FACTORS INFLUENCING ESCHERICHIA COLI O157 COLONIZATION OF THE
GASTROINTESTINAL TRACT OF FEEDLOT CATTLE

by

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M.S., Kansas State University, 2009
M.S., Ecole d'Ingénieur de Purpan, 2009

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Animal Sciences and Industry
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2012

Abstract

The first chapter of this dissertation reviews factors affecting *E. coli* O157:H7 prevalence in the gastrointestinal tracts of cattle. Chapter 2 assessed *E. coli* O157:H7 ability to use bovine intestinal mucus and its constituents as substrates for growth *in vitro* in the presence and absence of fecal inoculum and exogenous enzymes. Whole mucus supported the greatest pathogen growth ($P < 0.05$), but all components tested were able to sustain *E. coli* growth. Chapter 3 evaluated the impact of crude glycerin feeding on *E. coli* O157 fecal shedding by cattle fed growing and finishing feedlot diets with corn or a combination of corn, distiller's grains, and soybean hulls. Increasing levels of crude glycerin decreased incidence of *E. coli* O157 in growing cattle (linear effect, $P < 0.01$) and tended to do so in finishing cattle fed corn-based diets ($P < 0.06$). No effect of glycerin was observed in finishing cattle fed the byproduct-based diets ($P > 0.05$), highlighting potential for glycerin use as a means for controlling fecal prevalence of *E. coli* O157 in cattle fed conventional grain-based diets. Chapter 4 evaluated transportation and lairage effects on fecal shedding of *E. coli* in feedlot cattle by mimicking transport to the abattoir. Shedding patterns were influenced by transportation, with significantly lower *E. coli* O157 prevalence in transported animals 4 hours after transit ($P < 0.05$). Additional post-transit samplings are, however, needed to confirm effects of transport stress on pathogen prevalence and shedding patterns. The experiment summarized in chapter 5 evaluated the potential for utilizing fecal long-chain fatty acid (LCFA) profiles as an indicator of *E. coli* O157 status. Out of 39 LCFA evaluated, only eicosapentaenoic acid (EPA) concentration was associated with presence of the pathogen ($P < 0.02$). The final chapter assessed the impact of dietary menthol, up to 0.3% of diet DM, on antimicrobial resistance in commensal *E. coli*. Menthol addition affected prevalence of tetracycline resistant *E. coli,* but contrary to our hypothesis, increased their

occurrence after 30 days of treatment ($P < 0.006$). No hypothesis on mechanism responsible for this increase could be made from the present study.

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Acknowledgements

I wish to thank first and foremost my parents, Christine and Didier Apercé, without who this thesis would not have been possible. I thank them for their moral and financial support during my extended student life, their love and understanding despite the distance. Mum, Dad, I promise that this is my last diploma, for now at least.

I would like to express my deepest appreciation to my advisor, Dr. Drouillard, for his guidance and supervision over the course of my program. I thank him for giving me the opportunity to learn and gain hands on experience at the feedlot and in the Preharvest Food Safety Laboratory. These few years have been intense and eventful; I definitely learned more than I could have expected when starting my PhD.

I also would like to thank Dr. Minton who advised me during my master program at Kansas State University and who introduced me to Dr. Drouillard. He was always there when needed despite his extremely busy schedule.

I wish to express my sincere thanks to the other members of my committee, Dr. Edwards-Callaway, Dr. Thippareddi and to my outside chair Dr. Govind for providing me with valuable advices and comments on my dissertation work.

I also wish to thank Dr. Scott and Dr. Nagaraja for guiding me in my last project, as well, as Javier Vinasco and Raghu Amachawadi who helped me with PCR work and MIC analysis.

I would like to show my gratitude to all of Dr. Drouillard graduate students who I worked hand in hand with and who taught me patience and perseverance. Particular thanks to Cadra Van Bibber-Krueger, Kevin Miller, and Christian Alvarado, for keeping the many cattle processing days, the long runs from 500 alley, the pounds and pounds of fecal matter scooped, and the hours

Chapter 1 Literature review

INTRODUCTION

Foodborne diseases are estimated to cause 76 million illnesses in the USA each year, resulting in 5,200 deaths and an estimated public health cost of 10 to 83 billion dollars annually (Bavaro, 2012). Intensification of agriculture and expansion of large scale retail and distribution systems has created a substantial vector for rapid and wide-spread bacterial outbreaks. *E. coli* O157:H7 is notorious for important food recalls across the USA, following more or less severe gastrointestinal infections. These events are costly for the food industry and greatly affect consumer confidence. Despite considerable efforts spent in past decades to better comprehend the pathogen and provide efficient means of intervention, factors leading to colonization, proliferation, and shedding of *E. coli* O157:H7 are still poorly understood. Ruminants, mainly cattle, have been recognized as an important reservoir for the pathogen, and research has focus on factors influencing pathogen proliferation in their gastrointestinal tracts (GIT).

This literature review intends to give an overview of current knowledge on *E. coli* O157:H7 colonization of cattle GIT and on the role played by intestinal mucus as a substrate of growth. Concurrently, this chapter will summarize data available on the effects of grain replacement by byproducts in feedlot diets on *E. coli* O157:H7 shedding. Finally, implication of transport stress on *E. coli* O157:H7 proliferation in cattle GIT will be analyzed using newly developed principles of microbial endocrinology.

E. COLI O157:H7 FROM FOOD OUTBREAKS TO CATTLE COLONIZATION

Commensal and pathogenic *E. coli* of the human GIT

Escherichia coli is a genus of the *Enterobacteriaceae* family. *E. coli* are mesophilic Gram negative bacteria, bile-tolerant, lactose fermenting and commensal inhabitants of the GIT of mammals. In spite of their general commensal status, some *E. coli* strains can be harmful to their host. Six classes of intestinal pathotypes have been recognized (Kaper et al., 2004): enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffusely adhesive *E. coli* (DAEC), enteroaggregative *E. coli* (EAEC), and enterohaemorrhagic *E. coli* (EHEC); yet their identification remains challenging, as pathogenic and commensal *E. coli* share similar biochemical and ecological features.

Enterohaemorrhagic E. coli: Characteristics and mode of action

EHEC have the capacity to produce Shiga toxin and to carry a locus of enterocyte effacement (LEE) pathogenicity island encoding for attaching effacing (AE) lesions (Figure 1-1).

Figure 1-1 Enterohaemorrhagic *Escherichia coli* virulence factors
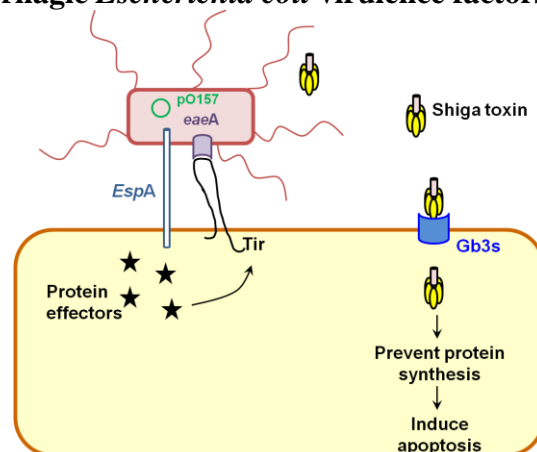
EHEC were first described in 1982 during a severe bloody diarrhea outbreak in Oregon and Michigan (Riley et al., 1983). This bacteria has since been shown to trigger hemorrhagic colitis,

non-bloody diarrhea, and hemolytic uremic syndrome in humans; the latter being in some cases lethal. Shiga toxins are EHEC key virulence factors and are found as two types, Stx1 and Stx2, both of which consist of a pentamer of identical B subunits that allow binding to cell surface glycolipid receptors (globotriaosylceramides; Gb3), and an A subunit, that enzymaticaly cleaves ribosomal RNA (Johannes and Romer, 2010). Stx2 is epidemiologically more important than Stx1 (Boerlin et al., 1999); however, release of either of these toxins by bacteria in the human colon lumen can induce disruption of protein synthesis, apoptosis of epithelial cells, and damage to colonic tissues. Hemolytic uremic syndrome develops when toxins enter the bloodstream and reach the kidneys, causing damage to endothelial cells and impairing vascular function (Kaper, 2005). The locus of enterocyte effacement, another EHEC characteristic trait, encodes a type III secretion system, protein effectors, and an intimin (*eae*A). Protein effector EspA forms a hollow filamentous extension into host epithelial cells, allowing translocation of other effectors into the cell (Figure 1-1). Once within the host cell, protein effector Tir acts as a receptor on cell membranes for *eae*A intimin, resulting in attachment effacement of the EHEC (Roe et al., 2003). Over one hundred EHEC serotypes sharing these characteristics have been discovered (Bettelheim, 1998); however, focus has been on *E. coli* O157:H7, thus EHEC are often referred to as EHEC O157 or EHEC non-O157 serotypes.

Focus on E. coli O157:H7

E. coli O157:H7 is characterized by the lack of β-glucoronidase and its inability to ferment sorbitol, whereas most other *E. coli* do (Viazis and Diez-Gonzalez, 2011). In addition, *E. coli* O157:H7 is resistant to acidity, as low as pH of 2.5 (Castanie-Cornet et al., 1999), and only requires 1 to 100 organisms to induce infection in humans (Paton and Paton, 1998). Although

this pathogen has been the subject of intense investigation for decades, 8% of patients infected with *E. coli* O157:H7 still develop HUS and 5% do not survive (Garg et al., 2003; Tarr, 2009).

E. coli O157 transmission to humans

Human infections can occur in many ways, but pathogens most frequently originate from the GIT of ruminants. Cases of bloody diarrhea have been observed in children after petting goats at a zoo (Goode et al., 2009), demonstrating risk for direct ingestion of fecal pathogens. Nevertheless, *E. coli* O157:H7 remains mostly a food-borne pathogen found in various food products, including meat, vegetables, and drinking water. In 2011, *E. coli* O157:H7 was responsible for recall of 1 million pounds of beef product, representing 59% of the total beef product recalled by the Food Safety and Inspection Services (FSIS) for potential hazard to human health in the US (FSIS, 2011). Contamination of meat occurs at slaughter when fecal deposits present on hides come into contact with carcasses as hides are removed (Arthur et al., 2004). Meat products then carry *E. coli* O157:H7, and if not properly cooked will contain viable pathogenic organisms capable of causing illness in consumers after ingestion (Wiegand et al., 2009). Contamination of vegetables and water is indirect, but also linked to ruminants. Excreted *E. coli* O157:H7 in cattle feces have the ability to survive in extra-intestinal environments for up to 3 months (Nicholson et al., 2005; Semenov et al., 2007). Over this period, pathogens can contaminate water sources (Vernozy-Rozand et al., 2002; Wang and Doyle, 1998) and spread to surrounding environments by runoff (Jaffrezic et al., 2011). Contaminated manure and irrigation water can lead to *E. coli* O157:H7 contamination of leafy vegetables such as spinach, sprouts, lettuce, and others (Charkowski et al., 2002; Mootian et al., 2009; Olaimat and Holley, 2012). Moreover, insects and wildlife can further contribute to crop contaminations (Renter et al., 2001;

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Talley et al., 2009). Although the incidence of contamination of leafy vegetables with *E. coli* O157:H7 has been increasing, meat contaminations remain the predominant vector and mitigation procedures have been developed to limit risk of outbreaks.

Control of E. coli O157:H7 in meat processing

Post-harvest interventions are considered to be most effective means of reducing pathogen loads in meat products. Since hides are the major source of contamination within abattoirs (Antic et al., 2010; Arthur et al., 2004), researchers have developed interventions that reduce risk of meat product contamination during hide removal. Many of these processes are based on hides wash with organic acids (Baird et al., 2006), cetylpyridinium chloride (Bosilevac et al., 2004), sodium hydroxide (Bosilevac et al., 2005a), or ozonated water (Bosilevac et al., 2005b). Hides washes have been shown to successfully reduce *E. coli* O157:H7 prevalence on hides of freshly slaughtered cattle and to limit carcass contamination during hide removal. Fecal contaminations that occur subsequent to hide removal can be controlled by steam vacuuming (Huffman, 2002), hot water wash (Bosilevac et al., 2006), and steam pasteurization (Phebus et al., 1997) of intact carcasses after evisceration. Intervention on final meat products are more challenging, as they should not alter shelf life or sensory attributes of meat products. Recently developed irradiation techniques using E-beam technology have demonstrated promising reductions in occurrence of *E. coli* O157:H7 in meat products without altering sensory qualities (Arthur et al., 2005). In spite of all these precautions, *E. coli* O157:H7 outbreaks still occur, thus underscoring the necessity to further reduce pathogen loads in market ready cattle to support post-harvest interventions. To do so it is imperative to understand colonization processes of *E. coli* O157:H7 in the GIT of ruminants.

