S; 3.9 g/kg of DM vs. 7.2 g/kg of DM) found in wheat DDGS are lower than the levels found in corn DDGS; however, wheat DDGS has higher levels of crude protein (CP; 393.2 g/kg of DM vs. 320.1 g/kg of DM), dry matter (DM; 937.6 g/kg vs. 914.4 g/kg), P (9.1 g/kg of DM vs. 7.7 g/kg of DM), and Ca (1.8 g/kg of DM vs. 0.5 g/kg of DM) (Ortin and Yu, 2009). Due the high levels of CP in DDGS, it is typically used as

a protein source in diets. Although the exact nutritional composition of DDGS varies depending on the ethanol plant, the overall energy content of corn DDGS is greater than that of wheat or a blended DDGS (Ortin and Yu, 2009).

Furthermore, the amino acid digestibility varies not only between corn and wheat DDGS but between samples of the same grain (Stein and Shurson, 2009). Lysine digestibility varies the most between samples due to possible heat damage during processing as well as greater amounts of dietary fibre in non-corn DDGS (Stein and Shurson, 2009).

2.4.3.2. DDGS and E. coli

In recent years, increased production in the ethanol industry has resulted in a supply of DDGS which can be incorporated into cattle diets, providing an excellent source of protein (Klopfenstein et al., 2008). Interestingly, DDGS supplementation has been shown to increase the pathogens harbored by cattle more specifically *E. coli* O157:H7 shedding (Callaway et al., 2009; Paddock et al., 2013). Feeding dried distiller's grains (DDG) to calves experimentally inoculated with *E. coli* O157:H7 resulted in increased O157:H7 shedding and persistence in the feces (Jacob et al., 2008c). Further research confirmed the observation that wet or dry DG caused increased shedding of O157:H7 in naturally colonized animals (Jacob et al., 2008a; Jacob et al., 2008b). Feeding corn and sorghum-based DG in steam flaked corn diets resulted in significant shifts in the microbial community structure, mainly impacting *Ruminococcacea* and *Prevotella* (Rice et al., 2012). Jacob et al. (2010) found that cattle fed 40% DG had more high shedders of *E.* coli O157:H7 than cattle fed 20% or 0% DG. This observed

difference was attributed to possible physiological changes and different microbial populations as a result of diet or the displacement of starch from the diet (Jacob et al., 2010). Paddock et al. (2013) tested the starch displacement theory by feeding DDG while keeping starch content in the diet the constant through the addition of pure starch and found that starch had no impact on the increased shedding of *E. coli* O157:H7 associated with feeding DDG. The proposed explanation was that either starch is not a factor in O157:H7 shedding or inclusion of pure starch did not accurately replicate a high starch diet (Paddock et al., 2013). The survival of *E. coli* O157:H7 incubated in fecal and rumen contents with 40% corn or wheat DDGS was found to be increased in comparison to fecal and rumen contents with 100% barley grain (Yang et al., 2010). The increased survival of *E. coli* O157:H7 may be due to alterations in microbial populations (Yang et al., 2010). The exact mechanism by which DG increases *E. coli* O157:H7 shedding is currently unknown.

2.4.4. Fasting prior to slaughter

The fasting of cattle prior to slaughter can have an impact on microbial populations and more specifically, O157:H7 shedding. Fasting results in a depression of VFA concentrations in the rumen and hindgut which in turn alters the microbial population (Harmon et al., 1999). However, in New Zealand, fasting heifers for 24 hrs was shown to increase rumen and fecal *E. coli* shedding (Jacobson et al., 2002). In another study there was no increase in fecal shedding of O157:H7 after 48 hrs of fasting; however, after re-feeding the fasted cattle, there was a significant increase in O157:H7 shedding (Buchko et al., 2000). A possible explanation for these finding was that the high

pH, low VFAs, and changes in the microflora in the hindgut may have produced conditions which favour the growth of *E. coli* O157:H7 (Buchko et al., 2000). Collectively, these findings suggest that fasting may increase the overall *E. coli* abundance, but O157:H7 shedding has been variable.

2.4.5. Overwintering strategies

Over the last several years, rising production costs have led producers to look for cost effective methods of feeding their cattle throughout the winter. Extensive grazing systems provide an alternative to the traditional intensive production dry lot system (Klein; MAFRI, 2008). Bale grazing systems provide economic advantages without compromising animal health or production (Klein; MAFRI, 2008). The advantages of an extensive bale-graze system include less wear on equipment, manure being deposited directly on the field, reduced labour requirements, improved fertility, and reduced concentration of manure at a single location (Klein; MAFRI, 2008). A typical set up for bale grazing is a fenced paddock containing approximately 25 bales per acre with bales placed 40 ft apart and each row of bales separated by a moveable electric fence (Klein; MAFRI, 2008). A 3- or 4- day supply of bales is provided at one time and after these are consumed, the fence is moved to provide access to the next set of bales (Klein; MAFRI, 2008). Portable shelters are necessary so that the cattle have protection from the wind throughout the winter (Klein; MAFRI, 2008). Cows may require additional energy or protein supplements depending on their nutritional requirements. Monitoring quality and availability of feed, as well as the condition of the animal is critical in a successful bale grazing system (Klein; MAFRI, 2008).

2.5. METABOLIC STATE OF THE ANIMAL

2.5.1. Subacute ruminal acidosis

Subacute ruminal acidosis (SARA) is a metabolic disorder that occurs throughout the dairy industry. This condition results from feeding diets high in concentrates that undergo rapid fermentation in the rumen causing a build up of organic acids, which in turn depresses rumen pH (Kleen et al., 2003; Krause and Oetzel, 2006; Stone, 2003). The altered rumen conditions cause changes in microbial populations as well as an increased rate of passage (Kleen et al., 2003). Symptoms of SARA include decreased DM intake, diarrhea, depression of milk fat, and increased incidence of laminitis (Kleen et al., 2003; Plaizier et al., 2008; Stone, 2003).

2.5.2. Subacute ruminal acidosis and *E. coli*

High concentrate diets which induce SARA also supply increased substrates in the diet to support microbial growth. The rapid growth of gram negative bacteria is believed to cause inflammation due to increased LPS levels in the rumen, as well as the small and large intestines (Gozho et al., 2007; Khafipour et al., 2009a; Khafipour et al., 2009b). An increase in *E. coli* abundance and virulence has been observed in the rumen of SARA-induced cows fed a grain pellet diet compared to an alfalfa pellet diet (Khafipour et al., 2011). This large influx of starch in the rumen, associated with diets high in concentrates, may pass to the colon where bacteria break it down into VFAs, maltose and maltodextrins (Callaway et al., 2003; Jacob et al., 2009; Khafipour et al., 2009b; Russell et al., 2000a). The provision of increased substrate as well as decreased pH may increase the shedding of acid-resistant *E. coli* by cattle (Diez-Gonzalez et al.,

1998; Russell et al., 2000a). Formulating diets which do not induce SARA may not only improve feed efficiency but also food safety through a reduced pathogen load.

2.6. ENVIRONMENTAL INFLUENCES ON *E. COLI* SHEDDING

2.6.1. Seasonality of *E. coli* shedding

Time of year can have an impact on shedding of pathogenic bacteria by cattle, with the greatest prevalence of O157:H7 shedding observed in summer (Chapman et al., 1997; Hancock et al., 1997; Synge et al., 2003; Van Donkersgoed et al., 1999). Interestingly, there is a correlated increase in human illness during the summer months (Chapman et al., 1997). In warmer months, the proximity of grazing animals to wild geese impact O157:H7 prevalence (Synge et al., 2003). Thus, it appears that O157:H7 shedding is influenced by a combination of factors, and understanding these elements may help to develop control programs.

2.6.2. Stress

An animal's response to stress may result in changes in pathogen abundance and shedding from the gastrointestinal tract. During periods of acute stress, catecholamines (adrenalin and noradrenalin) are released by the enteric nervous system and this can increase pathogenic *E. coli* populations in the gastrointestinal tract (Freestone et al., 2008). Noradrenalin has been shown to increase intestinal mucosal adherence and secretory response by *E. coli* O157:H7 (Freestone et al., 2008). Furthermore, adrenalin and noradrenalin have been shown to elevate expression of virulence genes such as *eae, espB, espA, stx1,* and *stx2* in this bacterium (Chen et al., 2006; Dowd, 2007; Lyte et al., 1996). Cattle may experience stress during transportation and handling, especially if the

animal associates the experience with a fear response (Grandin, 1997; von Borell, 2001). If animals experience stress during transportation, the release of catecholamines may up-regulate expression of virulence genes associated with *E. coli* O157:H7 pathogenesis (Chen et al., 2003; Dowd, 2007). During transportation and handling it is critical that we consider animal welfare as well as the potential consequences a stress response can have on pathogenic *E. coli* populations.

2.7. WASTE MANAGEMENT PRACTICES AND PRESENCE OF *E. COLI*

2.7.1. Survival of *E. coli* in manure

Pathogenic bacteria that are shed in bovine feces can contaminate not only water due to runoff but also food crops due to irrigation with contaminated water (Avery et al., 2004; Callaway et al., 2004). The factors that influence the survival of *E. coli* outside the host include soil type, rainfall, UV radiation, temperature, in-soil predation by other microorganisms, the physiological status of the microbes, and strain variability (Avery et al., 2004). *E. coli* has been shown to survive longer at cooler temperatures (4°C) than at warmer temperatures (>20°C) (Avery et al., 2004; Jiang et al., 2002; Kudva et al., 1998; Wang et al., 1996). Wang et al. (1996) found *E. coli* survived 42- 49 days at 37°C, 49-56 days at 22°C, and 63 to 70 days at 5°C. The properties of soil that influence bacterial survival include soil composition, pH, oxidation reduction potential, water activity, and microbial interactions within the soil (Jiang et al., 2002). Jiang et al. (2002) also reported *E. coli* survival in unautoclaved soil for 34-193 days at a range of temperatures and manure concentrations. From these findings, we can assume that the pathogenic bacteria survive outside the host long enough to infect another host (animal or human) or

contaminate waterways via runoff. In Walkerton, Ontario (2000) an estimated 2300 people became ill and seven died due to exposure to *E. coli* O157:H7 and *Campylobacter jejuni* present in drinking water which was contaminated by farm runoff (Hrudey et al., 2003). These findings underscore the importance of understanding pathogen survival and methods for reducing pathogen shedding by cattle to ensure public safety.

2.7.2. Horizontal transfer of *E. coli*

The fecal-oral route of transmission between cattle is thought to be the most probable mechanism of *E. coli* O157:H7 transfer (Chase-Topping et al., 2008). In the presence of supershedders (animals that shed unusually high numbers of O157:H7) the other cattle in the herd exhibit higher degrees of pathogen shedding (Cobbold et al., 2007). Housing cattle in confined pens has also been associated with increased shedding of *E. coli* O157:H7 in comparison to grazed cattle (Synge et al., 2003). In a housed environment, susceptible cows are more likely to come into contact with fecal matter or objects contaminated with *E. coli* O157:H7 (Chase-Topping et al., 2008). It is not unreasonable to assume that an extensive environment (such as bale grazing) for rearing cattle would reduce horizontal transfer of pathogens between animals as a consequence of reduced animal density.

Since water troughs are used by the whole herd, they are an avenue by which bacteria can be transmitted from one animal to another. *E. coli* O157:H7 has been found to survive in water troughs and to be in sufficiently high numbers to persist and infect new animals (Rice and Johnson, 2000; Shere et al., 2002; Van Donkersgoed et al., 2001). The ability of *E. coli* to survive in an aquatic environment such as a water trough, may

explain why animals within a herd tend to share the same O157:H7 strain (LeJeune et al., 2001; Renter et al., 2003; Shere et al., 2002). Water troughs can be contaminated with feces or saliva containing *E. coli* from cattle or possibly from wild birds, insects, and other animals (LeJeune et al., 2001; Renter et al., 2003; Shere et al., 2002; Van Donkersgoed et al., 2001). Once *E. coli* O157:H7 is in the water trough, it can survive for weeks or months providing ample opportunity for new animals to become infected (LeJeune et al., 2001). Possible methods to mitigate trough transmission may involve treating water with chlorine or ozone to reduce *E. coli* survival (Callaway et al., 2004).

2.8. CONCLUSIONS

Pathogenic *E. coli* found in livestock manure is a concern for animal and public health. In the gastrointestinal tract of cattle, enterohaemorrhagic *E. coli* can survive and persist resulting in continual shedding of the bacterium in the feces (Callaway et al. 2004). People may come in contact with this potentially harmful bacterium by ingesting contaminated drinking water, food crops, or by direct contact with fecal matter. Understanding the pathogenesis of *E. coli* strains which inflict disease is critical for developing surveillance and control techniques. An on-farm approach to reduce the pathogen load can serve as a preventative measure to reduce *E. coli* numbers prior to shipping. Identifying management practices that have the largest impact on the abundance and shedding of pathogenic *E. coli* is a crucial component of on-farm management practices directed at preventing transmission to humans.

3.0 RESEARCH HYPOTHESES AND OBJECTIVES

3.1. HYPOTHESIS

There are several on-farm management practices that can influence the abundance of pathogenic *E. coli* shed by cattle, including diet and housing. Overwintering beef cattle are housed either in an extensive grazing system such as bale grazing or in an intensive dry lot system. During the overwintering period, cows are often supplemented with an energy (barley) or a protein source (DDGS) to meet the nutrient demands of maintenance and pregnancy. Similarly, high-producing dairy cows are fed diets high in concentrates to meet the nutrient demands associated with lactation as well as those required to support the growing fetus. Due to the readily fermentable nature of these diets, cows commonly develop subacute ruminal acidosis. It is intuitive that the diet fed provides bacteria in the hind gut with nutrients for growth. We hypothesized that diets with higher nutrient density (i.e. supplementation of barley or DDGS to overwintering beef cattle and a grain-based diet in dairy cattle) will result in increased fecal *E. coli* abundance, virulence, and invasive ability. The overwintering environment will not affect *E. coli* abundance, virulence, and invasive ability since the cows in the extensive system will be concentrated around the bales, provided bedding, shelter, and free access to water therefore resulting in similar environmental conditions that are present in the intensive system.

3.2. OBJECTIVES

The objective of these studies was to determine the effect of dietary nutrient density (barley supplemented, DDGS supplemented, forage, grain and alfalfa pellet diets) and the influence of overwintering environments (intensive vs. extensive) on fecal *E. coli* populations in cattle. Specifically, the objectives were i) to assess fecal *E. coli* abundance in fecal grab samples from beef cattle in overwintering environments (intensive vs. extensive) and dairy cattle using selective media ii) to select and test morphologically distinct *E. coli* isolates for the presence of genes associated with virulence factors including adhesins, aggregation factors, toxins, pathogenicity islands, autotransporters, and capsule synthesis and iii) to evaluate these isolates for their ability to invade HT-29 cells.

4.0 MANUSCRIPT I

Impact of nutrient density on the abundance and virulence properties of *Escherichia coli* shed from beef cattle in overwintering environments

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4.1 ABSTRACT

The objective of this study was to determine the effect of nutrient density and overwintering housing strategy on *E. coli* populations in beef cattle during two, 21-day periods. Sixty second-trimester beef cows were assigned to three replicated feeding strategies, each with ten cows. Four groups of cows were fed using a bale-grazing strategy while the other two groups were fed in dry-lot pens. During period 1, two pens in the bale-graze system received grass hay supplemented with dried distiller's grains with solubles (DDGS; 2.5 kg/cow every third day), while two pens in the bale grazing system and the two pens in the dry lot system received grass hay. During period 2, the DDGS-supplemented cows remained on the same diet while the other four pens received barley in order to meet NRC requirements for cattle in the third trimester of pregnancy. Three cows in each pen were randomly selected and individual fecal samples were collected and cultured on selective media. *E. coli* were enumerated and three isolates were selected for PCR analysis to detect the presence of 18 genes encoding a range of virulence factors. These same isolates were tested for their ability to invade human adenocarcinoma epithelial cell line HT-29. There were no significant differences in fecal *E. coli* abundance between the treatments (*P*-value=0.33). However, *E. coli* isolates recovered from cows in period 1 receiving DDGS had a greater number of isolates harboring *cnf* (cytotoxic necrotizing factor) gene (*P*-value=0.05). Analysis of the virulence data by diet revealed that DDGS supplemented cows had a greater number of isolates with of *vt1* (verocytotoxin 1) and *cnf* and the least number of *E. coli* isolates with *fliC* (flagellin structural gene). Isolates recovered from cows supplemented with barley were significantly more invasive than those recovered from cows supplemented with DDGS

and tended to be more invasive than the isolates recovered from non-supplemented cows. The data suggest that increasing nutrient density of the diet may affect the abundance of *E. coli* shed in the feces and increase the prevalence of *E. coli* harbouring particular virulence genes that mediate adhesion and invasion of epithelial surfaces.

Key words: beef cattle, *Escherichia coli* abundance, dried distiller's grains with solubles, virulence genes, cell invasion, overwintering

4.2 INTRODUCTION

The shedding of pathogenic *E. coli* from livestock is a major concern with respect to food safety. Since cattle are a known reservoir of enterohaemorrhagic *E. coli* (EHEC), in particular serotype O157:H7, numerous studies have focused on the control of this pathogen (Cobbold and Desmarchelier, 2004; Edrington et al., 2009). There are also a number of other serogroups of EHEC (O26, O111, O103, and O145) which are harbored by cattle associated with human infections, ranging from watery diarrhea to potentially fatal haemolytic uremic syndrome (HUS) (Caprioli et al., 2005; Pearce et al., 2004; Rhoades et al., 2009). For pathogenic *E. coli* to cause illness, they must attach to epithelial surfaces (EHEC) or invade cells, as exhibited by enteroinvasive *E. coli* (Croxen and Finlay, 2010). Attachment to epithelial surfaces is often mediated by fimbriae or pili, and once attached, an array of proteins and virulence factors are produced leading to colonization and manipulation of host responses, thereby causing disease (Croxen and Finlay, 2010).

There are several on-farm management practices that can influence the abundance of pathogenic *E. coli* shed by cattle, including diet quality (Callaway et al., 2009) and housing (Synge et al., 2003). Cattle receiving high grain diets tend to shed greater numbers of all *E. coli* (including O157:H7) compared to cattle receiving forage-based diets (Callaway et al., 2009; Krause et al., 2003; Russell et al., 2000a). Cattle also shed greater numbers of pathogenic *E. coli* when they are fed poor quality forage versus high quality forage (Callaway et al., 2009). One possible explanation for the difference in fecal shedding is the reduced pH and higher volatile fatty acid (VFA) concentrations in the

gastrointestinal tract which are associated with high-grain diets (Cobbold and Desmarchelier, 2004). The combination of low pH and increased VFAs may reduce EHEC adherence to the gut wall, resulting in increased bacterial shedding (Cobbold and Desmarchelier, 2004). Supplemental DDGS may alter the hindgut microflora (Rice et al., 2012) possible resulting in the increased shedding of *E. coli* in the feces (Jacob et al., 2010). Inconsistencies in pathogenic *E. coli* shedding have been reported which may be due to batch variation in DDGS production (Callaway et al., 2009).

Other factors which may influence O157:H7 shedding include stress due to fasting (Cray et al., 1998). Catecholamines (adrenalin and noradrenalin) are released by the enteric nervous system during periods of stress and this can have an effect on pathogenic *E. coli* populations in the gastrointestinal tract (Freestone et al., 2008). Noradrenalin has been shown to increase intestinal mucosal adherence and secretory response by *E. coli* O157:H7 (Freestone et al., 2008). Stress may be induced as a consequence of temperature (heat and cold) or handling and transportation (Dargatz et al., 1997; Grandin, 1997; von Borell, 2001). From a livestock management perspective, different types of housing as well as animal handling practices are likely to influence the level of stress experienced by the animal. Many cattle producers in western Canada now house their cattle in extensive overwintering sites where cattle graze bales rather than in a dry lot.

The focus of our research was to examine the influence of overwintering environments (dry lot housing and bale grazing) and diet (increased nutrient density) on *E. coli* abundance, diversity and invasiveness of selected isolates. We hypothesized that

barley and DDGS supplementation would increase the abundance, diversity and invasiveness of *E. coli*, while environment would not affect the *E. coli* abundance, diversity, or invasiveness since the cows in the extensive system will be concentrated around the bales, provided bedding, shelter, and free access to water therefore resulting in similar environmental conditions that are present in the intensive system.

4.3 MATERIALS AND METHODS

Animal experiment

The trial was conducted at the University of Manitoba Glenlea Research Station from January 4 to March 3, 2010. Sixty second-trimester beef cows were assigned to three replicated feeding strategies, each with ten cows. Four groups of cows were fed using a bale-grazing strategy while the other two groups were fed in dry lot pens. During period 1, two pens in the bale graze-system received grass hay supplemented with dried distiller's grains with solubles (DDGS; 2.5 kg/cow every third day) while two pens in the bale-grazing system and the two pens in the dry lot system received grass hay. During period 2, the DDGS supplemented cows remained on the same diet while the other four pens received barley (1 kg/cow/day) in order to meet NRC requirements for cattle in the third trimester of pregnancy. Three cows in each pen were randomly selected and individual fecal samples were collected by gloved hand. Animal handling and care procedures used in this study were approved by the University of Manitoba Animal Care Committee according to the guidelines established by the Canadian Council on Animal Care.

Feed analysis

The nutrient composition was determined for the dietary components fed to cows in the overwintering environments (Table 1).

Table 1. Dry matter and nutrient composition of feedstuffs fed to cows in the overwintering environments

	Dietary Components		
	Hay	DDGS	Barley
n [z]	10	2	1
DM, %	84.6	94.8	90.7
Nutrient	% of DM		
CP	8.80	30.3	17.8
NDF	62.3	38.8	16.5
ADF	37.6	14.0	4.50
Ca	0.54	0.12	0.13
P	0.11	0.99	0.45
K	0.71	1.26	0.54
Mg	0.27	0.37	0.14
Energy	Mcal kg^{-1}		
GE	4.21	5.31	4.58
NE_m [yx]	1.29	1.67	1.81

[z] Hay is the average of composited bale cores from the 4 extensive treatment replicate pens over 2 periods plus composited bales cores from the intensive treatment over 2 periods; DDGS is the average of composited samples from 2 periods; barley is 1 composited sample from 1 period.
[y] Net energy for maintenance (NRC 2001)
[x] NE_m = ((1.37 * [ME]) – (0.138 * [ME2]) + (0.0105 * [ME3]) – 1.12), where ME = ([DE]*0.82) and DE = 3.44-(0.022*[ADF])

Sample preservation

Fecal samples were collected once per period and the same animals were used in both periods (3 animals/treatment/pen). A total of 36 gloved fecal samples were stored in sterile, 120 ml screw cap specimen containers (Fisher Scientific, Pittsburgh, PA) and kept

on ice until they were transported to the laboratory for processing. All collected fecal samples were stored at -20°C.

Isolation and enumeration of *E. coli*

A 1g subsample of feces in 9 ml of 2% buffered peptone water (Becton, Dickson and Company, Sparks, MD) was added to wells of the sterile 2 ml, 96-well dilution plate. Serial dilutions were created by adding 900 µl of buffered peptone water and 100 µl of sample to each well, thereby serially diluting the sample from 10^{-2} to 10^{-6}. A 10-µl aliquot of the diluted sample was plated on Levine Eosin Methylene Blue agar (EMB, BD, Sparks, MD). Following the absorption of the 10 µl sample, the plates were inverted and placed in a 37°C incubator for 18 hrs. *E. coli* colonies were selected on the basis of morphology (dark green, shiny). Three morphologically distinct *E. coli* colonies were selected and streaked for isolated colonies on EMB agar. These *E. coli* isolates were subjected to gram stain analysis and tested for a negative oxidase reaction (BD Dryslide Reagent Slides, BD, Sparks, MD), positive indole production (Kovacs reagent, Remel, Lenexa, KS), and negative citrate utilization (Simmons Citrate Slant, Oxoid Inc., Nepean, ON, Canada) to confirm that they were all *E. coli*.