Cattle gastrointestinal tract and *E. coli* O157:H7

Colonization of cattle GIT by E. coli O157:H7

E. coli O157:H7 have been shown to persist throughout the GIT of ruminants, and have been isolated from the esophagus, rumen, omasum, duodenum, jejunum, ileum, cecum, colon, gall bladder, and terminal rectal junction. Despite an apparent ubiquitous presence of the pathogen in the GIT of cattle, *E. coli* O157:H7 seem to prefer the lower tract ecosystem (Cray and Moon, 1995; Grauke et al., 2002). Recent works have identified the terminal rectal mucosa as an important site for *E. coli* O157:H7 colonization (Naylor et al., 2003). Specificities of this site are still unclear, although the authors hypothesized that abundance of lymphoid follicles could be responsible for the tropism. Peyer's patches, which also are rich in lymphoid follicles, have been shown to be site of predilection for *Salmonella enterica* serovar Typhimurium, *Listeria monocytogenes*, and *Shigella* spp. (Jensen et al., 1998; Vazquez-Torres and Fang, 2000), and similar mechanisms could exist at the terminal rectal mucosa. Presence of *E. coli* O157:H7 in bovine GIT and observation of attaching effacing lesions on the mucosa (Naylor et al., 2005; Phillips et al., 2000) without inducing pathogenicity in animals remained an enigma for a long time. Stx receptors appear to be present in colonic crypts of the cattle GIT (Hoey et al., 2002). Absence of Shiga toxin receptors on ruminant blood vessels and limited access to cryptic receptors by toxins (Schuller et al., 2004; Stamm et al., 2008) have been presented as explanations for the absence of enterotoxicity in cattle (Pruimboom-Brees et al., 2000). This lack of pathogenicity makes detection of *E. coli* O157:H7 even more challenging and further emphasizing the need to determine component(s) of GIT that maintain and promote pathogen growth.

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Intestinal mucus as E. coli O157:H7 substrate of growth

Previous studies have shown that bovine intestinal mucus supports bacterial colonization and can selectively influence composition of the bacterial population (Deplancke and Gaskins, 2001), yet little is known of nutrients and metabolic pathways used by *E. coli* (Miranda et al., 2004).

Intestinal mucus structure

Mucus acts as lubricant and physical barrier within the GIT, protecting epithelial cells from unwanted invasion (Deplancke and Gaskins, 2001), but also represents a substrate for bacterial growth (Atuma et al., 2001). Intestinal mucus is comprised of mucins, glycoproteins, glycolipids, epithelial cell debris, and electrolytes (Conway et al., 2006). Mucin molecules are large complexes of heterogeneous glycoproteins linked by disulfide bonds. Mucin subunits are formed by a protein backbone that is rich in serine and threonine, which allow attachment of O-linked oligosaccharides. Calf intestinal mucin is composed of 47.4% carbohydrates, namely galactose, N-acetylglucosamine, N-acetylgalactosamine, fucose, mannose, and sialic acids, and 52.6% proteins (Montagne et al., 2000). Mucins are divided in two groups – neutral and acidic – depending on the charge of carbohydrate groups they carry. Acidic mucins are more resistant to bacterial degradation due to the presence of a sialic acid or sulfate groups on the oligosaccharide chain, classifying them as non-sulfated or sulfated mucin, respectively (Deplancke and Gaskins, 2001). Mucin type prevalence is influenced by GIT location, and acidic mucins are most commonly found within the large intestine in rats and humans (Deplancke et al., 2002). Dietary factors also appear to influence mucin composition. High fiber diets have been observed to increase proportions of acidic mucin (Montagne et al., 2004) in rat models. Microflora can, as

well, modulate mucin composition as shown by higher ratio of neutral to acidic mucins found in germ-free animals compared to conventional animals (Szentkuti et al., 1990).

Intestinal mucus degradation by E. coli O157

Degradation of mucin complexes by bacteria is accomplished through the actions of multiple enzymes. Proteases are first in line and effectively alter mucus from a viscous to a more fluid state. Endoglycosidases can then act upon internal bonds to release oligosaccharide fragments. Further degradation of oligosaccharide fragments, by glycosidases and beta-galactosidases, is only possible after sialic acid removal by neuraminidases (Corfield et al., 1992). Lack of polysaccharide-degrading enzyme activity in *E. coli* (Chang et al., 2004) thus limits carbohydrate utilization by the bacteria to mono- or disaccharides (Mayer and Boos, 2005). The serotype O157:H7 lacks, in addition, neuraminidase activity (Hoskins et al., 1985), and consequently has limited capability to degrade complex mucin molecules. Hence, presence of other anaerobes within the GIT that have capacity to degrade mucin polysaccharides into smaller fragments is primordial to ensure *E. coli* O157:H7 growth (Jones et al., 2008).

Competition between E. coli O157 and microflora for mucus

Access to nutrients is the key factor to ensure GIT colonization by *E. coli*. Presence of pathogenic bacteria in the GIT, such as *E. coli* O157:H7, will depend on the principle of competitive exclusion (Tkalcic et al., 2003), whereby other microflora can effect changes in pathogen prevalence by limiting access to substrates of predilection. Environments with lower complexity are, indeed, more readily colonized by pathogens (Stecher et al., 2010). Bacteria have for a long time be thought to have a single preferred nutrient as a substrate of growth (Freter, 1988); however, this idea has been challenged by recent studies illustrating that *E. coli* relies on

a diverse range of nutrients for its growth (Chang et al., 2004), having successfully propagated with galactose, mannose, gluconic acid, glucuronic acid, and galacturonic acid as substrate (Alpert et al., 2009; Fox et al., 2009). Fucose, even if a minor component of intestinal mucus (Montagne *et al.*, 2000), appears important for bacterial development, as colonization of bovine rectal mucus by *E. coli* mutant lacking the catabolic pathway for L-fucose was largely decreased (Snider et al., 2009). Ability of *E. coli* O157:H7 to use multiple substrates is certainly an advantage when it comes to competition and colonization of the bovine GIT; however, specificities of this competitive advantage are still unknown. Regardless of the nature of mucus component(s) supporting pathogen growth, there is a growing body of evidence illustrating that *E. coli* O157:H7 can colonize and thrive on intestinal mucus. Other factors, such as mucus secretion/availability, may also play an important role in pathogen prevalence in feedlot cattle.

Manipulation of intestinal mucus secretion by E. coli O157

Goblet cells and mucus secretion

Mucus is synthesized in goblet cells and is either secreted as a baseline or, when needed, excreted in granule form into the intestinal lumen (Forstner and Forstner, 1994; Sharma et al., 2009). Areas of the GIT rich in goblet cells are associated with a thicker mucus layer (Brunsgaard, 1998), and goblet cells generally are more abundant in the colon and rectum of most species (Specian and Oliver, 1991). These findings corroborate heterogeneity found in mucus thickness across the human and rat GITs, where mucus was thickest within the colon (Atuma et al., 2001; Freitas et al., 2002; Robbe et al., 2004). Bovine mucus is believed to display similar patterns, and this heterogeneity could be responsible for part of *E. coli* O157:H7 tropism

to the terminal colon (Robbe et al., 2004; Snider et al., 2009), which may be characterized by the presence of greater amounts of mucus as substrate for growth.

Mucus secretion regulation

Mucin secretion is regulated by epithelial cell signaling factors, dietary factors, and also microbial signaling molecules and fermentation products (Dharmani et al., 2009; McGuckin et al., 2011). Cytokines releases by epithelial cells increase mucin secretion and goblet cell proliferation (Blanchard et al., 2004; Deplancke and Gaskins, 2001) in GIT to reinforce physical barrier protection in presence of potential invaders. Diets fed to animals also influence mucus secretion. Rats fed high fiber diets have higher goblet cell proliferation rate and increased mucus production when compared to rats receiving traditional diets that contain lesser amounts of fiber (Montagne et al., 2004). Moreover, microbial fermentation of food within the GIT releases volatile fatty acids (VFA), such as acetate and butyrate, which positively affect release of mucus within the intestinal lumen (Barcelo et al., 2000; Sakata and Setoyama, 1995; Shimotoyodome et al., 2000). Therefore, observations of lower numbers of goblet cells and reduced mucus production in germ-free rats (Kandori et al., 1996) comes with no surprise, and further underscores the important role played by microflora regulating mucus secretion. Virulence factors expressed by pathogenic bacteria also can modulate mucus secretion. Lipopolysaccharide (LPS) and flagellin present on most Gram negative bacteria are able to activate the transcription factor, nuclear factor κB (NFκB), inducing the production of mucin (Hedemann et al., 2009). Likewise injection of guanylin, a polypeptide produced by goblet cells similar to *E. coli* enterotoxins, is able to trigger rapid secretion of mucus by rat goblet cells (Furuya et al., 1998), and similar effects are observed with enterotoxins when applied to the intestinal lumen (Moon

and Whipp, 1971). Secretion regulation systems are intended to protect the GIT from pathogenic bacteria; however, *E. coli* O157:H7 appears to be able to exploit these systems to benefit its own substrate requirement.

In spite of the abundance of research that has focused on food-borne pathogens, factors influencing colonization of the ruminant GIT by *E. coli* O157:H7 is still poorly understood. Continuing research efforts aimed at understanding mechanisms and factors influencing *E. coli* O157:H7 prevalence in cattle are thus needed.

NEW FACTORS AFFECTING *E. COLI* O157 COLONIZATION IN CATTLE

There are many factors affecting growth and survival of *E. coli* O157:H7 within the GIT of cattle, but this thesis will focus on two emerging factors, namely the effect of biofuels byproduct inclusion in cattle diets and their influence on *E. coli* O157:H7 shedding, and the impact of transport stress on prevalence of *E. coli* O157:H7 in market ready cattle.

E. coli O157 and byproduct-based diets

Biofuels, such as ethanol and biodiesel, are defined as a renewable energy sources produced from natural materials (Demirbas, 2009). Throughout the last decade production of biofuels across the world has expanded considerably (Figure 1-2). Ethanol production worldwide grew from 17,371 million liters in 2000 to 109,573 millions of liters in 2010, 57% of which were produced in the United States (R.F.A., 2012). World biodiesel production also increased from 882 million liters in 2000 to 19,476 million liters in 2010, with Europe being the main producer (55%), and the United States accounting for approximately 7% of world production (EIA, 2010).

Figure 1-2: Historic of US ethanol (solid line) and biodiesel (broken line) production. Data from (EIA, 2010).
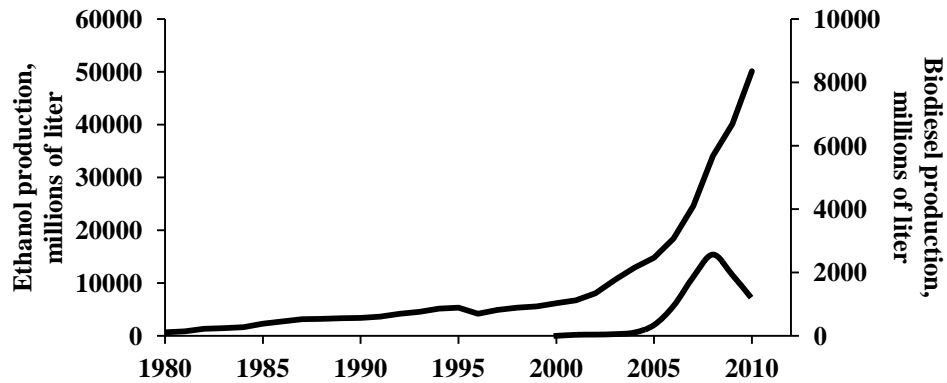
Both ethanol and biodiesel are obtained from crops that are, also, used as feeds for animal production. Expansion of the biofuels industries has, therefore, diverted use of these crops from feed to energy supply, and in so doing has contributed to increases in cereal grain prices (Figure 1-3).

Figure 1-3: US price history of corn (+), wheat (■), and soybeans (▲). Data from (USDA, 2012)
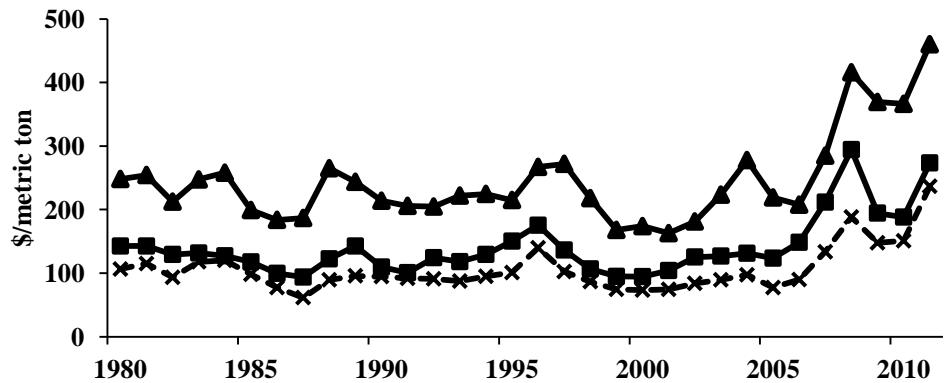
Nevertheless, the conversion of cereal grains into ethanol, also, yields large quantities of the byproduct, distiller's grain, approximately one-third of the weight of the original grain. Distiller's grain is, frequently, priced at a discount relative to cereal grains in addition to being a valuable source of both protein and energy, and thus is attractive to feedlots as an economical alternative to traditional cereal grains.

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Distiller's grain a by-product of the ethanol industry

Distiller's grains originate from corn, sorghum, barley, and wheat, which is dictated in large part by geographical location of the ethanol production facility. In the dry mill ethanol process, grains are first cleaned, grounded in a hammermill, and water is added to create a mash (Figure 1-4).

Figure 1-4: Ethanol production by dry mill process. From (ICM, 2012).
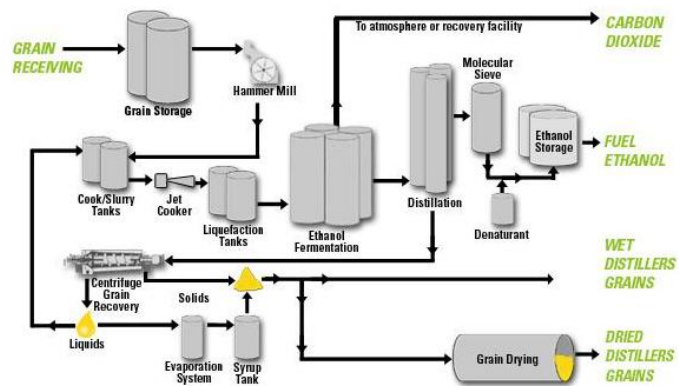
Enzymes are added and the mash is heated to break down starches and to facilitate liquefaction. The liquefied product is sterilized, yeast is added, and the resulting mixture undergoes fermentation, producing ethanol and carbon dioxide as major fermentative end products. The fermented mixture, referred to as whole stillage, is then distilled to recover ethanol The remaining portion is centrifuged to separate the liquid and particulate fractions, yielding liquid "thin stillage" and wet cake fractions. The thin stillage is concentrated by evaporation, producing a thick syrup, referred to as condensed solubles. The wet cake and solubles are mixed and merchandised in the wet form as wet distiller's grain with solubles (WDGS), or are dried to approximately 10% moisture to yield dried distiller's grain with soluble (DDGS; (Stock et al., 2000). Twenty five kilograms of corn yields approximately yields 10.4 liters of ethanol and 7.6

kg of distiller's grains using this process (Dinneen, 2008). WDGS and DDGS obtained are concentrated in non-starch nutrients making them a good source of energy, protein and fiber for animals (Spiehs et al., 2002). Moreover, addition of solubles to wet cake makes the final product palatable to animals (Klopfenstein et al., 2008).

Distiller's grain as a grain replacement in cattle diets.

Distiller's grains frequently are included in cattle diets as an energy or protein replacement. Substitution of part of the corn, 0 to 30%, with DDGS or WDGS has yielded satisfactory animal performance and meat quality. Cattle fed up to 30% distiller's grains had ADG, DMI and G:F similar to animals fed diets without by-products (Depenbusch et al., 2009; Klopfenstein et al., 2008). Furthermore, inclusion of distiller's grains had no apparent effect on carcass characteristics and sensory analysis (Depenbusch et al., 2009; Jenschke et al., 2007; Roeber et al., 2005). Comparable performance results and overall lower price of distiller's grains (USDA, 2012) when compared to corn (Figure 1-5) make this byproduct very appealing for the cattle feeding industry.

Figure 1-5 Price history of corn (■) and distiller's grains (Δ) in the US. Data from (USDA, 2012).

- Increased *E. coli* O157 prevalence with distiller's grains

Multiple studies have revealed higher fecal shedding of *E. coli* O157 in cattle fed WDGS, DDGS, and brewer's grains when compare to traditional cereal-based diets. A large study across 12 US feedlots reported animals fed brewer's grains to be 6 times more likely to shed pathogens in their feces when compared to steers fed diets without brewer's grains (Dewell et al., 2005a). In 2007, a study on terminal rectum colonization rate showed a strong correlation between *E. coli* O157:H7 carriage and increasing levels of distiller's grains in animal diets (Peterson et al., 2007). These results were confirmed with *E. coli* O157:H7 inoculated calves fed 25% of DDGS who had increased prevalence of pathogen compared to control (Jacob et al., 2008b). Moreover, commensal *E. coli* O157 and inoculated *E. coli* O157 were shown to survive longer in feces of cattle fed 20% WDGS or higher (Varel et al., 2008). Similarly, pathogen hide counts from cattle receiving WDGS were, also, higher than counts from animals receiving no byproduct (Wells et al., 2009), illustrating increased survival capacity of the pathogen in the presence of distiller's grains in diets. Increases in pathogen shedding have been noted for both wet and dried distiller's grains (Jacob et al., 2010). Considering the effect of distiller's grains inclusion on *E. coli* O157 prevalence and the increased risk of contamination at slaughter, a study was done to analyze the effect of a reduction of distiller's grains inclusion prior to harvest. Results showed complete removal or partial removal (70% to 15%WDGS) of by-product from diet 28 days prior harvest was ineffective as an intervention, but a 56-day withdrawal period did decrease prevalence of the pathogen in feces and hides at slaughter (Wells et al., 2011). Effect of distiller's grains on *E. coli*

O157 appears, therefore, to be reversible but advantage(s) provided to the pathogen by byproduct inclusion in diets are still unclear.

- Reasons for *E. coli* O157 increase

A few hypotheses have been made to explain higher prevalence of *E. coli* O157:H7 in cattle fed diets including distiller's grains. The main idea being that substitution of cereal grains with distiller's grains alters digestive characteristics, and thus hindgut ecology. During ethanol production processes most of the starch is converted to ethanol, leaving only trace quantities of starch in distiller's grains. Consequently, ingestion of byproduct diets will lead to a lower amount of starch reaching the hindgut and to an overall higher pH that is more favorable to proliferation of *E. coli* O157:H7 (Berg et al., 2004; Fox et al., 2007; Jacob et al., 2008b). This relationship was, however, not confirmed by *in vitro* and *in vivo* studies that found no correlation between fecal pH, diets, and *E. coli* O157 prevalence (Depenbusch et al., 2009; Wells et al., 2009; Yang et al., 2010). It also has been postulated that the relative increase in protein content of WDGS and DDGS compared to corn could have an effect on concentrations of VFA and branched amino acids (Klopfenstein *et al.*, 2008). Indeed, only part of proteins are degraded in the rumen, and greater supply of protein reaching the hindgut will increase microbial fermentation and production of VFA and branched amino acids. Bacteria have been shown to benefit from VFA and branched amino acids, which could favor pathogen survival in hindgut. Once again, experiments failed to verify this hypothesis (Huntington et al., 2006). Likewise, L-lactate concentrations have been investigated, as it is known to have antimicrobial properties (McWilliam Leitch and Stewart, 2002) and lower levels of this product in feces could benefit *E. coli* O157 persistence. Concentration of L-lactate in feces of cattle fed WDGS were, indeed,

significantly decreased when compared to concentration in feces of cattle fed corn-based diets (Varel et al., 2008; Wells et al., 2009); however, despite a significant decrease L-lactate concentrations still averaged 3.72 mM, which presumably is far below inhibitory concentrations previously observed, 50 to 200mM, and thus is an unlikely explanation for increased prevalence of *E. coli* O157 in feces of cattle fed distiller's grain. Distiller's grain-based diets provide nutrients with different characteristics than cereal-based diets, and these differences may cause shifts in digestion and in microflora selection (Yang et al., 2010). Changes in microflora could make *E. coli* O157:H7 more competitive and allow its establishment in the gastrointestinal tract of cattle, but this hypothesis has yet to be tested. Another hypothesis is that specific components within distiller's grains may benefit, directly or indirectly, growth of the pathogen, facilitating its colonization of the bovine GIT (Dewell et al., 2005a; Jacob et al., 2008a; Viazis and Diez-Gonzalez, 2011). Analysis of distiller's grains composition revealed the presence of glycerin, ranging from 3.5 to 10% on DM basis, in addition to carbohydrates and proteins (Kingsly et al., 2010; Wu, 1994), which could potential play a role in pathogen establishment. Glycerin found in distiller's grains is a secondary metabolite of yeast fermentation during ethanol production, but also is a byproduct resulting from the biodiesel industry, and occasionally is used as a corn replacement.

Glycerin and Escherichia coli O157

Glycerin a by-product of biodiesel production

Crude glycerin is a co-product of the biodiesel industry obtained through transesterification of vegetable oils or animal fats with methanol in presence of a catalyst. Each liter of biodiesel produced will lead to the synthesis of 0.079 kg of crude glycerol (Thompson

and He, 2006). Crude glycerin is used for multiple purposes, including pharmaceuticals, cosmetics, food and beverage, and chemical industries. Glycerin is generally recognized as safe (GRAS) for use in animal feed as long as methanol content represents less than 0.5% (FDA, 2004). Excess methanol remaining in crude glycerol can affect its potential as a feed supplement which is why refinement treatments are often applied to meet the 0.5% requirement (Yang et al., 2010). Crude glycerin obtained through transesterification processes usually contains 80% glycerin, 0.5% methanol or less, 5% salts (sodium chloride), 1.2% organic substances other than glycerin, and water, making it an excellent source of energy for cattle.

Glycerin as a feed additive

Glycerin is a glycogenic compound that was first used in dairy production to overcome ketosis (Johnson, 1954). Most research on this compound originates from this field and inclusion of up to 10% glycerol in dairy cattle diets repeatedly has proven to have no deleterious effects on performance (Schröder and Südekum, 1999), and may improve milk production when included at 5.6% or less of the diet (Bodarski et al., 2005; DeFrain et al., 2004). Despite differences between dairy and feedlot production systems, results derived from dairy cows can be extrapolated to growing and finishing cattle. Indeed, inclusion of 10% crude glycerin in feedlot diets containing rolled corn and distiller's grains improved feed efficiency by 19% (Pyatt *et al.*, 2007). Parsons et al. (2009) and Trabue et al. (2007) observed similar benefits on efficiency with 12 and 10% inclusion rates. Other authors observed no impact of glycerin on animal efficiency (Elam et al., 2003; Mach et al., 2009). In several of these studies a decreases in dry matter intake (DMI) have been observed with increasing levels of glycerin in diets (Elam et al., 2003; Parsons et al., 2009; Pyatt et al., 2007). Carcass characteristics and meat sensory properties were also

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assessed and 8% or less of glycerin inclusion appeared to have no deleterious effects on overall meat quality (Mach et al., 2009; Parsons et al., 2009). These results show potential for crude glycerin as a replacement for cereal grains in feedlot diets. Moreover, rapid development of the biodiesel industry led to sharp declines in market value of crude glycerin, with prices decreasing from \$0.55/kg in 2004 to between \$0.07 and 0.10/kg in 2006 (Johnson and Taconi, 2007), thus presenting glycerin as an economical substitution for cereal grains. Biodiesel byproduct inclusion in feedlot cattle diets is fairly recent, so little is known of its effects on *E. coli* O157:H7 prevalence.

Glycerin inclusion in cattle diets and Escherichia coli O157 prevalence

Important information can be derived from studies on glycerol metabolism within the rumen and from observations made on its effect on rumen microflora to try to understand possible effects on *E. coli* O157:H7 shedding in feedlot cattle.

- Glycerol metabolism in the rumen

Glycerol is found in cell wall phospholipids or in lipidic storage of seeds (Roger 1992). Once in the rumen, glycerol is rapidly metabolized by bacteria, mainly *Selenomonas* (Krehbiel, 2008), to produce mostly propionate (Johns, 1953). Propionate is a precursor for gluconeogenesis and is able to supply 32 to 73% of the energy demands of ruminants (Rico *et al.*, 2012), which is why glycerol was first used as a remedy for ketosis. Propionic acid, however, is not the only product of glycerol metabolism (Garton *et al.*, 1961). Part of ingested glycerol is directly absorbed by the rumen epithelium as evidenced by the presence of glycerol in plasma after dosing ruminally cannulated bulls with glycerin (Kijora *et al.*, 1998). The remaining glycerol is converted to carbon dioxide and volatile fatty acids, and the volatile acids produced

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are still a subject of debate. Wright showed production of acetate (Wright, 1969), whereas Remond and Kijora demonstrated production of butyric acids at the expense of acetic acid (Kijora et al., 1998; Remond et al., 1993). More recent studies showed no butyrate production (Trabue *et al.*, 2007) or a decrease in butyrate and valerate concentrations with increasing inclusion of crude glycerin in the diet (AbuGhazaleh et al., 2011; Lee et al., 2011; Parsons and Drouillard, 2010). These changes in final products of glycerol metabolism are likely related to differences in rumen microflora and differences in basal diets among experiments, and may also reflect the potential for adaptation of the rumen microorganisms to presence of glycerol. After few days of glycerol feeding, bacteria seem to be able to metabolize glycerol without any lag time (Ferraro *et al.*, 2009).

- Glycerol effect on rumen fermentation and microflora

Despite potential microbial adaptation, glycerol appears to affect ruminal microflora in many ways. Consistent observation of a decrease in cellulolytic activity in the rumen has been reported (Paggi et al., 2004; Roger et al., 1992). *In vitro* assay showed that addition of 5% glycerol inhibited growth and activity of *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* (Roger *et al.*, 1992) two rumen cellulolytic bacteria. *Butyrivibrio fibrisolvens* DNA concentrations also were depressed by replacement of 30% of corn substrate by glycerin in a continuous fermentation, leading the authors to speculate that glycerin was preventing attachment of bacteria to fiber and, hence, impairing their proliferation and persistence (AbuGhazaleh *et al.*, 2011). Related observations were done in feeding studies where apparent digestibility of neutral detergent fiber was decreased by increasing amount of glycerin incorporated in diets (Donkin et al., 2009; Parsons and Drouillard, 2010). In addition to its effect

on cellulolytic bacteria, glycerol was found to decrease proteolytic activity by 20% when added to culture media at concentration of 50mM or more (Paggi *et al.*, 1999). Bacterial protein synthesis was, as well, decreased by infusion of glycerol into the rumen of bulls (Kijora *et al.*, 1998). Furthermore, glycerin appears to affect rumen pH which in turn can affect rumen microflora; however like with distiller's grains observations are not always consistent. *In vivo* and *in vitro* assays related a decrease in rumen pH with increasing levels of glycerin (Kijora et al., 1998; Lee et al., 2011), whereas others failed to detect any effect of glycerol on pH (Rico *et al.*, 2012) or, in contrast, observed an increase in pH (Parsons and Drouillard, 2010). Regardless of the exact effects of glycerin in the rumen, its capacity to affect rumen microorganisms and pH can easily affect rumen equilibrium state, thus impacting pathogens such as *E. coli* O157:H7.