DNA extraction

Following confirmation, the three *E. coli* isolates from each fecal sample were inoculated into 10 ml of Luria-Bertani broth (LB, BD, Sparks, MD) and grown at 37°C for 16 hrs. The *E. coli* cells were harvested by centrifugation at 3,000 × g for 5 min. The supernatant was discarded and the pellet resuspended in 565 µl of 0.5 X Tris-EDTA

buffer (5 mM of Tris and 0.5 mM EDTA, pH 8). Subsequently, 30 µL of 10% sodium dodecyl sulfate (SDS) and 3 µL of proteinase K (20 mg/ml) were added to the tubes which were incubated for 1 hr at 37°C. Following incubation, 100 µL of 5M NaCl and 80 µL of 10% cetyl trimethylammonium bromide (CTAB) were added to each tube and mixed by repeated tube inversion followed by incubation for 10 min at 65°C. A 600-µL aliquot of phenol-chloroform-isoamyl alcohol (25:24:1) was added to each tube, mixed, and centrifuged at 10,000 × g for 5 min at 4°C. The supernatant was transferred to a new 1.5-ml tube, mixed with 600 µL of chloroform-isoamyl alcohol (24:1) and centrifuged at 10,000 × g for 5 min at 4°C. The supernatant was transferred to a fresh 1.5-ml tube, mixed with an equal volume of isopropanol and placed in a -20°C freezer for 10 min to precipitate nucleic acids. Tubes were centrifuged at 10,000 × g for 10 min to pellet the DNA. The DNA was washed with 70% ethanol and centrifuged again under the same conditions. After air drying, the DNA pellet was dissolved in 100 µL of Tris-EDTA buffer (pH 8.0) and stored at -20°C.

Detection of virulence genes

The PCR primers were designed to amplify a set of 18 virulence genes (Table 2), reported to be associated with adhesins, aggregation factors, toxins, pathogenicity islands, autotransporters, and capsule synthesis in virulent *E. coli*. The PCR reactions contained 2 µL of chromosomal DNA, 2.5 µL of 10X EconoTaq Buffer (Lucigen Corporation, Middleton, WI), 1.25 µL $MgCl_2$ (50mM) (Fisher Scientific, Fairlawn, NJ), 0.5 µL of each primer (25 pmol), 0.5 µL of 10mM nucleotide mix (Fisher Scientific, Fairlawn, NJ), 0.1 µL of EconoTaq polymerase (Lucigen Corporation, Middleton, WI), and Milli-Q water to

reach a volume of 25 μL. The thermal cycling conditions (Table 3) were as follows: 1 cycle of initial denaturation (94°C, 2 min), followed by 32 cycles of denaturation (94°C, 1 min), annealing (1 min) and extension (72°C, 1 min), and finally an extension cycle (72°C, 5 min).

Table 2. Selected primers used to target virulence characteristics of pathogenic *E. coli*

Target	Primer	Primer sequence (5''' to 3''')	Fragment size	Annealing temp (°C)	Reference
E. coli virulence genes[1]					
SPATE	SPATE1	GAGGTCAACAACCTGAACAAACGTATGGG	617	57	Kotlowski et al., 2007
	SPATE2	CCGGCACGGGCTGTCACTTTCCAG			
fimH	FimHf	CTGGTCATTCGCCTGTAAAACCGCCA	550	58	Kotlowski et al., 2007
	FimHr	GTCACGCCAATAATCGATTGCACATTCCCT			
agn43	Ag43F	TGACACAGGCAATGGACTATGACCG	317	67	Kotlowski et al., 2007
	Ag43R	GGCATCATCCCGGACCGTGC			
aggR	aggRf	GAGTTAGGTCACTCTAACGCAGAGTTG	377	61	Kotlowski et al., 2007
	aggRr	GACCAATTCGGACAACTGCAAGCATCTAC			
aidA	AIDA1	TATGCCACCTGGTATGCCGATGAC	545	69	Kotlowski et al., 2007
	AIDA2	ACGCCCACATTCCCCCAGAC			
pap	PapF	CCGGCGTTCAGGCTGTAGCTG	97	65	Kotlowski et al., 2007
	PapR	GCTACAGTGGCAGTATGAGTAATGACCGTTA			
sfaD-E	SfaF	CGGAGGAGTAATTACAAACCTGGCA	408	64	Martin et al., 2004
	SfaR	CTCCGGAGAACTGGGTGCATCTTAC			
afaE	AfaF	TATGGTGAGTTGGCGGGGATGTACAGTTACA	271	58	Kotlowski et al., 2007
	AfaR	CCGGGAAAGTTGTCGGATCCAGTGT			
cnf	CnfF	AGTACTGACACTCACTCAAGCCGC	930	62	Kotowski et al., 2007
	CnfR	GCAGAACGACGTTCTTCATAAGTATCACC			
vt1	Vt1F	CGCATAGTGGAACCTCACTGACGC	91	64	Kotlowski et al., 2007
	Vt1R	CATCCCCGTACGACTGATCCC			
vt2	Vt2F	CGGAATGCAAATCAGTCGTCACTCAC	265	65	Kotlowski et al., 2007
	Vt2R	TCCCCGATACTCCGGAAGCAC			
hlyA	HlyAF	TGCAGCCTCCAGTGCATCCCTC	355	63	Kotlowski et al., 2007
	HlyAR	CTTACCACTCTGACTGCGATCAGC			
eae	EaeF	CCAGGCTTCGTCACAGTTGCAGGC	300	66	Kotlowski et al., 2007
	EaeR	CGCCAGTATTCGCCACCAATACC			
fliC	FliC1	CAAGTCATTAATACMAACAGCC	900-2600	55	Machado et al., 2000
	FliC2	GACATRTTRGAVACTTCSGT			
crl	Crl1	TTTCGATTGTCTGGCTGTATG	250	58	Ewers et al., 2007
	Crl2	CTTCAGATTCAGCGTCGTC			
cgsA	CgsAf	ACTCTGACTTGACTATTACC	200	55	Maurer et al., 1998
	CgsAr	AGATGCAGTCTGGTCAAC			
ompC	OmpCF	GCAGGCCCTTTGTTCGATA	1236	59	Oteo et al., 2008
	OmpCR	GCCGACTGATTAATGAGGGTTA			
malX	MalXF	GGACATCCTGTTACAGCGCGCA	922	58	Ewers et al., 2001
	MalXR	TCGCCRCCAATCACAGCCGAAC			

[1] SPATE, serine protease autotransporter; *fimH*, D-mannose specific adhesion or type 1 fimbriae; *agn43*, antigen involved in *E. coli* autoaggregation; *aggR*, adhesin of aggregative adherence fimbria I; *aidA*, adhesin involved in diffuse adherence; *pap*, pap pili subunit located on pathogenicity island II; *sfa*, S-fimbriae minor subunit; *afaE*, AfaE-III afimbreal adhesin involved in diffuse adherence; *cnf*, cytotoxic necrotising factors cnf1 and cnf2; *vt1* and *vt2*, verocytotoxins 1 and 2; *hlyA*, α hemolysin; *eae*, intimin; *fliC*, flagellin; *crl*, curli regulatory gene; *cgsA*, curli structural gene which encodes curlin subunit; *ompC*, outer membrane protein C; *malX*, pathogenicity-associated island marker for UPEC CFT073

HT-29 cell invasion assays

All *E. coli* isolates were tested for their ability to invade the intestinal cell line HT-29. Monolayers of HT-29 cells were maintained in RPMI 1640 medium (Gibco, Invitrogen Corporation, Grand Island, N.Y) supplemented with 10% (vol/vol) fetal calf serum (Invitrogen, CA) at 37°C and 5% CO_2. Cells were added to 12-well plates at a concentration of 4 x 10^5 cells/ml and incubated at 37°C in RPMI until a monolayer was formed (approximately 2 days). The established monolayers were washed three times with 1X phosphate buffered saline (PBS). Bacterial cultures were grown in LB broth plus 0.4% glucose and diluted to obtain a concentration of 1 x 10^7 cfu/ml in RPMI media, as determined by optical density. A 1-ml aliquot of bacterial suspension was added to each of the monolayers and incubated for 3 hrs at 37°C and 5% CO_2. Following incubation, the medium was removed by aspiration and the monolayer of cells was rinsed 5 times with 1X PBS. To kill any extracellular bacteria, monolayers were incubated with 1 ml of RPMI containing gentamicin (100 μg/ml) for 1 hr. The media was removed by aspiration, cells were rinsed 5 times with 1X PBS and then lysed via the addition of 200 μl of 1% Triton X-100 in sterile water. Ten-fold dilutions of lysed cells were made and a 10-μl aliquot of each dilution was spread onto LB agar plates in duplicate. The plates were incubated at 37°C for 24 hrs, after which the number of colonies was recorded. Invasion experiments were conducted in triplicate along with a negative control.

Statistical analysis

E. coli abundance data was analyzed using the MIXED procedure of SAS (SAS Ins. 2008). The model statement included treatment and period as fixed effects with cow

as a random effect. Virulence data was analyzed using the MIXED and GLIMMIX procedure of SAS (SAS Ins. 2008) with treatment, period, and treatment by period as fixed effects and cow by treatment, period by treatment, and colony by cow by isolate as random effects for Model 1. Virulence data was re-analyzed (Model 2) using the MIXED and GLIMMIX procedure of SAS (SAS Ins. 2008) with data sorted by diet type and environmental parameters. Period effects were excluded since they were not significant in Model 1. The Model 2 statement included feed type as a fixed effect and cow by feed and cow by feed by isolate as random effects. Statistical analysis for HT-29 invasion assay was conducted using Model 1 and 2 as previously described, except that isolate was no longer required in the model statement. Multivariate discriminant analysis was performed on virulence data using JMP 8 to establish the association between virulence genes and diet (SAS Ins. 2008). Statistical differences were considered significant if $P < 0.05$. All assumptions associated with the statistical analysis were met.

4.4. RESULTS

Effect of overwintering environments on the abundance of *E. coli* and coliforms

There was no *E. coli* isolated from the rumen fluid. This is not surprising since *E. coli* primarily attach to epithelial surfaces (Croxen and Finlay, 2010). The abundance of *E. coli* in fecal grab samples ranged from log 3.29 to log 5.13 cfu/g (Table 3); however, there were no significant differences between treatments as well as across periods. Using Model 2 (considering diet type only), the supplementation of either barley or DDGS had no effect on *E. coli* abundance compared to non-supplemented cows.

Table 3. *E. coli* abundance in fecal samples collected from six pregnant beef cows in two overwintering environments

	Period 1			Period 2				
Parameter	BG[1]	DDGS[1]	DL[1]	BG*	DDGS	DL*	Std. Err.[2]	*P*-value
log cfu[3]/g	4.68	3.29	5.06	5.13	4.80	5.02	0.57	0.33

*denotes cows receiving barley supplementation

[1]BG: Bale Graze; DDGS: Bale Graze with Dried Distillers Grain with Solubles supplementation; DL: Dry Lot

[2]Standard error of the mean

[3]Colony forming units

Virulence gene prevalence in *E. coli* isolates

A total of 103 isolates (Period 1 = 52; Period 2 = 51) were evaluated for 18 virulence genes (Table 3). We discovered a significant difference in the prevalence of *cnf* (cytotoxic necrotizing factor, which induces prominent stress fiber formation and increases adherence to epithelia) amongst the 5 treatments (Table 4; *P*-value = 0.05). Using statistical Model 2, there were significant differences regarding the prevalence of

fliC (flagellin structural gene), *vt1* (verocytotoxin 1), and *cnf* (Table 4; *P*-value < 0.05), with DDGS-supplemented cows having a greater number of isolates with *cnf* and *vt1* and fewer isolates with *fliC*. No significant differences were observed between isolates from bale grazed or barley supplemented cows.