- Glycerol effect on *E. coli* O157:H7

As of today very few studies are available to assess the effect of this byproduct on *E. coli* O157:H7 shedding in cattle. Most of the research has focused on distiller's grains inclusion and little attention has been given to glycerol. The presence of significant quantities of glycerol in final WDGS or DDGS has, however, brought to our attention a potential role played by glycerin in the increase of pathogen prevalence in byproduct-based diets. Literature relates two studies conducted on this topic. The first one evaluated the effects of glycerin inclusion at 10% to diets containing dry rolled corn, steam flaked corn and DDGS on prevalence of *E. coli* O157:H7 in feces of inoculated calves. Researchers observed no effect of glycerin inclusion on pathogen prevalence (Paulus *et al.*, 2011). The second experiment used naturally infected cattle fed a combination of DDGS and glycerin in a steam-flaked corn and hay grass diet, and once again did not observe any effect of glycerin inclusion on *E. coli* O157:H7 prevalence in cattle feces

(Jaderborg *et al.*, 2011). Despite the absence of effect of glycerin inclusion in these two experiments, it would be interesting to investigate the relationship between pathogen prevalence and glycerin inclusion in feedlot diets.

As previously discussed, diet has an important impact on fecal shedding of *E. coli* O157:H7, and constant adaptation of animal production to crop availability brings new challenges to mitigate the risk of food product contamination. A recent development in microbial endocrinology has highlighted a cross talk between bacteria and their host during stressful events, which may ultimately benefit the proliferation of pathogens. Such phenomenon could anile all efforts made pre-harvest to limit *E. coli* O157:H7 shedding in cattle, as cattle undergo a certain level of stress immediately before slaughter.

Escherichia coli O157 and transport stress

The following paragraphs are a review of stress definition and quorum sensing principles and their possible relevance to pathogen shedding in market ready cattle.

Notion of stress

Definition of stress

−All of the vital mechanisms, however varied they may be, have always one goal, to maintain the uniformity of the conditions of life in the internal environment […]− (Bernard, 1878), this notion is known as homeostasis (Cannon, 1932). Any stimuli capable of disrupting this equilibrium is considered a stressor (Mostl and Palme, 2002) whether it is pleasurable or not. In today's animal husbandry settings, it is important to minimize stress to improve animal welfare, productivity, and product quality. Cattle stressors are divided in two main categories:

physiological (hunger, thirst, fatigue, injury, thermal extremes) and psychological (handling, restraint, novelty). Weaning, castration, and transport are highly stressful events for animals. This literature review will focus on transport stress and its impact on animal physiology and bacterial susceptibility.

Transport stress

Numerous factors are involved in the induction of transport stress (Nielsen et al., 2011) and animals have different capacity to cope with it depending on their genetics and history (Hahn and Becker, 1984). Significant among these factors is the impact of feed and water depravation. Cattle are usually taken off feed 12 hours or more before transport to limit gut fill (Warriss et al., 1995) and may have limited, or no, access to water over the course of the journey, impacting normal control of pathogenic bacteria in the rumen and increasing risk for gastrointestinal infections (Hogan et al., 2007). Cattle are, also, exposed to psychological stressors, including handling by humans, commingling with unfamiliar animals, and through exposure to new environments. These factors disrupt the social organizations among cattle populations and create novelty (Grandin, 1997), both of which are recognized as stressors. Another major factor is the fact that animals are prevented from lying down during transport. Ruminants spend on average 12 hours laying every day (Munksgaard et al., 2005), and preventing them from doing so induces fatigue. Finally, cattle are exposed to large bacterial loads and potentially novel species or strains of bacteria during transit, after their arrival at the abattoir, within lairage areas and stunning boxes, all of which are sources of contamination for *E. coli* O157:H7 (Avery et al., 2004). All of these factors contribute to the overall transport stress and will ultimately disturb homeostasis,

triggering the central nervous system and immune system to work toward reinstatement of animal equilibrium.

Stress physiology in cattle

Nervous system response to stress

The nervous system will be the first affected by stressful conditions. In cattle, two types of responses can be observed: fight or flight response or general adaptation response (Figure 1-6).

Figure 1-6: Neuroendocrine response to stress. Adapted from (Matteri *et al.*, 2000)
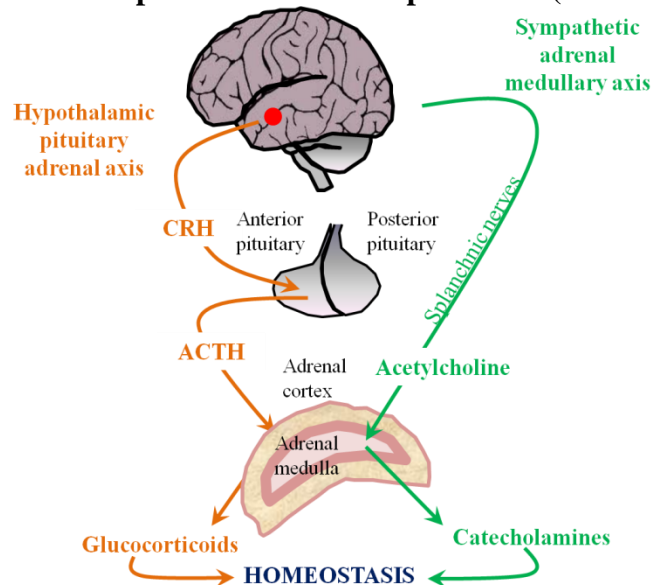
The first response relies on the activation of the sympathetic adrenal medullary axis (SAM). Splanchnic nerves release acetylcholine which induces synthesis and secretion of the catecholamines, epinephrine and norepinephrine, by the adrenal medulla into peripheral tissues. Catecholamines prepare the body for an intense effort, such as fight or flight, by redirecting energy to muscle and accelerating heart rate. This response is rapid but has short lasting effects. General adaptation response relies on actions of the hypothalamic pituitary adrenal axis (HPA).

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Corticotropic-releasing hormones (CRH) are synthesized in the hypothalamus and released into portal blood, inducing synthesis and release of adrenocorticotropic hormone (ACTH) by the anterior pituitary. Presence of elevated concentrations of ACTH activates production and release of the glucocorticoids, cortisol and corticosterone, by the adrenal cortex into the circulatory system (Matteri et al., 2000). Glucocorticoids stimulate conversion of fat and protein, in liver, to their metabolites as precursors for glucose production to fulfill energy needs of animals during stress. General adaptation response is slower than the fight or flight response, but can be maintained for a longer period of time.

Immune system response to stress

Immune system, as neuroendocrine system, is involved in maintaining homeostasis in the body. Immunity is composed of innate and adaptive immunity. Innate immunity is the non-specific first line of defense against pathogens (Figure 1-7). It occurs quickly, allowing time for the organism to develop adaptive immunity specific to an invader. Innate immunity relies on recognition of highly conserved structures in microorganisms: pathogen-associated molecular patterns (PAMPs).

Figure 1-7: Immune response to stress.
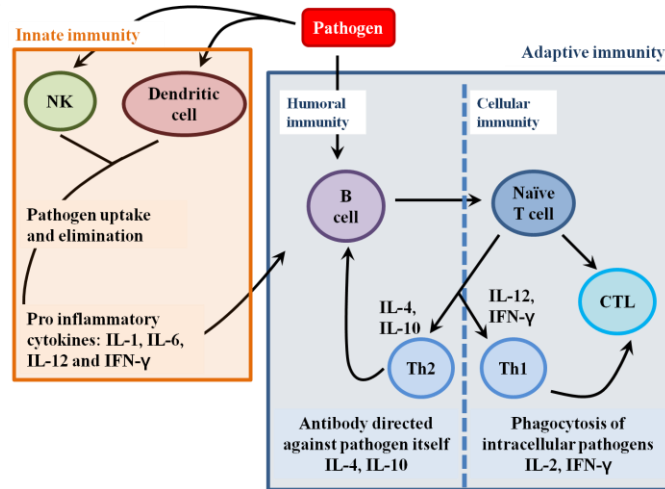
Macrophages and neutrophils recognize PAMPs and initiate killing. Simultaneously, natural killer cells target pathogen infiltrated cells and secrete pro-inflammatory cytokines (Il-1, IL-6, and IL-12), allowing the activation of acute phase response and adaptive immunity. Adaptive immunity is specific to an antigen previously encountered. The B cells recognize, bind, and ingest the specific antigen to present it to naïve T cells. The T cells then mature into either T helpers cells (Th), expressing $CD4^+$ proteins, or cytotoxic T lymphocyte cells (CTL), expressing $CD8^+$ proteins. There are two kinds of Th − Th1 and Th2 − which are responsible for different immune functions and produce different cytokines. Th1 are involved with CTL in cellular adaptive immunity, also known as cell-mediated immunity. These lymphocytes target cells that have been infiltrated by specific antigens and induce apoptosis without using antibodies. On the other hand, Th2 are involved in humoral adaptive immunity, also known as antibody-mediated immunity. The Th2 stimulate B cell differentiation into plasma cells which produce specific antibodies against free pathogens. Differentiation of T cells towards Th1 or Th2 depends on the nature of initial innate response (Janeway and Medzhitov, 2002). Coffman demonstrated that IFN-γ inihibits differentiation of naïve T-cells toward Th2, whereas synthesis of IL-4 and IL-10 inhibited differentiation into Th1 (Coffman, 2006).

Nervous system/Immune system cross talk during stress

Glucocorticoids produced by the nervous system during stress have an effect on cytokines synthesis and release. Glucocorticoids increase production of immunosuppressive cytokines, such as IL-10, and decrease the synthesis of pro inflammatory cytokines, such as IL-12, IL-2, and IFN-γ (Hickey et al., 2003; Richards et al., 2000). Similar observations have been made with catecholamines, which increase IL-10 secretion but inhibit IL-12 (Elenkov et al.,

1996). Cytokines also are able to stimulate the HPA axis, increasing glucocorticoid concentrations (Salak-Johnson and McGlone, 2007). In this manner, overstimulation of the immune system during stress can be prevented (Wiegers et al., 2005). Conversely, glucocorticoids by their capacity to regulate cytokine production can induce a shift in maturation of T cells from Th1 to Th2, resulting a shift from cellular immunity to humoral immunity. Glucocorticoids also can suppress cytotoxic T lymphocyte (CTL) proliferation and neutrophil function (Elenkov, 2002), which impairs cellular and innate immunity. Multiple studies on transport stress showed an increase in neutrophil (Blecha et al., 1984; Dixit et al., 2001) and lymphocyte counts (Ishizaki et al., 2005) in cattle, but also a loss of function of these cells in these animals (Stanger et al., 2005; Weber et al., 2001). Many authors attribute immunosuppression in cattle after transport to elevated glucocorticoid concentrations (Dixit et al., 2001; Grandin, 1997; Mackenzie et al., 1997; Mormede et al., 1982). All of these observations underline a cross talk between the neuroendocrine and immune systems, which has important repercussions for capacity to resist bacterial infections, as bacteria have been shown to sense eukaryotic signals through quorum sensing.

Stressed animal and bacteria

Quorum sensing and interkingdom signaling

The ability of bacteria to communicate is called quorum sensing. Bacteria metabolize hormone like compounds called autoinducers (AI) using LuxS enzymes. Genes encoding for LuxS are found in commensal and pathogenic bacteria (Clarke and Sperandio, 2005). The AI are aromatic compounds, hydrophobic by nature, that are not able to cross cell membranes (Hughes and Sperandio, 2008). Adrenergic receptors for AI, such as QseC and QseE, are found on

bacterial membranes (Chen et al., 2006; Kendall and Sperandio, 2007). The AI-3/LuxS system notifies bacteria when they reach a favorable environment to proliferate and, in some cases, to attach (Clarke and Sperandio, 2005). Binding of AI-3 to the QseC receptor activates phosphorylation of the two-component quorum sensing system QseBC, which in turn activates the two-component system QseEF, the LEE genes, the motility genes flagella regulon (FlhDC), and Shiga toxin genes (Hughes and Sperandio, 2008).

Pathogens, such as *E. coli* O157:H7, have developed the ability to hi-jack signals from eukaryotic cells to serve their own needs. One example of the inter-kingdom signaling is revealed in the capacity of catecholamines secreted during stress (i.e., epinephrine and norepinephrine) to substitute the autoinducer 3 (Clarke and Sperandio, 2005) and activate phosphorylation of the two-component quorum sensing system QseBC of *E. coli* O157:H7, thereby stimulating an increase in motility, adherence, and virulence (Figure 1-8).

Figure 1-8: Interkingdom signaling. Adapted from (Hughes and Sperandio, 2008).
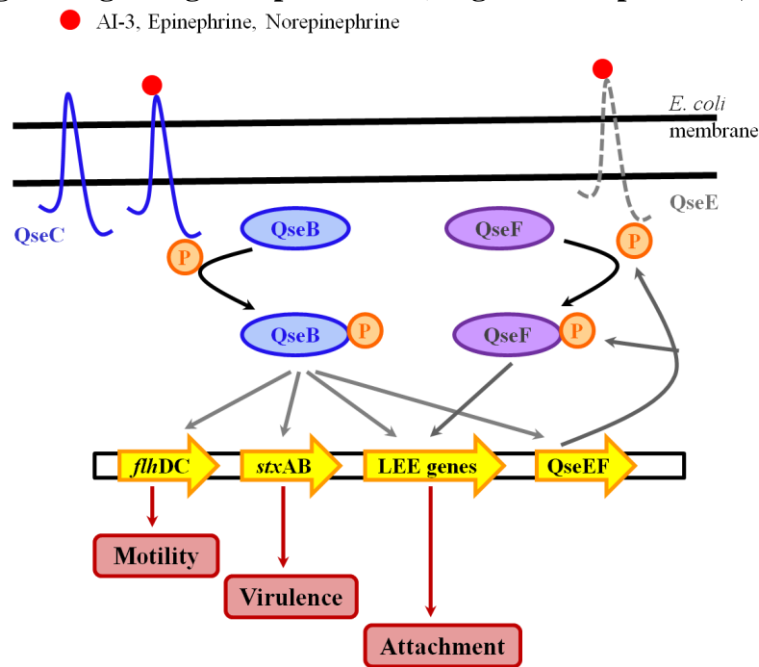
Norephineprhine and bacterial growth

Simultaneously, the production and release of norepinephrine can be beneficial to the growth of the pathogens in iron sequestering environments. Pathogenic bacteria need iron to express virulence. Iron-sequestering glycoproteins (lactoferrins and transferring) are secreted in the GIT to prevent pathogens virulence. Norepinephrine releases Fe^{3+} bound to lactoferrin and transferrin, making iron available for bacterial uptake (Freestone and Lyte, 2008; Freestone et al., 2007).

E coli O157 prevalence during stress transport

Effect of stress on the neuroendocrine and immune systems and the ability of bacteria to utilize eukaryotic signals for their own needs are likely to induce changes in prevalence of *E. coli* O157:H7 in transported cattle, thus presenting a higher risk of contamination at slaughter. As previously noted, glucocorticoid and catecholamine secretion will render animals more susceptible and will support *E. coli* O157:H7 growth and shedding. Studies have illustrated an increase in detection of *E. coli* O157:H7 following transportation (Arthur et al., 2007; Bach et al., 2004; Dewell et al., 2005b; Dewell et al., 2008), while others did not reveal any significant effect of transport (Barham et al., 2002; Fegan et al., 2009; Minihan et al., 2003) on pathogen shedding. Discrepancies in the results come in part from the many factors involved in transport stress, as well as from difficulties associated with obtaining samples during transport, and finally from the differences of sampling and analysis techniques used in the various publications. Despite conflicting results, quorum sensing and interkingdom signaling represent potentially important mechanisms to take into account when studying *E. coli* O157:H7 colonization of cattle

GIT and in attempting to design interventions aimed at limiting bacterial loads in market ready cattle.

SUMMARY

In summary of this chapter, *E. coli* O157:H7 is an important foodborne pathogen that is yet to be fully understood. Cattle are undoubtedly a main reservoir for this pathogen but bacterial tropism and competitive growth of *E. coli* O157:H7 is still unclear. Moreover, adaptation of animal production systems to changing agriculture has brought to light new challenges, such as the increase of pathogen prevalence in cattle receiving ethanol byproduct-based diets which has so far no concrete explanation. Potential for glycerin to be a key player in this effect will be further investigated in this dissertation. Impact of quorum sensing and eukaryote signaling during transport stress will also be further evaluated in regards to their impact on *E. coli* O157 fecal shedding patterns.

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Chapter 2 Capacity of the bovine intestinal mucus and its components to support growth of *Escherichia coli* O157:H7

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Running head: *E. coli* O157:H7 growth in bovine intestinal mucus

ABSTRACT

Colonization of the gastrointestinal tract of cattle by Shiga toxin-producing *Escherichia coli* increases the risk of contamination of beef carcasses at slaughter. Our study aimed to shed more light on the mechanisms used by *E. coli* O157:H7 to thrive and compete with other bacteria in the gastrointestinal tract of cattle. We evaluated bovine intestinal mucus and its constituents *in vitro* for their capacity to support growth of *E. coli* O157:H7 in the presence or absence of feces, with and without various enzymes. Growth of *E. coli* O157:H7 was proportionate to the amount of mucus added as substrate, whereas the quantity of total anaerobic organisms was unchanged. Growth of *E. coli* O157:H7 was similar for small and large intestinal mucus as substrate, and was partially inhibited with the addition of fecal inoculum to cultures, presumably due to competition from other organisms. Whole mucus stimulated growth to the greatest degree compared with other evaluated compounds, but the pathogen was capable of utilizing all substrates to some extent. Addition of enzymes to cultures failed to affect growth of *E. coli* O157:H7 except for neuraminidase, which resulted in greater growth of *E. coli* O157 when combined with sialic acid as substrate. We concluded that *E. coli* O157 has the capacity to utilize small or large intestinal mucus, and growth is greatest with whole mucus rather than individual mucus components. There are two possible explanations for these findings: (i) multiple substrates are needed to optimize growth, or (ii) a component of mucus not evaluated in this experiment is a key ingredient for optimal growth of *E. coli* O157:H7.

Keywords: *Escherichia coli* O157:H7, intestinal mucus

IMPLICATIONS

These studies offer insight into the potential of intestinal mucus and its components to promote growth of *E. coli* O157:H7 in the gastrointestinal tract of cattle. Factors that influence intestinal mucus secretion in cattle may be important determinants of *E. coli* O157:H7 colonization rates.

INTRODUCTION

Escherichia coli are commensal copiotroph bacteria found in the intestinal mucus layer (Montagne *et al.*, 2000, Moller *et al.*, 2003, Naylor *et al.*, 2003) of mammalian digestive tracts (Ihssen and Egli, 2005). Some strains of *E. coli* are pathogenic to humans, such as the well known Enterohemorrhagic (**EHEC**) *E. coli* O157:H7. Infection with EHEC often originates from the ingestion of contaminated food products. *Escherichia coli* O157:H7 is non-pathogenic to cattle, and cattle are recognized as an important reservoir for the pathogen. Exposure in human populations can occur either directly through contact with cattle or their waste products or indirectly through water, meat, or other food products that have been contaminated by cattle feces. To control contamination in the food chain, it is essential to understand how this pathogen is able to grow and compete with other bacteria in the bovine gastrointestinal tract. Previous studies have shown that bovine intestinal mucus supports bacterial colonization and can selectively influence composition of the bacterial population (Deplancke and Gaskins, 2001), yet little is known of the nutrients and metabolic pathways used by *E. coli* (Miranda *et al.*, 2004). Intestinal mucus comprises mucins, glycoproteins, glycolipids, epithelial cell debris, and electrolytes (Conway *et al.*, 2006). Degradation of the complex mucin components to simpler, more readily fermentable substrates requires multiple enzymes; for example, proteases convert

the mucus from a viscous to a fluid state, and endoglycosidases act at internal sites to release oligosaccharide fragments. Sialic acid is then removed from these fragments by neuraminidases, allowing degradation of the remaining chain by glycosidases and beta-galactosidases (Corfield *et al.*, 1992). *Escherichia coli* does not produce polysaccharide-degrading enzyme (Chang *et al.*, 2004), thus restricting its capacity for carbohydrate utilization to mono- or disaccharides (Mayer and Boos, 2005). Moreover, *E. coli* O157:H7 lacks neuraminidase activity (Hoskins *et al.*, 1985), and therefore has limited ability to degrade complex mucin molecules. The organism therefore depends on other anaerobes in the gastrointestinal tract to degrade mucin polysaccharides and release fragments beneficial for their growth (Jones *et al.*, 2008). To be maintained in the gastrointestinal tract of cattle, *E. coli* O157:H7 need to compete with other bacteria to colonize the mucus layer. In the 1980s, researchers believed that bacteria have a single preferred nutrient as a substrate of growth (Freter, 1988); however, recent studies have illustrated that *E. coli* relies on a diverse range of nutrients for its growth (Chang *et al.*, 2004), allowing EHEC to proliferate in cattle and be shed in their feces, thus providing opportunity for contamination of carcasses at harvest. This series of *in vitro* experiments was conducted to gain insight into the mechanisms used by *E. coli* O157:H7 to thrive and compete with other bacteria of the gastrointestinal tract of cattle in the presence or absence of feces and various enzymes.

MATERIAL AND METHODS

Intestinal mucus harvest

Intestinal tissues were collected from freshly harvested cattle and immediately transported to the Preharvest Food Safety Laboratory (Kansas State University, Manhattan, KS, USA). Sections of the ileum and colon were excised with sterile scissors and washed with a

HEPES-Hanks buffer (pH 7.4) to remove digesta. Mucus was collected from each of the sections by gently scraping the epithelium with a sterile microscope slide. Harvested mucus was centrifuged twice at 27,000 x g for 20 min to remove cellular debris and impurities. Supernatant containing the crude mucus was dialyzed overnight at 4°C in HEPES-Hanks buffer, lyophilized and stored at -20°C.

Bacterial strains

Five Shiga toxin-producing *E. coli* O157:H7 strains (STEC), supplied by Dr. Nagaraja (Department of Diagnostic Medicine and Pathobiology, Kansas State University, Manhattan, KS, USA), were used in this experiment. Mutants resistant to nalidixic acid (Nal^R) were obtained by serial transfer into Luria-Bertani broth (LB; Neogen, Inc., Baltimore, MD, USA) containing increasing concentrations of nalidixic acid (from 0.2 to 50 mg/L; Sigma-Aldrich, St. Louis, MO, USA) following the procedure outlined by Schamberger *et al.* (2004).

Bacterial inoculum preparation

All five Nal^R STEC were plated onto blood agar containing 5% sheep's blood and were incubated overnight at 37°C. A single colony was selected from each plate and grown overnight at 37°C in 10 mL tryptic soy broth (**TSB**) to be transferred to five other 9-mL TSB tubes. After 18 h incubation at 37°C, the five broths were combined in equal proportions to create a five-strain cocktail of Nal^R STEC.

Fecal inoculum preparation

Feces were collected by rectal palpation from a steer fed a concentrate-based diet and transported to the Preharvest Food Safety Laboratory in a pre-warmed thermos. Feces were

blended in an Osterizer blender for 60 s with McDougall's buffer (6 mL/g of feces) under a stream of CO_2 and strained through two layers of cheesecloth.

In vitro fermentation assay

Fecal inoculum, at 10^4 CFU/mL, or a similar amount of McDougall's buffer was added to the tubes containing the substrates to be tested. Tubes were inoculated with 10^3 CFU/mL of the five-strain bacterial inoculum, gassed with CO_2, capped with butyl stoppers fitted with Bunsen valves, and incubated on a shaker at 40°C. A volume of 100 µL was extracted from each fermentation tube after 0, 6, 8, 12, and/or 24 h and diluted into 900 µL of Butterfield's phosphate buffer (pH 7.2). Subsequent dilutions (100 µL) were plated onto MacConkey sorbitol agar with cefixime (0.05 mg/L), potassium tellurite (2.5 mg/L) and nalidixic acid (50 mg/L; CTN-SMAC). Plates were incubated at 37°C for 24 h, and non-sorbitol fermenting colonies were enumerated. Dilutions also were plated on tryptic soy agar (TSA) and coliforms/*E. coli* Petrifilm plates to enumerate total anaerobic bacteria, coliforms and commensal *E. coli*. The TSA plates were inoculated with 100 µL of the cultures and incubated at 40°C in a Coy rigid anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) containing 90% N, 5% CO_2 and 5% H. Petrifilm plates were inoculated with 1 mL of the cultures and incubated at 37°C under aerobic conditions.

Nitrogen and organic matter content of mucus

Organic matter (**OM**) contents of mucus harvested from the small and large intestine were determined by ashing the samples (Undersander *et al.*, 1993). Briefly, mucus samples were dried in aluminum pan overnight at 105°C to determine dry matter content. Pans were then heated to 450°C overnight, slowly cooled and transferred to a desiccator to be weighted. Organic matter percentage was obtained by subtracting ash content from 100. A bicinchoninic acid assay

(BCA, Thermo Scientific, Rockford, IL, USA) was performed to assess CP contents on those samples. *In-vitro* fermentation performed in this study was carried out in tubes containing mucin from the small and large intestine with an equal level of OM, 4.4 mg of OM/mL (7.5 and 9.2 µg of protein/mL, respectively), if not stated otherwise.

Increasing concentrations of mucus

Increasing concentration of mucus from the small intestine (0, 0.5, 1.0, 2.0, 4.4, 10, and 15 mg OM per mL of inoculum) were tested in an *in-vitro* fermentation assay as described previously.

Mucus and mucus components

Mucin from the ileum and the colon (4.4 mg of OM/mL), as well as mucus components such as the lipid L-alpha-phosphatidylserine, (1 mg/mL; CAS number: 840032P, Avanti Polar Lipids Inc., Alabaster, AL), and the carbohydrates, D-gluconic acid (CAS number: G9005), D-glucuronic acid (CAS number: 6556-12-3), N-acetyl-D-glucosamine (NAG; CAS number: 7512-17-6), D-galacturonic acid (CAS number: 91510-62-2), sialic acid (CAS number: 131-48-6), galactose (CAS number: G5388) and mannose (10 mg/mL; CAS number: 3458-28-4, Sigma-Aldrich, St. Louis, MO, USA), were tested as growth substrate in the *in-vitro* fermentation assays.