Multivariate discriminant analysis, was used to group virulence gene occurrence by diet type (Bale, Barley supplemented, and DDGS supplemented; Figure 1). The largest spatial separation occurred between the DDGS supplemented group and the other two groups. DDGS isolates had a positive association with *cnf*, *fimH* (D-mannose specific adhesion or type 1 fimbriae), and *SPATE* (genes encoding a serine protease autotransporter). The separation between the mean circles suggests that there is a difference in *E. coli* diversity associated with diet.

Table 4. Distribution of virulence genes found in *E. coli* isolated from fecal samples collected from three pregnant cows fed three diets in two overwintering environments

	% *E. coli* isolates with gene												
	Model 1[2]								Model 2[2]				
	Period 1			Period 2									
Genes[1]	BG[3]	DDGS[3]	DL[3]	BG*	DDGS	DL*	Std. Err.[4]	*P*-value	Bale[5]	DDGS	Bar[5]	Std. Err.	*P*-value
n[6]	18	17	17	16	18	17			35	35	33		
fimH	94.9	94.4	88.8	94.4	94.4	89.7	6.3	0.99	91.4	94.1	91.2	4.5	0.88
cgsA	100.0	100.0	100.0	93.8	94.0	94.5	4.2	0.99	100.0	97.3	94.0	2.6	0.29
ompC	94.7	94.6	94.4	87.9	83.0	89.1	7.0	0.95	94.4	89.2	88.6	5.2	0.68
fliC	100.0	82.2	88.7	94.0	81.2	100.0	7.6	0.57	94.4[a]	83.0[b]	97.1[a]	4.6	0.05
malX	49.8	47.9	49.0	24.1	14.8	32.3	14.7	0.80	48.6	32.4	29.4	8.1	0.22
Crl	100.0	99.9	100.0	93.4	88.0	100.0	3.3	0.26	100.0	100.0	96.9	2.9	0.67
aidA	94.4	82.5	94.1	99.7	75.4	86.6	9.5	0.81	94.4	80.8	94.3	5.6	0.23
agn43	94.4	88.4	83.1	99.6	88.0	94.5	7.3	0.79	88.6	88.2	97.1	4.6	0.41
hlyA	55.8	52.8	53.1	43.7	29.4	33.4	12.1	0.88	54.3	41.2	38.3	8.7	0.40
Pap	9.7	9.7	26.2	37.3	41.0	31.3	12.3	0.35	19.2	25.4	35.0	9.1	0.46
vt1	0.0	12.1	0.0	0.0	0.0	0.0	2.6	0.07	0.0[a]	11.1[b]	0.0[a]	2.2	0.01
vt2	94.8	82.6	65.5	82.1	78.5	68.4	11.5	0.64	80.1	80.3	74.5	8.4	0.86
SPATE	100.0	100.0	82.4	87.4	82.4	82.4	7.2	0.37	91.4	91.2	85.3	5.3	0.67
Cnf	33.3[ab]	76.5[a]	17.6[ab]	12.5[b]	41.2[ab]	11.1[b]	9.7	0.05	25.7[a]	58.8[b]	11.8[a]	7.1	0.01
afaE	5.4	0.3	5.4	20.3	35.6	11.1	9.4	0.27	5.7	17.5	14.7	5.9	0.38

*Denotes cows received barley supplementation

[1]Virulence genes *eae, sfaD-E,* and *agg* were not detected in any of the isolates

[2]Model 1: treatment, period, and treatment by period used as fixed effects; Model 2: diet set as the fixed effect

[3]BG: Bale Graze; DDGS: Bale Graze with DDGS supplementation; DL: Dry Lot

[4]Standard error of the mean

[5]Bale: comprised of cows without any supplementation; Bar: comprised of cows with supplemental barley

[6]A minimum of 2 and a maximum of 3 isolates were selected per cow

[a,b] Means within a row with different superscripts are significantly different ($P < 0.05$)

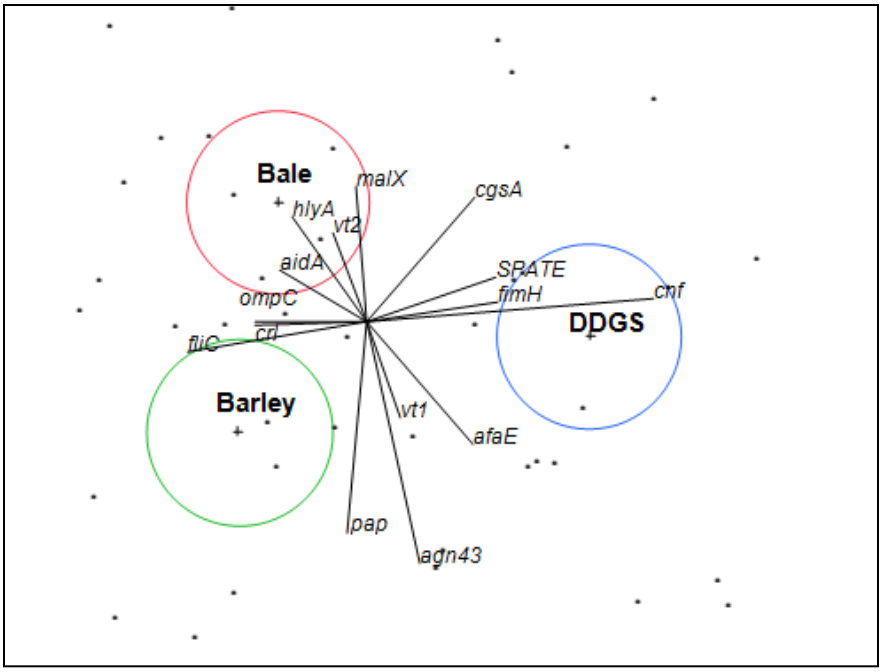

Figure 1. Discriminant analysis of virulence gene prevalence amongst *E. coli* isolates separated by diet (Bale: all cows receiving no supplementation, Barley: all cows receiving barley supplementation, DDGS: all cows receiving DDGS supplementation)

HT-29 cell invasion assays

A total of 103 isolates were tested for their ability to invade the human adenocarcinoma epithelial cell line HT-29 (Table 5). Using Model 1, there was no significant difference between the invasive ability of isolates recovered from BG, DL, or DDGS supplemented cattle. Re-analyzing the data using Model 2 revealed that isolates recovered from barley supplemented cows were significantly more invasive than those from cattle receiving DDGS (Table 5; *P*-value=0.05) and tended to be more invasive than isolates from cows receiving no supplementation.

Table 5. Abundance of *E. coli* isolates recovered following HT-29 cell invasion assays

	Model 1[1]								Model 2[1]				
	Period1			Period 2									
Parameter	BG[2]	DDGS[2]	DL[2]	BG*	DDGS	DL*	Std. Err.[3]	*P*-value	Bale[4]	DDGS	Bar[4]	Std. Err.	*P*-value
n[5]	18	16	17	15	15	17			35	35	33		
log cfu[6]/ml	3.68	3.45	3.58	3.88	3.48	3.89	0.18	0.46	3.63^{ab}	3.40^{b}	3.88^{a}	0.13	0.05

*Denotes cows receiving barley supplementation

[1]Model 1: treatment, period, and treatment by period were used as fixed effects; Model 2: diet set as the fixed effect

[2]BG: Bale Graze; DDGS: Bale Graze with DDGS supplementation; DL: Dry Lot

[3]Standard error of the mean

[4]Bale: comprised of cows without any supplementation; Bar: comprised of cows receiving supplemental barley

[5]A minimum of 2 and maximum of 3 isolates were selected per cow

[6]Colony forming units

[a,b] Means within a row with different superscripts are significantly different ($P < 0.05$)

4.5 DISCUSSION

Nutrient density has been shown to increase pathogenic *E. coli* populations including the shedding and prevalence of O157:H7 by cattle (Hovde et al., 1999; Jacob et al., 2009; Jacob et al., 2008a; Russell et al., 2000a). Focusing specifically on one strain of pathogenic *E. coli*, however, excludes the numerous other pathogenic strains harbored by cattle (Pearce et al., 2004; Rhoades et al., 2009). It has been suggested that assessing total *E. coli* abundance provides a better understanding of interaction between diet and the presence of pathogenic bacteria (Bettelheim et al., 2005). For this reason we enumerated total *E. coli* abundance and selected isolates for further analysis rather than focusing specifically on one strain.

Supplementation of either barley or DDGS did not increase *E. coli* abundance compared to cows receiving no dietary supplementation. This is contradictory to a number of studies which have found that providing DDGS or barley significantly increases *E. coli* shedding by cattle (Gilbert et al., 2005; Jacob et al., 2008a; Su et al., 2011). Gilbert et al. (2005) reported that a roughage diet consisting of Rhodes grass hay reduced *E. coli* numbers and virulence factors associated with EHEC when compared to a grain finishing diet consisting of 80% sorghum grain. Jacob et al. (2008a), observed that addition of 25% DDG to a diet consisting of steam-flaked corn and corn silage, led to an increase in the prevalence of *E. coli* O157:H7 in the feces. Su et al. (2011) compared the effect of backgrounding diets containing 40% tempered rolled barley to a diet with 40% corn or wheat DDGS and found that *E. coli* abundance in the feces was higher when the 40% barley diet was fed. A possible explanation for this discrepancy is that the previous

studies focused on diets containing grain or DDGS as a main dietary component and not as a supplementary source of energy or protein. A limited amount of DDGS and barley was offered (3.5 kg/cow every third day and 1 kg/cow/day, respectively) in the current study as it was fed to meet the nutrient requirements of second and third trimester beef cows throughout the winter.

Cows supplemented with DDGS had significantly more isolates with the virulence gene *cnf* (cytotoxic necrotizing factor, which induces prominent stress fiber formation and increased adherence to epithelia). A greater number of differences were discovered between isolates when grouped by diet alone, excluding environmental differences. Specifically, isolates from DDGS-supplemented cows had a lower prevalence of *fliC* (flagellin structural gene) and a higher prevalence of *vt1* (verocytotoxin 1) and *cnf* when compared with isolates recovered from cattle receiving barley or hay. Our findings support the notion that diet increases diversity of *E. coli* isolates, likely resulting in the proliferation of certain subpopulations while reducing others. Bettelheim et al. (2005) reported that a roughage or roughage and molasses diet resulted in a greater occurrence of the α-haemolysin gene (*hlyA*) amongst *E. coli* isolates compared to a high grain diet. Similarly, Gilbert et al. (2005) observed a reduction in genes encoding shiga toxin (*stx1*), α-haemolysin gene (*hlyA*), and intimin (*eae*) in fecal *E. coli* isolates recovered from cattle receiving a roughage or roughage and molasses diet compared to a high grain diet. The proposed explanation presented by Bettelheim et al. (2005) and Gilbert et al. (2005) was that the dietary factors (carbohydrates; sucrose and cellulose) may have affected *E. coli* subpopulations. Feeding non starch polysaccharides

to swine has been shown to modulate the prevalence of *E. coli* harboring particular virulence factors and pathways for butyrate production (Hopwood et al., 2002; Metzler-Zebeli et al., 2010). Inclusion of carboxymethyl cellulose, a non starch polysaccharide, increased the prevalence of *E. coli* producing certain virulence factors, specifically heat labile enterotoxin (Metzler-Zebeli et al., 2010). In the present study the impact of diet on *E. coli* was confirmed by discriminant analysis which revealed distinct groupings based solely on feed type. Our data suggest that the prevalence of *E. coli* sub populations possessing virulence genes associated with human pathogenesis may be influenced by dietary factors such as nutrient density.

The *E. coli* isolates were also characterized with respect to their ability to invade human adenocarcinoma epithelial cell line HT-29, which has been used as a model for *E. coli* invasiveness (Kim et al., 1998; Martin et al., 2004; Neeser et al., 1989). It is difficult to compare results from this study to those of previous studies which focused on specific serotypes and not on a diverse set of *E. coli* isolates. Other studies using the HT-29 cell invasion model typically selected specific *E. coli* isolates from human samples based on serotype or gene presence then proceeded to conduct invasion assays (Boudeau et al., 1999; Martin et al., 2004). Whereas our objective was to test for the overall invasiveness of *E. coli* shed by cattle and the influence of diet. *E. coli* recovered from cows receiving barley were significantly more invasive than those from DDGS supplemented cows and tended to be more invasive than isolates from cows on a hay diet. There was no correlation between the presence of virulence genes and the invasiveness of isolates suggesting that there may be other genes that impact the ability of *E. coli* to invade

epithelial cells. The mechanism by which diet influences the invasiveness of *E. coli* subpopulations is unknown, but it may be related to the presence of metabolites in the hind gut providing a competitive advantage to particular *E. coli*.