Enzymes and enzyme inhibitors

All enzymes and inhibitors were added to tubes containing small or large intestinal mucin and McDougall's buffer to be subjected to a fermentation assay. A protease from bovine pancreas (CAS number: 9001-92-7), an endoglycosidase from *Elizabethkingia meningoseptica* (PNGase F; CAS number: 83534-39-8), a neuraminidase from *Clostridium perfringens* (CAS

number: 9001-67-6) and a lipase from *Candida antarctica* (CAS number: 9001-62-1) all were purchased from Sigma-Aldrich and tested at a concentration of one unit per milliliter of McDougall's buffer. Beta-galactosidase (CAS number: 9031-11-2; Sigma-Aldrich, St. Louis, MO, USA) and phenylethyl beta-D-thiogalactopyranoside (PETG; CAS number: P-1692; Invitrogen, Grand Island, NY) were inoculated at a concentration of 100 µM and 200 µM, respectively. Finally, bacterial protease inhibitor cocktail containing 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), EDTA disodium salt, bestatin, pepstatin A, and E-64 (P8465; Sigma-Aldrich, St. Louis, MO, USA) was added at two different concentrations, 0.25 and 2.5 mL/g of Nal^R *E. coli* O157:H7 culture.

Statistical analysis

Colony-forming unit (**CFU**) counts were transformed to the $\log_{10}$ scale. Fermentation assay statistical analysis was performed using the MIXED procedure of SAS (SAS 9.2, SAS Institute Inc., Cary, NC, USA). Sampling times, substrate types and presence or absence of fecal inoculum were included in the model as fixed effects. Effects of increasing level of small intestinal mucus on Nal^R *E. coli* O157:H7 and total anaerobes were analyzed using the MIXED procedure of SAS and linear contrast. Differences were considered significant at $P<0.01$ and $P<0.05$.

RESULTS

Mucus anatomical origin and composition

The analysis of the N and OM content of the mucus collected at the ileum and colon revealed slight differences in composition, with 1.068 µg of protein and 627 µg of OM per milligram of small intestinal mucus versus 0.910 µg of protein and 442.6 µg of OM per

milligram of large intestinal mucus. Figure 2-1 depicts the growth of Nal^R *E. coli* O157:H7 in the presence or absence of a fecal inoculum with small intestinal mucus, large intestinal mucus or no substrate overtime. It reveals no significant differences ($P>0.10$) in growth between the two mucus anatomical origins (ileum and colon). The bacteria increased from 10^3 CFU/mL of culture, at h 0, to 10^7–10^8 CFU/mL at h 8. An overall time effect was observed on the growth of the bacteria ($P<0.01$); however, there was no significant difference between h 8 and h 12, which drove us to use h 8 as a point of comparison. The presence of fecal inocula in the culture affected the growth of *E. coli* O157:H7 ($P<0.01$). The final concentration of bacteria decreased from 10^7 CFU/mL in tubes containing no fecal inoculum to 10^5 CFU/mL in tubes containing fecal inoculum.

Increasing concentrations of mucus

Growth of total anaerobic bacteria and Nal^R *E. coli* O157:H7 was tested with increasing levels of small intestinal mucus. Growth of total anaerobes was significantly influenced by the levels of substrate addition at h 6 and 12 (Table 2-1; SI concentration effect, $P<0.01$). Linear increase in anaerobe concentration was observed with increasing concentration of small intestinal mucus (linear effect, $P<0.01$). Cultures containing 15 mg of mucus OM/mL supported a 28% increase in anaerobe growth after 12 h incubation compared with culture receiving no mucus. Growth of Nal^R *E. coli* O157:H7 increased at every time point in response to increased concentrations of mucus (linear effect, $P<0.01$). Counts of Nal^R STEC counts were increased by 72% in the presence of 15 mg of mucus OM/mL of culture compared with tubes containing 0 mg of mucus/mL after 12 h of fermentation.

Mucus and mucus components

Figure 2-2a depicts the growth of *E. coli* O157:H7 after 8 h of anaerobic incubation with fecal inoculum and either whole mucus or selected components of mucus as substrates. With the notable exceptions of mannose and galactose, all substrates allowed the growth of *E. coli* O157:H7 in the presence of a fecal inoculum ($P<0.05$). Growth was numerically greatest with L-alpha-phostatidylserine and glucuronic acid as substrates but was not different from that obtained with whole mucus from the small or large intestines ($P>0.17$).

Figure 2-2b depicts growth of Nal[R] *E. coli* O157:H7 after 8 h of anaerobic incubation in the absence of fecal inoculum. With the exception of L-alpha-phosphatidylserine, all of the mucus components tested increased growth of the bacteria in comparison to the batch containing no substrate ($P<0.05$), but mucus originating from the large and small intestines supported greater growth than the individual mucus fractions ($P<0.05$). This is in contrast to results observed in the presence of fecal inoculum. Gluconic acid was the only single compound to yield growth similar to that obtained with whole large intestinal mucus ($P>0.09$) but still was less than that observed for whole small intestinal mucus ($P<0.0001$).

Figure 2-3 illustrates the growth of Nal[R] *E. coli* O157:H7 in response to small intestinal mucus or sialic acid substrates in the presence or absence of a fecal inoculum. Nal[R] *E. coli* O157:H7 growth was significantly lower in sialic acid than in mucus ($P<0.01$) in the absence of feces. When fecal inoculum was added to the culture with small intestinal mucus, there was a significant decrease in the growth of Nal[R] *E. coli* O157:H7 compared with the same culture without feces ($P<0.0001$). When fecal inoculum was added to the culture containing sialic acid, growth of Nal[R] *E. coli* O157:H7 increased compared with the same culture without feces

($P<0.0002$). The addition of neuraminidase to cultures containing sialic acid fermentation increased growth of the bacteria compared with tubes containing sialic acid with ($P<0.025$) or without feces ($P<0.0001$), but was still lower than in cultures containing small intestinal mucus as substrate ($P<0.0001$).

Enzymes and enzyme inhibitors

Figure 2-4 illustrates our attempt to evaluate the stimulatory effect of mucus-degrading enzymes on the growth of Nal[R] *E. coli* O157:H7. There was no effect of protease, endoglycosidase, lipase, beta-galactosidase, neuraminidase, or protease inhibitor addition to cultures (data not shown; $P>0.05$). Conversely, the addition of beta-galactosidase enzyme inhibitor increased the growth of Nal[R] *E. coli* O157:H7 cultured with either small or large intestinal mucus ($P<0.05$).

DISCUSSION

Previous studies have shown heterogeneity in mucus composition and thickness across the human and rat gastrointestinal tracts (Atuma *et al.*, 2001, Freitas *et al.*, 2002, Robbe *et al.*, 2004). Bovine mucus is believed to display a certain level of heterogeneity between the different sections of the intestine, and this diversity is considered partly responsible for the bacterial tropism (Robbe *et al.*, 2004, Snider *et al.*, 2009). Our analysis of the ileum and colon mucus composition did not reveal any major differences in N or OM content; moreover, Nal[R] *E. coli* O157:H7 was equally capable of growing on large and small intestinal mucus. These observations led us to postulate that heterogeneity in bovine mucus is either less important than in other species or that differences in composition do not appreciably influence bacterial growth.

The growth of Nal^R *E. coli* O157:H7 was highly modulated by the presence or absence of feces in the assay. In presence of fecal inoculum, Nal^R *E. coli* O157:H7 growth was reduced by at least 2 log units, which likely is due to competition for nutrients between fecal bacteria and our introduced strains of Nal^R *E. coli* O157:H7. These observations are consistent with the principle of competitive exclusion (Tkalcic *et al.*, 2003), by which the presence of other bacteria in the medium limits the substrate availability to the pathogen, thus reducing its growth.

Another interesting observation was the linear increase in Nal^R *E. coli* O157:H7 and total anaerobes with the increasing level of small intestinal mucus. Small intestinal mucus at 15 mg of OM/mL supported a 72% increase in growth of Nal^R *E. coli* O157:H7 after 12 h incubation, whereas the total anaerobe counts increased by only 28%. Our results suggested that intestinal mucus may preferentially stimulate Nal^R *E. coli* O157:H7 or that these bacteria were better equipped to compete with other anaerobes for utilization of whole mucus.

Nal^R *E. coli* O157:H7 was able to grow on all mucus components tested in this experiment, indicating that the bacteria were able to metabolize all these compounds. As seen in previous studies (Fox *et al.*, 2009), galactose, mannose, gluconic acid, glucuronic acid, and galacturonic acid yielded the greatest growth, with gluconic acid numerically superior. Despite the ability of the pathogen to grow on all substrates, whole mucus stimulated the greatest degree of growth; therefore, it is likely that a combination of substrates may more closely meet requirements of the pathogen or, alternatively, that a component of mucus not tested in this experiment is a key ingredient for optimal growth of Nal^R *E. coli* O157:H7. For example, an *E. coli* mutant deficient in the catabolic pathway for L-fucose demonstrated a marked decrease in colonization of rectal mucus (Snider *et al.*, 2009). Similar results were also observed in mice and *in vitro* with *E. coli* MG1655 (Chang *et al.*, 2004, Fabich *et al.*, 2008). Because fucose is only a

minor component of the calf ileal mucus (Montagne *et al.*, 2000), it was not evaluated in this study but could be a key constituent supporting optimal growth of Nal^R *E. coli* O157:H7.

Cultures of Nal^R *E. coli* O157:H7 alone with sialic acid as substrate resulted in modest bacterial growth, whereas in the presence of fecal inoculums, growth of the pathogen closely resembled that obtained with whole mucus. The *E. coli* strains used in our experiment appeared to have limited ability to use sialic acid but seem to be able to use intermediate products or metabolites synthesized by other fecal bacteria in the degradation of sialic acid. This observation could partly explain why feeding distiller's grains to feedlot cattle stimulates *E. coli* O157:H7 shedding (Jacob *et al.*, 2008, Jacob *et al.*, 2009). Distiller's grains are rich in yeasts that contain a substantial percentage of sialic acid, up to 3% of the dry weight of yeast (Malhotra and Singh, 2006). In such conditions, it would be conceivable that sialic acid is the active component that stimulates proliferation of the pathogen in cattle fed dried distiller's grains.

One of our initial hypotheses was that *E. coli* O157:H7 would have little ability to use whole mucus to support its growth because the organism is not known to produce endoglycosidase (Chang *et al.*, 2004) or neuraminidase (Hoskins *et al.*, 1985), but our results suggest otherwise. First, Nal^R *E. coli* O157:H7 grew best on whole mucus rather than individual components. Secondly, addition of protease, endoglycosidase, lipase or neuraminidase to the medium did not improve growth of the pathogen. Only beta-galactosidase inhibitor had an effect on the growth of the bacteria and, surprisingly, increased the Nal^R *E. coli* O157:H7. Presence of β-galactosidase activity is used in chromogenic medium as a means of distinguishing *E. coli* O157:H7 from other *E. coli* that are β-galactosidase-negative and glucuronidase-positive. Therefore, we were expecting the addition of beta-galactosidase inhibitor to decrease growth of the organism. It is possible that galactosides are more stimulatory to growth of Nal^R *E. coli*

O157:H7 than degradation products derived from galactosides, or that the inhibitor itself was used as a source of nutrients by the bacteria. The latter explanation seems unlikely based on the very small amount of inhibitor (200 μM) added in this assay. Additional experiments are needed to further investigate this effect.

In conclusion, this series of experiments provided information regarding metabolism of mucus and mucus components by Nal^R *E. coli* O157:H7. We were, however, unable to identify a single component as a key stimulator or inhibitor of growth of these bacteria. Further investigation of the relationship between *E. coli* O157:H7 or other STEC and the gastrointestinal colonization of cattle is necessary to develop innovative and efficient preharvest intervention measures.