It is also important to note that the supplementation of DDGS occurred every third day to simulate that which occurs on-farm. Cattle are moved within a bale grazing site every third day with supplement provided at the same time to minimize labor costs. However, this pulse feeding of DDGS could cause instability in the hindgut microbiome resulting in instability in the bacterial populations in terms of nutrient availability. Post ruminal variations in pH, osmolality, and ammonia concentration varies less with six times a day feeding versus three times a day feeding (Le Liboux and Peyraud, 1999). Consequently, the *E. coli* populations may have been affected causing them to divest their energy from non-essential functions to those most critical for survival; hence the *E. coli* isolates recovered were the least invasive.

The biological significance of our results is difficult to determine since a small number of animals sampled (3 cows/pen; total of 18 cows/period) and the fact that only three isolates were selected from each sample due to physical limitations to conduct laboratory analysis in a timely fashion. The work done by Bettleheim et al. (2005) reported differences for virulence gene prevalence of greater than 25% across a large sample size (474 *E. coli* isolates). Only the virulence gene prevalence for cnf was greater than 25% and the largest difference in the invasion assays was 0.48 log. Since the observed differences between treatments for most of the virulence gene prevalence and invasion assays are relatively low, the biological significance is questionable even though

there are statistically significant differences between treatments. The *E. coli* virulence and invasion data has limitations for extrapolation due to the limited scope of our experiment (103 fecal *E. coli* isolates).

4.6 CONCLUSION

The findings from this study indicate that type of housing did not have an impact on the abundance, diversity, or the invasive ability of *E. coli*. The inclusion of barley and DDGS provided to the animals did not affect the abundance of *E. coli* but did result in a definite shift in *E. coli* sub populations shed in the feces. Diet also proved to be the most important factor influencing the prevalence of *E. coli* isolates possessing certain virulence genes as well as their ability to invade epithelial cells. It is difficult to conclude whether the *E. coli* isolates from cows supplemented with barley or DDGS are more pathogenic to humans since the presence or absence of virulence genes does not necessarily mean that the genes are expressed and it is also not clear whether the bacteria can survive passage through barriers present in the human gastrointestinal tract (Vanselow et al., 2005). Further research involving more in-depth feeding trials are required to determine the mechanisms by which diet influences the proliferation of *E. coli* subpopulations.

5.0 MANUSCRIPT II

Effect of alfalfa pellet and grain-induced subacute ruminal acidosis on the abundance and virulence properties of *Escherichia coli* in dairy cows

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5.1 ABSTRACT

The objective of this study was to determine the effect of nutrient density (starch) on the abundance and virulence properties of *E. coli* in dairy cattle. Six rumen and caecal-canulated, nonlactating, multiparous Holstein cows received the following: i) a 70% forage diet (control), ii) a diet with 34% of the dry matter (DM) replaced with grain pellets composed of 50% ground wheat and 50% ground barley (grain-based SARA challenge, GBSC), and iii) a diet with 37% of DM replaced with pellets containing ground alfalfa (alfalfa-pellet SARA challenge, APSC). Rumen, caecal, and rectum fecal samples were collected and cultured on selective media. *E. coli* were enumerated and three isolates were selected based on morphology. Isolates were subjected to PCR analysis to detect the presence of 18 genes encoding a range of virulence factors. Furthermore, isolates were tested for their ability to invade the human adenocarcinoma epithelial cell line HT-29. There were no significant differences in fecal and caecal *E. coli* abundance between the control, GBSC, and APSC treatments but more *E. coli* (5.45 log cfu/g) were present in caecal samples in comparison to fecal samples (5.08 log cfu/g) regardless of diet (*P*-value = 0.01). Since the difference between sampling location was only 0.37 log, it has little biological significance. The GBSC treatment resulted in a greater proportion of isolates harboring the *agg* gene (*P*-value = 0.01), which encodes the adhesin of aggregative adherence fimbria I, as well as the *hlyA* gene (*P*-value = 0.06) encoding α-haemolysin. Furthermore, *E. coli* isolates recovered from the GBSC cows were more invasive than cows receiving the other treatments (*P*-value = 0.03). Our findings suggest that the GBSC treatment may increase the prevalence of *E. coli*

harboring virulence genes that may be involved in adhesion and invasion of epithelial cells.

Key words: *Escherichia coli*, subacute ruminal acidosis, HT-29 cell invasion

5.2 INTRODUCTION

Subacute ruminal acidosis (SARA) is a common metabolic disorder in high-producing dairy cows. Typically, SARA occurs as a result of feeding diets that are insufficient in feed particle size and high in rapidly fermentable carbohydrates which leads to a build up of organic acids and depressed rumen pH (Kleen et al., 2003; Krause and Oetzel, 2006; Stone, 2003). Symptoms of SARA include decreased DM intake, diarrhea, depression of milk fat, and increased incidence of laminitis (Kleen et al., 2003; Plaizier et al., 2008; Stone, 2003). In addition, changes in microbial populations are associated with the increase in readily fermentable carbohydrates which support rapid growth of some gram positive bacteria (*Streptococcus bovis*) resulting in impaired rumination (Gozho et al., 2007; Khafipour et al., 2009a; Khafipour et al., 2009b). The large influx of starch associated with readily fermentable diets results in a greater proportion passing through the rumen to the colon where bacteria degrade the starch into volatile fatty acids (VFA), maltose and maltodextrins which can be utilized by *E. coli* (Callaway et al., 2003; Jacob et al., 2009; Khafipour et al., 2009b; Russell et al., 2000a). Li et al. (2012) observed a starch content of 7.4% DM in caecal digesta when cows were fed a high starch diet (33.7% DM) in comparison to a low starch (14.2% DM), forage diet which had starch levels of 2.8% DM in caecal digesta. An increase in *E. coli* abundance and virulence has been observed in the rumen of cows fed a highly fermentable grain-based diet to induce SARA (Khafipour et al., 2011). This is particularly important, as dairy cattle are a known reservoir of enterohemorrhagic *E. coli* (EHEC), in particular serotype O157:H7 (Hussein and Sakuma, 2005; Thran et al., 2001). Furthermore, high-grain diets have been associated with an increased abundance of generic and acid

resistant *E. coli* as well as increased fecal shedding of serotype O157:H7 (Dargatz et al., 1997; Diez-Gonzalez et al., 1998; Krause et al., 2003; Russell et al., 2000a). A possible explanation for lower *E. coli* abundance in forage-fed cattle is the associated increased pH of the rumen and hindgut as well as lower VFA concentrations; whereas grain-based diets have the opposite effect (Callaway et al., 2003; Jacob et al., 2009). The combination of greater substrate (simple sugars) availability for bacterial growth, low pH and elevated VFAs associated with these diets may result in the increased fecal shedding of pathogenic *E.* coli (Cobbold and Desmarchelier, 2004; Russell et al., 2000a).

The focus of our research was to examine the effect of grain and alfalfa pellet-induced SARA on *E. coli* abundance and virulence in dairy cattle. Potential virulence was assessed by screening for genes known to be associated with pathogenic *E. coli*. Furthermore, bacterial invasiveness was investigated by using the human adenocarinoma epithelial cell line HT-29.

5.3 MATERIALS AND METHODS

Animal experiment

Six rumen and caecal-cannulated, nonlactating, multiparous Holstein cows were chosen for this study. The experimental design was a 3 x 3 Latin square arrangement of treatments with 4-week experimental periods. During the first 3 weeks, all cows received a diet containing 70% (DM basis) forage and in the final week, cows received one of the following: i) a 70% forage diet (control), ii) a diet with 34% of the dry matter (DM) replaced with grain pellets comprised of 50% ground wheat and 50% ground barley (grain-based SARA challenge, GBSC), and iii) a diet with 37% of DM replaced with pellets comprised of ground alfalfa (alfalfa-pellet SARA challenge, APSC). Cows were fed ad libitum once daily at 0900 h and had unlimited access to fresh water. Rumen, caecal, and fecal samples were collected 6 h after feed delivery on day 5 of week 4. Animal handling and care procedures were approved by the University of Manitoba Animal Care Committee according to the guidelines established by the Canadian Council on Animal Care.

Feed analysis

Diet samples were collected twice weekly and pooled across sampling periods. The DM was determined by drying at 60°C for 48 h in a forced air oven. Dried samples were ground using a Wiley mill through a 1 mm screen (Thomas-Wiley, Philadelphia, PA) and stored at -20°C until analyzed by wet chemistry (Bhandari et al., 2007). The starch contents were determined using an enzymatic technique (AOAC, 1990). The nutrient composition was determined for all diets (Table 6). The most notable differences

between the diets was that the GBSC diet had a starch content 33.7% of DM and NFC at a level 50.4 % of DM, compared to the control which had a starch content of 14.2% and a NFC amount of 34.8% of DM.

Table 6. Ingredients and nutrient composition of the control diet and the diets fed during the alfalfa-pellet subacute ruminal ruminal acidosis (SARA) challenge and the grain-based SARA challenge (GBSC)

Item	Control	APSC	GBSC
Ingredients, % of DM			
Barley silage	40	33	28
Alfalfa hay	30	---	8
Supplement	30	30	30
Wheat-barley pellets	---	---	34
Alfalfa pellets	---	37	---
Nutrient composition			
DM, %	54.3	69.0	61.6
CP, % of DM	16.1	16.0	16.0
NDF, % of DM	35.6	34.5	22.9
ADF, % of DM	23.3	22.6	15.2
NFC[1], % of DM	34.8	39.0	50.4
Starch, % of DM	14.2	15.9	33.7
Crude fat, % of DM	3.6	3.2	3.3
Ash, % of DM	9.9	7.3	7.4
Ca, % of DM	0.85	0.13	0.64
P, % of DM	0.35	0.35	0.41
Mg, % of DM	0.35	0.3	0.29
K, % of DM	0.21	0.18	1.25
Na, % of DM	0.27	0.32	0.28

[1]NFC = 100 – (NDF % + CP % + crude fat % + ash %)

Rumen, caecal, and fecal sample collection

Rumen samples were collected via the rumen canula from the ventral sac. Samples were strained using a cheese cloth technique (Li et al., 2012). Caecal digesta samples were collected from the caecal cannula and fecal samples were collected from

the rectum using a gloved-hand technique. All samples were stored at -20°C prior to analysis.

Isolation and enumeration of *E. coli* from rumen and caecal digesta

A 1g subsample of feces in 9 ml of 2% buffered peptone water (Becton, Dickson and Company, Sparks, MD) was added to wells of a sterile 2 ml, 96-well dilution plate. Serial dilutions were created by adding 900 µl of buffered peptone water and 100 µl of sample to each well, thereby serially diluting the sample from 10^{-2} to 10^{-6}. A 10-µl aliquot of the diluted sample was plated on Brilliance *E. coli* and coliform agar (CM0956, Oxoid Ltd., Basingstoke, Hampshire, England). Following the absorption of the 10 µl sample, the plates were inverted and placed in a 37°C incubator for 18 hrs. Bacterial colonies were selected on the basis of morphology (dark purple). Three morphologically distinct colonies were streaked for isolated colonies on Brilliance *E. coli* and coliform agar. Suspect *E. coli* were subjected to gram stain analysis and tested negative for oxidase (BD Dryslide Reagent Slides, BD, Sparks, MD), positive for indole production (Kovacs reagent, Remel, Lenexa, KS), and negative for citrate utilization (Simmons Citrate Slant, Oxoid Inc., Nepean, ON, Canada).

DNA extraction

After the isolates were confirmed to be *E. coli*, single colonies were inoculated into 10 ml of Luria-Bertani broth (LB, BD, Sparks, MD) and grown at 37°C for 16 hrs. The *E. coli* cells were harvested by centrifugation at 3,000 × g for 5 min. The supernatant was discarded and the pellet resuspended in 565 µl of 0.5 X Tris-EDTA buffer (5 mM of

Tris and 0.5 mM EDTA, pH 8). Subsequently, 30 µL of 10% sodium dodecyl sulfate (SDS) and 3 µL of proteinase K (20 mg/ml) were added to the tubes which were incubated for 1 hr at 37°C. Following incubation, 100 µL of 5M NaCl and 80 µL of 10% cetyl trimethylammonium bromide (CTAB) were added to each tube and mixed by repeated tube inversion followed by incubation for 10 min at 65°C. A 600-µL aliquot of phenol-chloroform-isoamyl alcohol (25:24:1) was added to each tube, mixed, and centrifuged at 10,000 × g for 5 min at 4°C. The supernatant was transferred to a new 1.5-ml tube, mixed with 600 µL of chloroform-isoamyl alcohol (24:1) and centrifuged at 10,000 × g for 5 min at 4°C. The supernatant was transferred to a fresh 1.5-ml tube, mixed with an equal volume of isopropanol and then placed in a -20°C freezer for 10 min to precipitate nucleic acids. Tubes were centrifuged at 10,000 × g for 10 min to pellet the DNA. The DNA was washed with 70% ethanol and centrifuged again under the same conditions. After air drying, the DNA pellet was dissolved in 100 µL of Tris-EDTA buffer (pH 8.0) and stored at -20°C.

Detection of virulence genes

The PCR primers were designed to amplify a set of 18 virulence genes (Table 7), reported to be associated with adhesins, aggregation factors, toxins, pathogenicity islands, autotransporters, and capsule synthesis in virulent *E. coli*. The PCR reactions contained 2 µL of chromosomal DNA, 2.5 µL of 10X EconoTaq Buffer (Lucigen Corporation, Middleton, WI), 1.25 µL $MgCl_2$ (50mM) (Fisher Scientific, Fairlawn, NJ), 0.5 µL of each primer (25 pmol), 0.5 µL of 10mM nucleotide mix (Fisher Scientific, Fairlawn, NJ), 0.1 µL of EconoTaq polymerase (Lucigen Corporation, Middleton, WI), and Milli-Q water to

achieve a volume of 25 μL. The thermal cycling conditions (Table 7) were as follows: 1 cycle of initial denaturation (94°C, 2 min), followed by 32 cycles of denaturation (94°C, 1 min), annealing (1 min) and extension (72°C, 1 min), and finally an extension cycle (72°C, 5 min).

Table 7. Selected primers used to target virulence characteristics of pathogenic *E. coli*

Target	Primer	Primer sequence (5''' to 3''')	Fragment size	Annealing temp (°C)	Reference
E. coli virulence genes[1]					
SPATE	SPATE1	GAGGTCAACAACCTGAACAAACGTATGGG	617	57	Kotlowski et al., 2007
	SPATE2	CCGGCACGGGCTGTCACTTTCCAG			
fimH	FimHf	CTGGTCATTCGCCTGTAAAACCGCCA	550	58	Kotlowski et al., 2007
	FimHr	GTCACGCCAATAATCGATTGCACATTCCCT			
agn43	Ag43F	TGACACAGGCAATGGACTATGACCG	317	67	Kotlowski et al., 2007
	Ag43R	GGCATCATCCCGGACCGTGC			
aggR	aggRf	GAGTTAGGTCACTCTAACGCAGAGTTG	377	61	Kotlowski et al., 2007
	aggRr	GACCAATTCGGACAACTGCAAGCATCTAC			
aidA	AIDA1	TATGCCACCTGGTATGCCGATGAC	545	69	Kotlowski et al., 2007
	AIDA2	ACGCCCACATTCCCCCAGAC			
pap	PapF	CCGGCGTTCAGGCTGTAGCTG	97	65	Kotlowski et al., 2007
	PapR	GCTACAGTGGCAGTATGAGTAATGACCGTTA			
sfaD-E	SfaF	CGGAGGAGTAATTACAAACCTGGCA	408	64	Martin et al., 2004
	SfaR	CTCCGGAGAACTGGGTGCATCTTAC			
afaE	AfaF	TATGGTGAGTTGGCGGGGATGTACAGTTACA	271	58	Kotlowski et al., 2007
	AfaR	CCGGGAAAGTTGTCGGATCCAGTGT			
cnf	CnfF	AGTACTGACACTCACTCAAGCCGC	930	62	Kotowski et al., 2007
	CnfR	GCAGAACGACGTTCTTCATAAGTATCACC			
vt1	Vt1F	CGCATAGTGGAACCTCACTGACGC	91	64	Kotlowski et al., 2007
	Vt1R	CATCCCCGTACGACTGATCCC			
vt2	Vt2F	CGGAATGCAAATCAGTCGTCACTCAC	265	65	Kotlowski et al., 2007
	Vt2R	TCCCCGATACTCCGGAAGCAC			
hlyA	HlyAF	TGCAGCCTCCAGTGCATCCCTC	355	63	Kotlowski et al., 2007
	HlyAR	CTTACCACTCTGACTGCGATCAGC			
eae	EaeF	CCAGGCTTCGTCACAGTTGCAGGC	300	66	Kotlowski et al., 2007
	EaeR	CGCCAGTATTCGCCACCAATACC			
fliC	FliC1	CAAGTCATTAATACMAACAGCC	900-2600	55	Machado et al., 2000
	FliC2	GACATRTTRGAVACTTCSGT			
crl	Crl1	TTTCGATTGTCTGGCTGTATG	250	58	Ewers et al., 2007
	Crl2	CTTCAGATTCAGCGTCGTC			
cgsA	CgsAf	ACTCTGACTTGACTATTACC	200	55	Maurer et al., 1998
	CgsAr	AGATGCAGTCTGGTCAAC			
ompC	OmpCF	GCAGGCCCTTTGTTCGATA	1236	59	Oteo et al., 2008
	OmpCR	GCCGACTGATTAATGAGGGTTA			
malX	MalXF	GGACATCCTGTTACAGCGCGCA	922	58	Ewers et al., 2001
	MalXR	TCGCCRCCAATCACAGCCGAAC			

[1] SPATE, serine protease autotransporter; *fimH*, D-mannose specific adhesion or type 1 fimbriae; *agn43*, antigen involved in *E. coli* autoaggregation; *aggR*, adhesin of aggregative adherence fimbria I; *aidA*, adhesin involved in diffuse adherence; *pap*, pap pili subunit located on pathogenicity island II; *sfa*, S-fimbriae minor subunit; *afaE*, AfaE-III afimbreal adhesin involved in diffuse adherence; *cnf*, cytotoxic necrotising factors cnf1 and cnf2; *vt1* and *vt2*, verocytotoxins 1 and 2; *hlyA*, α hemolysin; *eae*, intimin; *fliC*, flagellin; *crl*, curli regulatory gene; *cgsA*, curli structural gene which encodes curlin subunit; *ompC*, outer membrane protein C; *malX*, pathogenicity-associated island marker for UPEC CFT073 (Ewers et al., 2007; Kotlowski et al., 2007; Martin et al., 2004; Maurer et al., 1998; Oteo et al., 2008).

HT-29 cell invasion assays

All *E. coli* isolates were tested for their ability to invade the intestinal cell line HT-29. Monolayers of HT-29 cells were maintained in RPMI 1640 medium (Gibco, Invitrogen Corporation, Grand Island, N.Y) supplemented with 10% (vol/vol) fetal calf serum (Invitrogen, CA) at 37°C and 5% CO_2. Cells were added to 12-well plates at a concentration of 4 x 10^5 cells/ml and incubated at 37°C in RPMI until a monolayer was formed (approximately 2 days). The established monolayers were washed three times with 1X phosphate buffered saline (PBS). Bacterial cultures were grown in LB broth plus 0.4% glucose and diluted to obtain a concentration of 1 x 10^7 cfu/ml in RPMI media, as determined by optical density. A 1-ml aliquot of bacterial suspension was added to each of the monolayers and incubated for 3 hrs at 37°C and 5% CO_2. Following incubation, the medium was removed by aspiration and the monolayer of cells was rinsed 5 times with 1X PBS. To kill any extracellular bacteria, monolayers were incubated with 1 ml of RPMI containing gentamicin (100 μg/ml) for 1 hr. The media was removed by aspiration, cells were rinsed 5 times with 1X PBS and then lysed via the addition of 200 μl of 1% Triton X-100 in sterile water. Ten-fold dilutions of lysed cells were made and a 10-μl aliquot of each dilution was spread onto LB agar plates in duplicate. The plates were incubated at 37°C for 24 hrs, after which the number of colonies was recorded. Invasion experiments were conducted in triplicate along with a negative control.

Statistical analysis

E. coli abundance data was analyzed using the MIXED procedure of SAS (SAS Ins. 2008). The model statement included diet and sample location as fixed effects

and cow as a random effect. The virulence gene data was analyzed using the MIXED and GLIMMIX procedure of SAS (SAS Ins. 2008). The model statement included diet and sample location as fixed effects and the cow and bacterial isolate as random effects. *E. coli* cell invasion data was analyzed using the MIXED procedure of SAS with diet as the fixed effect and cow and bacterial isolate as the random effects. Sample location was not included as we were unable to recover sufficient numbers of caecal *E. coli* isolates from the -80°C stocks. Multivariate discriminant analysis using JMP8 was performed on the virulence data in order to establish the association between virulence gene presence and diet (SAS Ins. 2008). Statistical differences were considered significant if $P < 0.05$. All assumptions associated with the statistical analysis were met.

5.4 RESULTS

Effect of SARA challenge on *E. coli* abundance

No *E. coli* was recovered from the rumen samples, nor was there a significant difference between the SARA challenges and the control (Table 8). However, a significant difference between *E. coli* numbers in fecal grab samples (5.08 log cfu/g) and caecal digesta (5.45 log cfu/g; *P*-value = 0.01) was observed. Since the difference between the two sampling areas is only 0.37 log, this result has little biological significance.

Table 8. *E. coli* abundance in caecal and fecal samples recovered from dairy cows with SARA

	Treatment				Location			*P*-value		
Parameter	Con[1]	APSC[2]	GBSC[3]	SEM[4]	Caecal	Fecal	SEM	Trt[5]	Loc[6]	Trt*Loc[7]
n[8]	10	11	11		14	18				
Log cfu[9]/g	5.33	5.19	5.27	0.18	5.45	5.08	0.14	0.91	0.01	0.36

[1]Con: control diet
[2]APSC: alfalfa pellet SARA challenge
[3]GBSC: grain based SARA challenge
[4]Standard error of the mean
[5]Treatment comparison
[6]Comparison of sampling locations (caecal*fecal)
[7]Treatment by location comparison
[8]Number of observations
[9]Colony forming unit

Diversity of *E. coli* isolates, virulence gene presence

A total of 75 *E. coli* isolates (23 caecal; 52 fecal) were tested for the presence of 18 virulence genes (Table 9) known to be associated with pathogenic *E. coli.* Compared to fecal isolates, caecal isolates had a significantly greater prevalence for *pap* (*P*-value = 0.02) encoding the Pap pili subunit and located on pathogenicity island II and tended to

have fewer isolates that harbored *SPATE* (*P*-value = 0.09) which encodes a serine protease autotransporter. The GBSC treatment resulted in a greater proportion of isolates positive for *agg* (*P*-value = 0.01), the gene for adhesin of aggregative adherence fimbria I in comparison to APSC treatment. This same treatment group tended to have a greater proportion of isolates positive for α-haemolysin (*hlyA*; *P*-value = 0.06) in comparison to the control and APSC treatment.

Multivariate discriminant analysis was used to cluster the occurrence of virulence genes based on dietary treatment (Figure 2). Circles representing the treatment group were separated from each other indicating that there were differences in the *E. coli* isolates recovered from cows fed the different diets. The GBSC group was separated from the others due to a positive association with *vt1*, *pap*, *sfa-DE*, and *agg*. The APSC group was distinct because of the presence of *cnf* and *fimH*, while the control diet grouping was characterized by a negative association with *malX* and *SPATE*.