ACKNOWLEDGEMENTS

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Figure 2-1 Growth of Nal^R *E. coli* O157:H7, with buffer (○), small (Δ) or large (□) intestinal mucus as a substrate in the presence (filled symbols) or absence of fecal inoculum (open symbols). SEM = 0.21, effect of mucus origin, P>0.01; effect of addition of fecal inoculum, P<0.01.
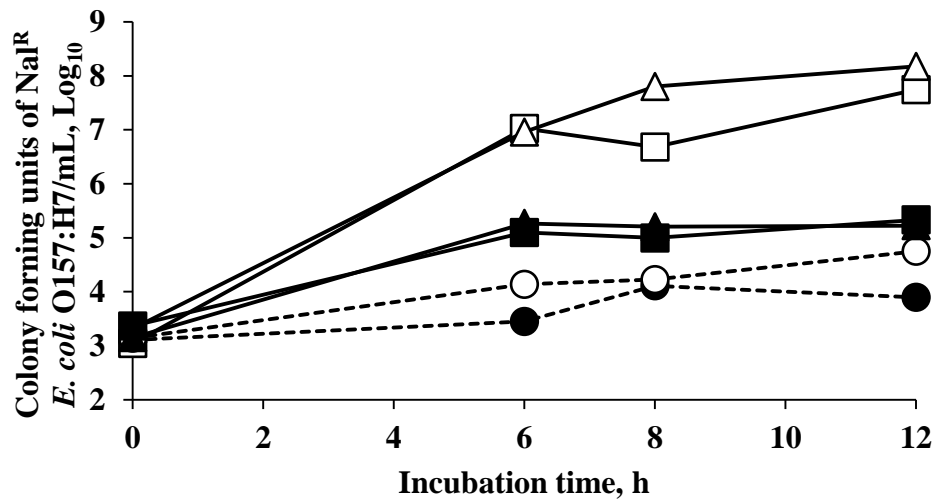
Table 2-1: Growth of Nal[R] *E. coli* O157:H7 and total anaerobes with increasing levels of small intestinal (SI) mucus over a 12-h fermentation at 40°C. SEM= 0.3.

| CFU/mL, Log$_{10}$ | Hour | SI mucus concentration, mg of OM/mL | | | | | | | P-value | |
		0	0.5	1	2	4.4	10	15	trt	linear
E. coli O157:H7	0	3.15	3.13	3.08	3.18	3.16	3.38	3.40	>0.05	>0.05
	6	4.08	4.29	4.86	4.66	5.01	4.90	5.74	<0.01	<0.01
	8	4.18	4.50	4.62	5.05	4.97	5.12	5.88	<0.01	<0.01
	12	4.64	4.73	4.64	4.90	5.06	5.15	5.84	<0.05	<0.01
Total anaerobe	0	6.82	6.82	6.82	6.82	6.82	6.82	6.82	>0.05	>0.05
	6	7.88	7.89	7.96	7.91	8.95	9.03	8.98	<0.01	<0.01
	8	8.57	8.57	8.13	8.45	8.56	8.73	8.86	>0.05	>0.05
	12	7.71	8.04	8.00	8.10	8.11	8.62	8.72	<0.01	<0.01

Figure 2-2: Growth of Nal^R *E. coli* O157:H7 after 8 h incubation at 40°C with mucus or single mucus components as a substrate for growth in the presence (a) or absence (b) of fecal inoculum. Means without common superscript are different, P<0.05.

a)
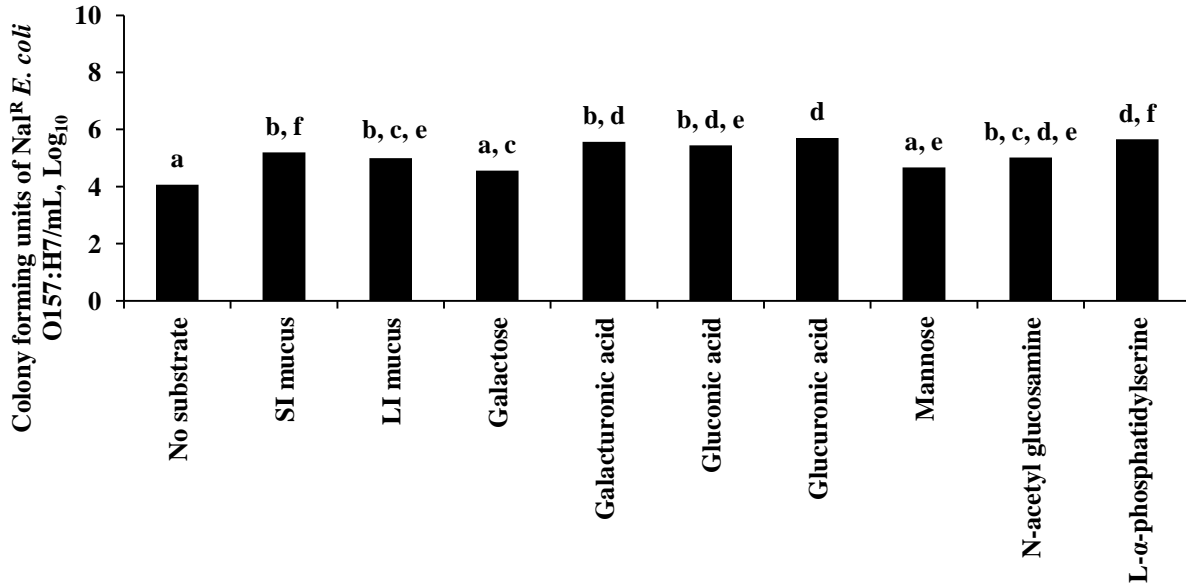
b)
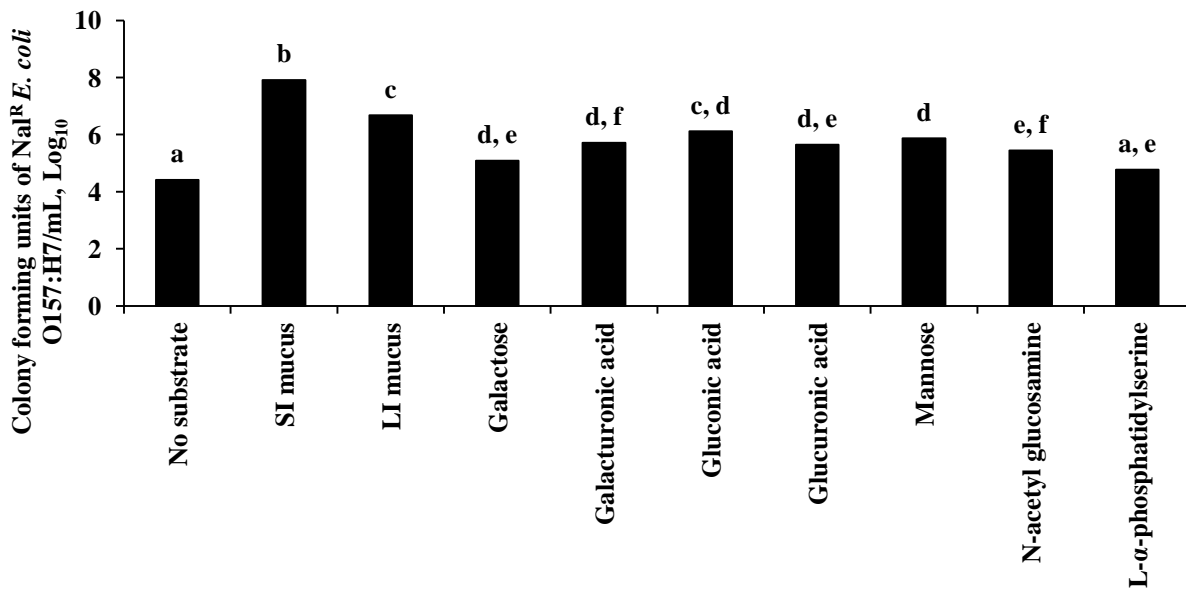
Figure 2-3: Growth of Nal[R] *E. coli* O157:H7 in response to small intestinal mucus (Δ) and sialic acid (○) in the presence (filled symbols) or absence of fecal inoculum (open symbols) and in response to sialic acid with neuraminidase (◊). Effect of time, P<0.01; effect of substrate, P<0.01; effect of addition of fecal inoculum, P<0.01; interaction between substrate and fecal inoculum, P<0.01.
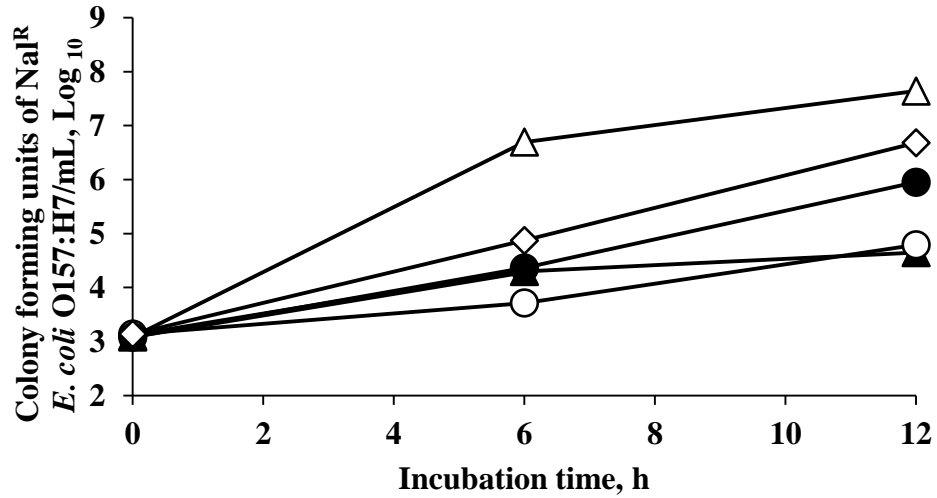
Figure 2-4: Growth of Nal[R] *E. coli* O157:H7 after 8h incubation at 40°C with no substrate, small (SI) or large (LI) intestinal mucus and in the presence or absence of beta-galactosidase inhibitor. Means without common superscript letters are different, P<0.05.
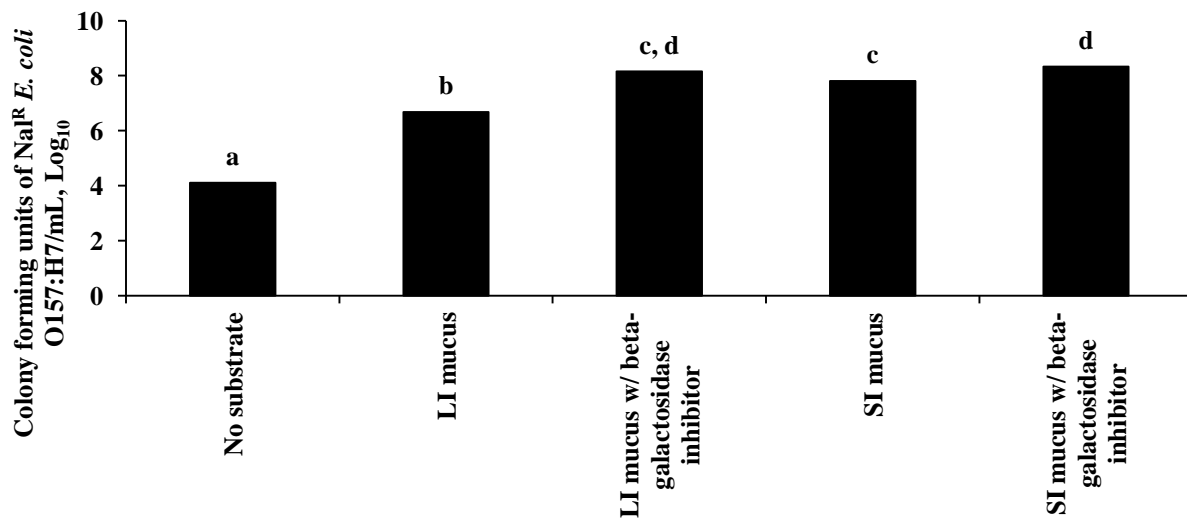
Chapter 3 Effect of crude glycerin on fecal shedding of *Escherichia coli* O157 in growing and finishing cattle.

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Running head: Glycerin impact on *E. coli* O157 fecal prevalence

ABSTRACT

Two experiments were conducted to evaluate the effects of crude glycerin feeding on *E. coli* O157 prevalence in feces of growing and finishing cattle. In study 1, crude glycerin was included at 0, 4 or 8% of dry matter in growing cattle diets comprised of dry-rolled corn, corn silage, alfalfa hay, and corn steep liquor. Heifers (n = 368; initial body weight = 234 ± 3.2 kg) were housed in partially covered, concrete-surfaced pens (36.5 m^2) with 7 to 8 animals per pen and 16 pens/treatment for the 90-day experiment. Study 2 was a finishing experiment with a 2 x 2 factorial treatment arrangement. Factor 1 consisted of the level of crude glycerin (0 or 2% of dry matter) and factor 2 was the presence or absence of a combination of soybean hulls and distiller's grains as grain substitutes. Heifers (n = 232; initial body weight = 427 ± 8.8 kg) were fed in pens containing 7 to 8 heifers each, with 8 replicates per treatment. Fresh fecal samples were taken from growing and finishing animals via rectal palpation. One gram of feces was incubated for 6 h at 40ºC in Gram negative broth with cefixime (0.05 mg/L), cefsulodin (10 mg/L), and vancomycin (8 mg/L) and then subjected to immunomagnetic separation (IMS) with *E. coli* O157 beads. Recovered beads were plated onto MacConkey agar with sorbitol, cefixime, and tellurite (CT-SMAC), and non-sorbitol fermenting colonies were tested for indole production and O157 antigen agglutination. Positive colonies for both tests were confirmed as *E. coli* O157 using the API 20E kit. Treatment effects and interactions were analyzed using Proc Glimmix of SAS. Fecal incidence rates of *E. coli* O157 were affected by sampling day in the growing study ($P<0.01$) but not in the finishing study ($P>0.1$). Increasing levels of crude glycerin decreased incidence of *E. coli* O157 in growing cattle (linear effect, $P<0.01$; 4.4, 3.2, and 1.8% for heifers fed 0, 4, and 8% glycerin, respectively) but not in finishing cattle ($P>0.05$) despite a strong tendency for a decrease in finishing cattle receiving corn-based diets (P=0.0597). There was no

interaction between WDGS inclusion and glycerin level ($P>0.1$) and no WDGS effect on prevalence of *E. coli* O157 in finishing cattle ($P>0.1$). Glycerin may be useful as a means for decreasing fecal prevalence of *E. coli* O157 in cattle, but effects may depend on the type of diet that is fed.