Table 9. Distribution of virulence genes found in *E. coli* isolated from caecal and fecal samples from dairy cows

	% *E. coli* isolates with gene										
Virulence	Caecal				Fecal				*P*-value		
Genes[1]	Con[2]	APSC[3]	GBSC[4]	SEM[5]	Con	APSC	GBSC	SEM	Trt[6]	Loc[7]	Trt*Loc[8]
n[9]	5	9	9		16	17	19				
fimH	97.6	100.0	88.6	9.9	84.7	100.0	95.3	6.7	0.52	0.77	0.56
ompC	75.2	99.3	94.9	6.8	89.2	89.9	82.0	5.6	0.27	0.58	0.12
fliC	40.1	43.8	44.1	19.2	37.1	62.7	47.3	12.8	0.71	0.65	0.79
malX	41.6	66.8	66.4	18.4	58.1	37.7	35.1	12.1	0.99	0.30	0.34
crl	100.0	100.0	100.0	0.0	100.0	100.0	100.0	0.0			
aidA	100.0	100.0	100.0	0.0	100.0	100.0	100.0	0.0			
agn43	78.2	95.8	100.0	9.4	94.3	93.7	93.2	7.3	0.16	0.93	0.46
hlyA	0.0	0.0	0.0	0.0	0.0[a]	0.0[a]	25.0[b]	4.9	0.06	0.09	0.06
sfaD-E	0.0	0.0	4.4	4.0	0.0	0.0	5.4	3.5	0.47	0.90	0.96
pap	43.5	17.4	32.3	10.7	0.0	8.4	15.8	7.9	0.50	0.02	0.24
vt1	0.0	2.1	19.8	7.8	0.0	6.0	1.4	5.5	0.41	0.34	0.17
vt2	5.1	0.0	0.0	10.9	10.9	13.0	5.9	7.7	0.85	0.20	0.87
SPATE	23.2	49.4	40.2	19.2	63.6	62.7	66.1	13.0	0.79	0.09	0.73
cnf	11.9	15.0	12.7	14.2	4.5	15.3	10.3	8.2	0.77	0.58	0.89
agg	9.8[ab]	0.0[a]	29.8[b]	10.7	16.5[ab]	6.1[a]	24.4[b]	8.5	0.01	0.71	0.80

[1]virulence genes *eae* and *afaE* were not detected in any of the isolates
[2]Con: control diet
[3]APSC: alfalfa pellet SARA challenge
[4]GBSC: grain based SARA challenge
[5]Standard error of the mean
[6]Treatment comparison
[7]Comparison of sampling locations (caecal*fecal)
[8]Treatment by location comparison
[9]Number of observations
[a,b] Means within a row with different superscripts are significantly different ($P < 0.05$)

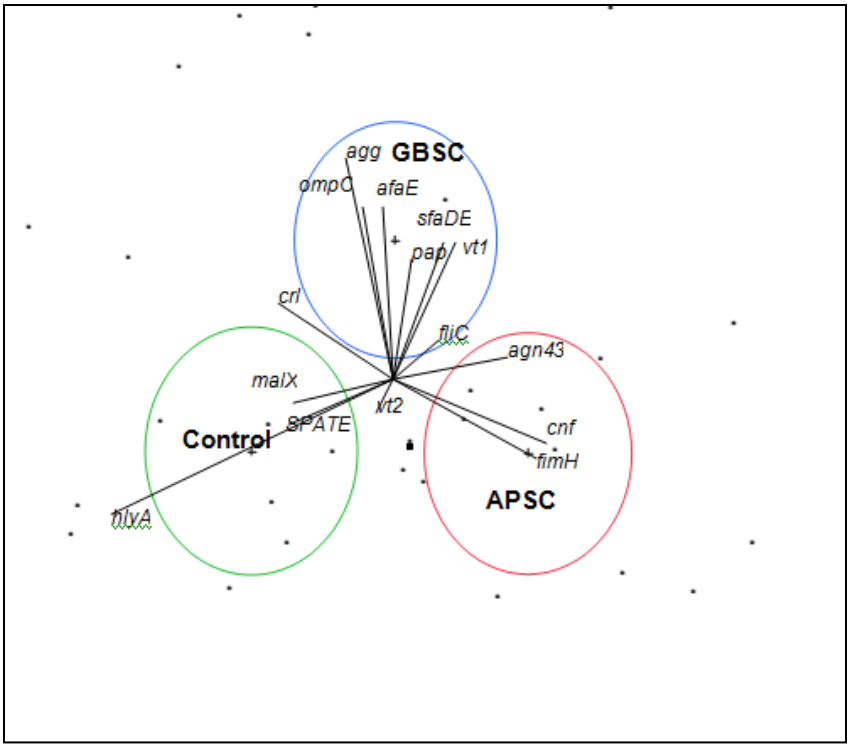

Figure 2. Discriminant analysis of virulence gene prevalence amongst *E. coli* isolated from dairy cow fecal samples

HT-29 cell invasion assays

Twenty-five *E. coli* isolates (control-5; APSC-10; GBSC-10) were tested for their ability to invade the human adenocarcinoma epithelial cell line HT-29. The number of *E. coli* recovered from the cell lysate ranged from 2.98 to 3.41 log cfu/ml (Figure 3). The GBSC isolates were found to be more invasive than the control and APSC isolates (*P*-value = 0.03).

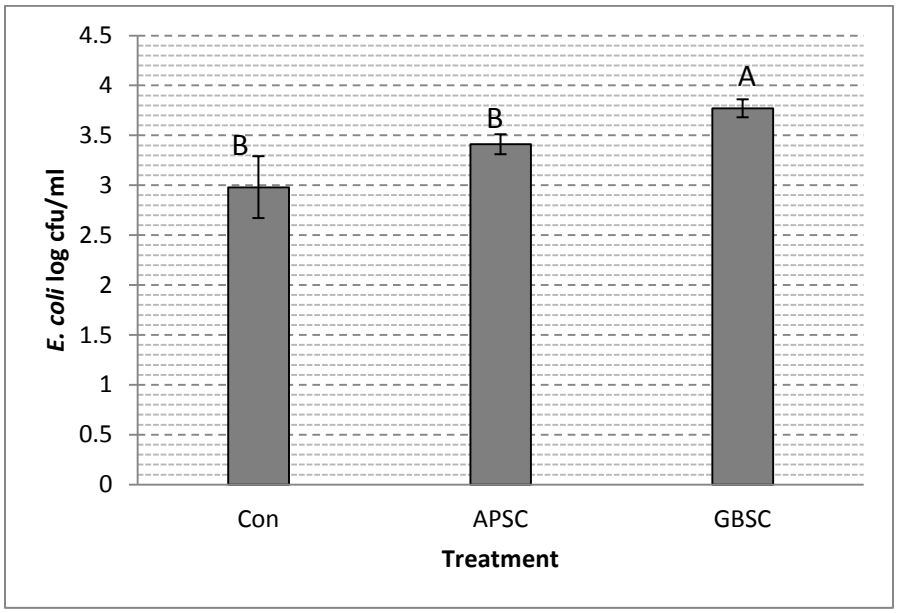

Figure 3. Average log *E. coli* abundance recovered from the cell lysate of the HT-29 cell invasion (*P*-value=0.05)

5.5 DISCUSSION

Inclusion of readily fermentable feed ingredients has been shown to increase *E. coli* abundance as well as shedding of EHEC O157:H7 and other pathogenic fecal *E. coli* (Bettelheim et al., 2005; Callaway et al., 2009; Gilbert et al., 2005; Russell et al., 2000a; Vanselow et al., 2005). Subacute ruminal acidosis is a common metabolic disorder in dairy cows and feedlot cattle. It results from a depression in rumen pH caused by feeding rapidly fermentable carbohydrate diets leading to organic acid accumulation (Kleen et al., 2003; Krause and Oetzel, 2006; Stone, 2003). Currently, little information is available regarding the impact of SARA on *E. coli* populations in the hindgut, and as such it is important to understand how pathogenic *E. coli* populations are affected by this condition (Nagaraja and Titgemeyer, 2007; Plaizier et al., 2008). An assessment of all commensal and pathogenic *E. coli* populations will provide insight regarding the interaction between diet and the presence of pathogenic *E. coli* compared to examination of a single serotype (Bettelheim et al., 2005).

This examination of all commensal and pathogenic populations of *E. coli* revealed that SARA had no effect on *E. coli* abundance in the caecal digesta and feces. We were unable to culture *E. coli* from the collected rumen samples, despite using a methodology comparable to that utilized by Khafipour et al. (2009), who demonstrated an increase in *E. coli* abundance in the rumen fluid with grain-induced SARA. Our results concur with several studies which have found the rumen environment to be inhibitory to *E. coli* growth (Rasmussen et al., 1993; Wallace et al., 1989; Wolin, 1969). There was a significantly greater abundance of *E. coli* in the caecal digesta compared to feces. Since

feces are more concentrated than caecal digesta one would expect *E. coli* should be concentrated as well. The GBSC *E. coli* isolates had a significantly higher prevalence of *agg* (adhesin of aggregative adherence fimbria I) carriage; similarly more bacteria harbored *hlyA* (α-haemolysin). These findings suggest that although overall abundance of *E. coli* did not differ, certain subpopulations of *E. coli* are favored when feeding a high-grain diet. Bettelheim et al. (2005) observed that a roughage or roughage and molasses diet resulted in a greater occurrence of fecal *E. coli* isolates carrying *hlyA* compared to a grain diet leading researchers to speculate that diet increased the prevalence of *E. coli* harboring this gene. Bettelheim et al. (2005) speculated that the dietary factors (high sucrose in the molasses and roughage diet or high cellulose in the roughage diet) may influence *E. coli* in ways other than using these substrates for growth possibly due to changes in the microflora. In the current study, discriminant analysis of the virulence data revealed distinct groupings of *E. coli* isolates based on diet type. This may suggest that the nutrient density of the diets is influencing the abundance of certain subpopulations of *E. coli*.

We conducted cell invasion assays on the human adenocarcinoma epithelial cell line HT-29 which has been used as a model to test the ability of *E. coli* to invade and attach to human epithelial cells (Kim et al., 1998; Martin et al., 2004; Neeser et al., 1989). The fecal *E. coli* isolates recovered from cows in the GBSC were significantly more invasive than the control diet and tended to be more invasive than isolates from the APSC. Although statistically significant, these findings need to be interpreted with caution as the magnitude of the difference was relatively small.

Changes in microbial populations are complex and potentially mediated by a myriad of factors, therefore it is difficult to determine the exact mechanism(s) by which diets affects pathogenic *E. coli* populations. In the current study, the diets used to induce SARA were readily fermentable; therefore, there was ample substrate (simple sugars) to support bacterial growth. The GBSC diet, for example, contained a large portion of starch (33.7% of DM) which may have increased substrate in the hindgut, thereby leading to a proliferation of specialized *E. coli* subpopulations, particularly those with invasive ability. Conversely in the control diet, there was reduced substrate availability and in this environment, a lower number of invasive *E. coli* were found. Although the underlying mechanisms remain elusive, our findings suggest that nutrient density influences the presence of invasive *E. coli* and may alter the presence of virulence genes, possibly leading to increased shedding of pathogenic *E. coli*.

The biological significance of our results is difficult to determine since a small number of animals sampled and the fact that only three isolates were selected from each sample due to physical limitations to conduct laboratory analysis in a timely fashion. The work done by Bettelheim et al. (2005) reported differences for virulence gene prevalence of greater than 25% across a large sample size (474 *E. coli* isolates). None of our observed virulence gene prevalence differences were greater than 25% and the largest observed difference for the invasion assays was 0.79 log. Since the observed differences between the virulence gene prevalence and invasion assays amongst treatments are relatively low the biological significance is questionable even though there are statistically significant differences between treatments. The *E. coli* virulence and invasion

data has limitations for extrapolation due to the limited scope of our experiment (23 caecal and 52 fecal *E. coli* isolates).

5.6 CONCLUSION

In summary, our findings demonstrate that nutrient density does not significantly change overall *E. coli* abundance in the feces but it does promote the growth of distinct subpopulations. We noted that the GBSC treatment resulted in more isolates carrying the virulence genes *agg* and *hlyA*. Furthermore, the GBSC *E. coli* were significantly more invasive than isolates from the other treatments. The means by which the *E. coli* subpopulations are affected is unknown. It may be related to the presence of increased starch availability providing a favorable niche for *E. coli*. This study further demonstrates that readily fermentable diets can influence fecal *E. coli* populations, increasing the presence of virulence genes and invasiveness of *E. coli* isolates shed by cattle. Our findings suggest that it may be possible to manipulate nutrient density such that it will minimize the abundance and shedding of pathogenic *E. coli*.

6.0 GENERAL DISCUSSION

The shedding of pathogenic *E. coli* from livestock is a major concern with respect to food safety. Over the past year the temporary closure of XL Foods Inc due to an outbreak of *E. coli* O157:H7 highlights the importance of controlling pathogenic bacteria (CFIA, 2013). The incorporation of concentrates or other readily fermentable feed stuffs into cattle diets has been shown to increase pathogenic *E. coli* populations including the shedding and prevalence of O157:H7 by cattle (Hovde et al., 1999; Jacob et al., 2009; Jacob et al., 2008a; Russell et al., 2000a). Although, this strain has received the most attention, it is important to note that there are numerous other pathogenic *E. coli* strains harbored by cattle (Pearce et al., 2004; Rhoades et al., 2009). Therefore, rather than focusing specifically on one strain of pathogenic *E. coli*, an inclusive approach to assessing total *E. coli* abundance was used to examine the interaction between nutrient density, overwintering environment (intensive vs. extensive) and the presence of pathogenic *E. coli*. Overall, housing did not influence the carriage and shedding of pathogenic *E. coli*, therefore the remainder of the discussion will focus on the impact of diet.

6.1 *E. coli* ABUNDANCE

In both the overwintering beef cattle and dairy cow experiments, we were unable to recover *E. coli* from the rumen which is in agreement with previous studies (Rasmussen et al., 1993; Wallace et al., 1989; Wolin, 1969). Conversely a study done by Khafipour et al. (2009), noted an increase in *E. coli* abundance in the rumen fluid associated with grain-induced SARA. Wolin et al. (1969) described that a pH of 6.0 and

rumen VFAs (60 μmol of acetate, 20 μmol of proprionate, and 15 μmol of butyrate) are inhibitory to *E. coli* growth. Another study demonstrated that pH ranging from 6.2 to 6.6 with the addition of rumen VFAs inhibits the growth of *E. coli* (Wallace et al., 1989). In our dairy cattle, the average ruminal pH ranged from 5.98 to 6.3 for the three treatments (Li et al., 2012). Since the growth of *E. coli* is likely inhibited under these conditions of low pH in the presence of VFAs, it provides a possible explanation for why we were unable to isolate *E. coli* from the rumen of the dairy cows. As pH was not measured in the beef trial, we can only speculate that a similar phenomenon occurred.

Neither supplementation of the beef cow diet with DDGS or barley nor provision of a grain-based pellet diet to dairy cattle increased the fecal abundance of *E. coli* in comparison to the forage-based diets. Gilbert et al. (2005) reported that a roughage diet consisting of Rhodes grass hay reduced *E. coli* numbers and virulence factors associated with EHEC when compared to a grain finishing diet consisting of 80% sorghum grain. Jacob et al. (2008a) observed that addition of 25% DDG to a diet consisting of steam-flaked corn and corn silage, led to an increase in the prevalence of *E. coli* O157:H7 in the feces. Su et al. (2011) compared the effect of backgrounding diets containing 40% tempered rolled barley to a diet with 40% corn or wheat DDGS and found that *E. coli* abundance in the feces was higher when the 40% barley diet was fed. Our results did not demonstrate an association between grain or DDGS inclusion in diets and increased fecal shedding of *E. coli* as in the aforementioned studies (Gilbert et al., 2005; Jacob et al., 2008a; Su et al., 2011). This could be due to the relatively low level of fermentable carbohydrates (grain) and protein supplementation (DDGS) included in the beef cattle

diets. In our studies, beef cows were fed 1 kg of barley/day which constitutes approximately 8% of the total diet on a DM basis and the dairy cows were fed a diet which contained ground wheat and barley (34 % of DM). Secondly, the amount of DDGS (2.5 kg) offered in the overwintering beef cattle study reflects diets fed to second and third trimester beef cows throughout the winter, which was fed every third day. The frequency of feeding cattle has a direct influence on the fermentation which occurs in the rumen. Post ruminal variations in pH, osmolality, and ammonia concentration varies less with six times a day feeding versus three times a day feeding (Le Liboux and Peyraud, 1999). Similarly, Robles et al. (2007) found smaller fluctuations with two times a day feeding in comparison to once per day feeding of a concentrate diet (Robles et al., 2007). In nutrient limiting or stressful situations, *E. coli* acquires mutations that allows them to enter a state where they are no longer dividing and are resistant to environmental stressors (Vulic and Kolter, 2001). The every third day feeding may have caused unstable growing conditions for bacteria in the hind gut and as a result, the *E. coli* populations did not increase in abundance in accord with the earlier findings.

6.2 PREVALENCE OF VIRULENCE GENES

Dietary components (non-starch polysaccharides) may influence the proportion of pathogenic bacteria as well as the number of isolates harbouring certain virulence genes. Feeding non-starch polysaccharides to swine has been shown to modulate the prevalence of *E. coli* harboring particular virulence factors and pathways for butyrate production (Hopwood et al., 2002; Metzler-Zebeli et al., 2010). Inclusion of carboxymethyl cellulose, a non-starch polysaccharide, increased the prevalence of *E. coli* producing

certain virulence factors, specifically heat labile enterotoxin (Metzler-Zebeli et al., 2010). Similarly, Hopwood et al. (2002) found that including carboxymethyl cellulose into swine diets resulted in increased fecal shedding of enterotoxigenic hemolytic *E. coli*. We observed that the prevalence of virulence genes harbored by *E. coli* isolates was influenced by diet. In beef cows supplemented with DDGS, fecal isolates had a lower prevalence of *fliC* (flagellin structural gene) and a higher prevalence of *vt1* (verocytotoxin 1) and *cnf* (cytotoxic necrotising factor) when compared to isolates recovered from cattle receiving barley or hay. A possible explanation for our findings is that DDGS creates a suitable environment in which *E. coli* harboring these virulence genes can thrive. The GBSC *E. coli* isolates had a significantly higher prevalence of carrying the *agg* gene carriage (adhesin of aggregative adherence fimbria I) and tended to harbor *hlyA* (α-haemolysin). The prevalence of these genes was not affected by the supplementation of barley to beef cows, probably as a result of the low level of grain provided (8% of DM) in comparison to that provided to dairy cows (34% of DM).

The impact of nutrient density on *E. coli* was confirmed by discriminant analysis which revealed distinct groupings based solely on feed type. Our data suggest that the prevalence of *E. coli* subpopulations possessing virulence genes associated with disease in humans can be influenced by the diet fed to cattle. Subpopulations of *E. coli* O157:H7 associated with clinical cases have increased prevalence of the *stx* (shiga toxin) gene, thereby increasing their virulence and the proportion of total clinical cases in which they are implicated (Manning et al., 2008).

6.3 *E. coli* INVASION CHARACTERIZATION

The expression of bacterial virulence genes can be influenced by environmental conditions, thereby permitting bacteria to select against unnecessary energy expenditure in order to optimize survival (Guiney, 1997). Environmental cues such as osmolarity, O_2 and CO_2 levels, pH, reactive oxygen and nitrogen compounds, nutrient availability, and inorganic ion concentration all act as signals to modulate virulence gene expression (Guiney, 1997). For example, *Salmonella* Typhimurium invasion of host cells is increased under conditions of an alkaline pH and high osmolarity (Galan and Curtiss, 1990; Guiney, 1997; Tartera and Metcalf, 1993). In the current study, it was found that providing supplemental barley or a grain-pellet diet increased the ability of the *E. coli* isolates to invade human adenocarcinoma epithelial cell line HT-29. *E. coli* recovered from beef cows receiving barley were significantly more invasive than those from DDGS supplemented cows and tended to be more invasive than isolates from cows on a hay diet. Similarly, *E. coli* isolates recovered from dairy cows in the GBSC were significantly more invasive than the control diet and tended to be more invasive than isolates from the APSC. The GBSC diet contained 33.7% (DM basis) of starch, which may have increased substrate availability in the hindgut for *E. coli*, leading to increased diversity and virulence gene expression associated with host cell invasion. Similarly, barley supplementation may have resulted in an increase in substrate availability. The pulse feeding of DDGS could cause instability in the hindgut microbiome resulting in fluctuations in the bacterial population. Consequently, the *E. coli* populations may have divested their energy from non-essential functions to those most critical for survival. This may, in part, account for the fact that *E. coli* isolates recovered from the DDGS-

supplemented cattle were the least invasive. Changes in microbial populations are expected to be complex involving a multitude of factors, making it difficult to determine the exact mechanism by which diet affects pathogenic *E. coli* populations.

6.4 PRACTICAL CONSIDERATIONS

The results of the current study reinforce the concept that diet influences the abundance and invasiveness of certain *E. coli* populations, indicating that carriage of pathogenic bacteria might be controlled through diet manipulation. The most notable affect was the inclusion of barley in the diets which led to a significant increase in invasiveness of the *E. coli* isolates. In the beef cattle overwintering trial, DDGS was used as a protein supplement and we observed reduced *E. coli* invasion compared to barley supplementation. However further investigation is required to confirm the impact of DDGS on *E. coli* shed in the feces. The *E. coli* virulence and invasion data has limitations for extrapolation since we only tested three isolates from each animal due to resource and time constraints and that there are animal to animal differences which can't be accounted for due to the small number of animals sampled (18 beef cows/period; 6 dairy cows/period). From a practical standpoint, reformulating diets (reducing nutrient density) to reduce pathogenic bacteria shedding will not be favored by producers if it affects the profitability of their operation. In order to initiate a change in current production practices, there has to be a monetary incentive or other substantial benefit such as abattoir demand for cattle shedding low levels of *E. coli*.

6.5 SUMMARY

In summary, our findings demonstrate that nutrient density may not significantly change overall *E. coli* abundance but may alter the proportion of isolates harboring virulence genes and having the ability to invade HT-29 cells. The invasive ability of isolates was increased when barley was supplemented at low levels (8% of DM), which was observed for the beef cows, or at higher levels (34% of DM), as seen in the GBSC dairy cows.

Thus it appears that increasing the starch content of the diet via barley supplementation may create a suitable intestinal environment for *E. coli* growth, diversification, and specialization. Our findings demonstrate that even if a particular diet does not cause a change in *E. coli* abundance, it may affect the virulence and invasiveness of *E. coli*, leading to a comparable number of *E. coli* shed but with increased shedding of pathogenic bacteria by cattle. The mechanism by which the *E. coli* subpopulations are affected is currently unknown, but it may be related to increased availability of starch or other nutrients, or changes in the overall microbial populations. Future research should be directed at understanding the molecular mechanisms underlying the influence of diet on the carriage and shedding of pathogenic *E. coli*.

7.0 CONCLUSION

The findings from the current study indicate that housing environment (intensive vs. extensive) does not have an impact on the abundance, diversity, or invasive ability of *E. coli*. Diet proved to be the most important factor influencing the prevalence of *E. coli* isolates possessing certain virulence genes as well as having the ability to invade epithelial cells. It is difficult to conclude whether the *E. coli* isolates from cows supplemented with barley or DDGS or fed a grain-based diet are more pathogenic to humans since the presence or absence of virulence genes does not reveal whether these genes are expressed or whether the bacteria can survive passage through barriers presented by the human gastrointestinal tract (Vanselow et al., 2005). Conducting further research examining the complex interaction between diet type and intestinal conditions favoring growth of pathogenic bacteria will enable the cattle industry to manage and manipulate diets to reduce carriage of pathogenic bacteria.

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