Keywords: Glycerin, *Escherichia coli* O157, distiller's grains

INTRODUCTION:

Expansion of biofuels production has diverted the use of corn, sorghum, wheat, and soybeans from livestock feed to energy feedstocks. Utilization of cereal grains and oilseeds for production of renewable fuels yields large quantities of co-products that can be used as animal feeds. Ethanol production from cereal grains generates distiller's grains, as a byproduct, which is merchandised in wet (WDGS) or dried (DDGS) forms. These byproducts commonly constitute 10 to 50% of cattle diets in the United States whilst maintaining acceptable animal performance and meat quality (Jacob *et al.*, 2010). Feeding distiller's grains has, however, been shown to increase prevalence of pathogenic bacteria, such as *E. coli* O157:H7, in cattle feces (Wells *et al.*, 2009, Jacob *et al.*, 2010, Wells *et al.*, 2011) and persistence in manure (Varel *et al.*, 2008, Varel *et al.*, 2010). This can increase the risk of hide contamination with pathogens (Wells et al., 2009), as well as indirect contamination of crops by fertilization with manure or though exposure to water runoff from confined animal feeding operations. The mechanism by which pathogen shedding increases in cattle fed distiller's grains remains unclear. It has been hypothesized that changes in the hindgut environment creates a more favorable environment for the pathogen (Berg *et al.*, 2004, Fox *et al.*, 2007, Klopfenstein *et al.*, 2008). Others suggested the presence of a compound in distiller's grain responsible for creating a selective niche for *E. coli* O157 (Dewell *et al.*, 2005, Jacob *et al.*, 2008, Viazis and Diez-Gonzalez, 2011). Analysis of distiller's grains has revealed the presence of 3.5 to 10% glycerin, which is formed by yeast during fermentation of carbohydrates (Kingsly et al., 2010; Wu, 1994). Crude glycerin also is a byproduct of the biodiesel industry and frequently is fed to swine, poultry, and ruminants as an alternative source of energy (Hampy *et al.*, 2008, Parsons *et al.*, 2009, Gunn *et al.*, 2010). Glycerin also has been shown to influence microbial populations in ruminants (Roger *et al.*, 1992, Paggi *et al.*, 2004,

AbuGhazaleh *et al.*, 2011). Based on these observations we chose to focus our work on the impact of glycerin on the prevalence of *E. coli* O157 in feces of feedlot cattle. Our objectives were to investigate the effects of glycerin on pathogen prevalence in cattle feces, and to determine if the impact of glycerin feeding would be influenced by feeding distiller's grains. For this purpose, we conducted two experiments: one in growing cattle fed diets containing 0, 4, or 8 % of glycerin, and another in finishing cattle fed diets with and without added distiller's grains and with 0 or 2% crude glycerin.

MATERIALS AND METHODS

Animals and Experimental designs

Study 1. Crossbred heifers (n=368; initial body weight 234 ± 3.2 kg) were randomly assigned to growing diets containing dry-rolled corn, corn silage, alfalfa hay, corn steep liquor and one of three levels of crude glycerin: 0, 4 or 8% of diet dry matter (Table 3-1). Diets were fed once daily *ad libitum*. Each treatment was represented by 16 pens (36.5 m^2) of cattle, each containing 7 to 8 animals. Approximately half of each pen, including the feed bunk, was covered by a corrugated steel roof. Pens were equipped with fenceline feed bunks (3.65 linear m), and a fenceline water fountain was shared between two adjacent pens. Fecal samples were obtained from individual animals in each pen by rectal palpation once each week for 6 weeks on days 55, 62, 69, 76, 83, and 90.

Study 2. Crossbred heifers (n=232; initial body weight 427 ± 8.8 kg) were stratified by weight and randomly assigned (within strata) to the previously described pens, using a total of 32 pens containing 7 to 8 animals each. The study utilized a 2 x 2 factorial treatment arrangement, with factor 1 consisting of the level of crude glycerin added to the diet (0 or 2%, dry matter basis),

and factor 2 consisting of type of basal diet (corn or corn plus soybean hulls and WDGS; Table 3-2). Pens within block were randomly assigned to treatment, thus providing 8 replicates per treatment. Cattle were fed once daily *ad libitum*. Fecal samples were collected on days 160, 164, 168, 172, 176, 180 and 184 after beginning of the treatment. On each sampling day, fresh fecal pats were obtained from 5 randomly selected heifers in each pen.

Crude glycerin analysis

Crude glycerin used in the diets was analyzed by a commercial laboratory and contained 14.3% moisture, 6.68% ash, 2.58% Na, 0.04% N, and less than 0.01% methanol. Composite samples of wet distiller's grains and corn silage also were analyzed and contained 7.2% and less than 0.1% of glycerol on a DM basis, respectively.

Escherichia coli O157 isolation

Samples were placed into plastic bags and stored on ice immediately after sampling, and rapidly transported 3 km to the Preharvest Food Safety Laboratory for analysis. Feces (1 g) were weighed and transferred to 9-mL of Gram negative broth (Difco, Franklin Lakes, NJ) containing 0.05 mg/L cefixime, 10 mg/L cefsulodin, and 8 mg/L vancomycin (GNccv) for a 6-hour incubation at 40°C. After incubation, samples from study 1 were thoroughly vortexed, and 1 mL of the inoculated GNccv broth was added to a sterile tube containing *E. coli* O157 specific Dynabeads (Invitrogen Dynal AS, Oslo, Norway) and subjected to immunomagnetic separation. GNccv tubes from study 2 were pooled by pen after incubation and subjected to immunomagnetic separation using a Pathatrix device (Matrix Microscience, Life Technologies, Grand Island, NY). *Escherichia coli* O157 beads resulting from both immunomagnetic separations were resuspended in 100 µL of phosphate buffer and plated onto two MacConkey

sorbitol plates (CT-SMAC) containing cefixime (0.05 mg/L) and potassium tellurite (2.5 mg/L). Up to 6 non-sorbitol fermenting colonies were selected from the CT-SMAC plate and inoculated into 5 mL Tryptic soy broth (TSB). Colonies were grown overnight at 37°C and tested for indole production. Indole-positive colonies were plated onto SMAC and further tested for O157 antigen agglutination (Oxoid, Hampshire, United Kingdom). Colonies positive for indole production and antigen agglutination were confirmed as *E. coli* O157 by Gram staining and API 20E (Biomerieux, Durham, NC).

Statistical analysis

Effect of glycerin inclusion on prevalence of *E. coli* O157 in study 1 was analyzed as binary data using Proc Glimmix of SAS (SAS Inst. Inc., Cary, NC). Levels of glycerin and sampling day were included in the model as fixed effects, while animal and pen were random effects. Linear and quadratic contrasts were analyzed for the different levels of glycerin. In study 2, effect of sampling day, glycerin, WDGS and the combination of glycerin and WDGS inclusion on prevalence of *E. coli* O157 were analyzed using Proc Glimmix. Glycerin levels, byproduct inclusion and sampling day were the fixed effects and weight strata the blocking factor.

RESULTS

Study 1.

There were no interactions between sampling day and glycerin levels ($P>0.1$), therefore only main effects are presented. Prevalence of *E. coli* O157 in fecal samples was influenced by sampling day ($P<0.01$). Figure 3-1 represents shedding of the pathogen over the experimental

period. Pathogen prevalence was low for the first two weeks, 1.3 and 0.8%, respectively. Prevalence then increased to 4.0% in the third week ($P<0.021$), peaking at 8.4% during the fourth week ($P<0.014$), and then going back down to 4.0% on the fifth week ($P<0.014$) to stabilize at 5.0% during the final week of sampling ($P=0.07$). Figure 3-2 illustrates the effect of glycerin on *E. coli* O157 prevalence. Fecal incidence rates of *E. coli* O157 were 4.4, 3.2, and 1.8% for heifers fed 0, 4, and 8% glycerin, respectively (linear effect of glycerin, $P<0.01$). The prevalence observed in heifers fed 8% glycerin was less than that of cattle fed 0% glycerin ($P<0.05$), while prevalence in cattle fed 4% glycerin was not different from other treatments ($P>0.18$).

Study 2.

In this experiment there was no overall sampling day effect ($P>0.1$). *E. coli* O157 fecal shedding, illustrated in Figure 3-3, was highest at the first sampling (23%), d 160, but rapidly decreased to 8.2% on day 164 and 168 ($P>0.1$). Prevalence reached its lowest point on day 172 (2.7%), which was significantly different from day 160 ($P<0.04$). On day 176, *E. coli* O157 shedding again increased to 16.9%, which tended to be different from the previous sampling date ($P<0.08$). For the last two sampling days, pathogen prevalence again declined, which likely is due to seasonality of prevalence. Presence of a high number of *E. coli* O157 negative samples in this experiment did not allow us to statistically characterize the sampling day by glycerin level by WDGS inclusion interactions. Analysis of the combination of glycerin inclusion and presence of WDGS in the diet was, however, possible and revealed the absence of an interaction ($P>0.1$). WDGS inclusion alone (Figure 3-4) had no effect of WDGS on prevalence of *E. coli* O157 ($P>0.1$). Heifers fed corn-based diets or diets containing corn with soybean hulls and WDGS but

without glycerin presented 11.64% *E. coli* O157 positive isolates over the course of the experiment. Heifers fed corn diets with 2% glycerin had a 2.7% pathogen prevalence and animals fed corn with soybean hulls and WDGS diets with 2% glycerin had a 10% prevalence; but the difference between these two treatments only tended to be significant (P=0.0957). There was a tendency for glycerin inclusion in the diet to decrease pathogen prevalence (P=0.0871), and pathogen prevalence, also, tended to differ between animals fed corn diets with and without glycerin (P=0.0597), with 2.7% and 11.6% prevalence, respectively. In diets containing soybean hulls and WDGS the difference in prevalence with the addition of 2% glycerin was not significant (10 vs. 11.6%, respectively; P>0.10).

DISCUSSION

Increases in prevalence of *E. coli* O157 have frequently been associated with addition of distiller's grains, wet or dried, in feedlot diets (Dewell *et al.*, 2005, Varel *et al.*, 2008, Jacob *et al.*, 2010). Nevertheless, explanations for this interaction are still unclear. It was first thought that replacement of part of the corn in the diet by DDGS or WDGS modified the amount of starch reaching the hindgut (Jacob et al., 2010). Such modification would, in turn, increase pH, potentially creating an environment that is more hospitable toward pathogen growth and survival (Berg *et al.*, 2004, Fox *et al.*, 2007). *In vitro* and *in vivo* studies have failed to establish a clear relationship among pH, diets, and *E. coli* O157 prevalence (Depenbusch *et al.*, 2009, Wells *et al.*, 2009, Yang *et al.*, 2010). Similarly, it has been hypothesized that differences in concentrations of VFA and branched amino acids might explain changes in pathogen prevalence. Distiller's byproducts contain much higher levels of protein compared to corn (Klopfenstein *et al.*, 2008), and only a portion of this protein is degraded in the rumen. Consequently, greater

supplies of protein reach the hindgut, potentially yielding increased levels of VFA and branched amino acids that could influence pathogen survival. Definitive evidence supporting this hypothesis is, however, lacking (Huntington et al., 2006). Concentration of L-lactate has also been proposed as a contributing factor, recognizing that L-lactate has antimicrobial properties (McWilliam Leitch and Stewart, 2002) and its rarefaction could benefit pathogen persistence in manure. Inclusion of WDGS in cattle diets has been shown to decrease significantly L-lactate concentration in feces compared to cattle fed corn (Varel *et al.*, 2008, Wells *et al.*, 2009); however, these concentrations, 3.72 mM on average, are far below the 50 to 200 mM concentrations that have been identified as having inhibitory effects, and cannot solely explain increased prevalence of *E. coli* O157 in feces. Other authors (Dewell *et al.*, 2005, Jacob *et al.*, 2008) suggested the presence of a compound in distiller's grains that would benefit, directly or indirectly, the pathogen. Previous publications have reported distiller's grains to contain between 3.5 and 10% glycerin on DM basis (Wu, 1994, Kingsly *et al.*, 2010). Glycerin is formed during yeast fermentation in the early stage of ethanol production. Its concentration in the final byproduct depends on ethanol process, grain type, yeast type, and treatment of distiller's grain used. In our experiment WDGS consumed by finishing heifers contained 7.2% glycerol, thus contributing 1% glycerol to the total diet. Feedlot diets with higher inclusion levels of byproducts could contain significant amount of glycerin. We hypothesized that glycerin would influence shedding of *E. coli* O157 in feces of cattle fed distiller's grains. Based on present results, inclusion of glycerin, in presence or absence of byproduct in the diet, did not increase pathogen prevalence. Addition of glycerin to growing diets significantly decreased the *E. coli* O157 shedding, and numerically decreased it in grain-based finishing diets. Based on these findings, glycerin could be a potential way to mitigate pathogen prevalence. Explanation of the

mechanism behind *E. coli* O157 prevalence reduction is, however, not yet understood. Glycerin is known to reduce cellulolytic activity in the rumen (Roger *et al.*, 1992, Paggi *et al.*, 2004). One percent excess glycerol inhibited growth and activity of *Ruminococcus flavefaciens* and *Fibrobacter succinogens* in an *in vitro* assay (Roger *et al.*, 1992). Likewise, replacing 30% of the corn by glycerin, in a continuous fermenter, reduced DNA concentrations of *Butyrivibrio fibrisolvens* by preventing its attachment to fiber (AbuGhazaleh *et al.*, 2011). Feeding studies corroborate with these observations, as apparent digestibility of neutral detergent fiber (Donkin *et al.*, 2009, Parsons and Drouillard, 2010) was decreased by increasing amount of glycerin incorporated into cattle diets. In addition, glycerol was found to decrease proteolytic activity by 20% when added to culture media at concentration of 50 mM or more (Paggi *et al.*, 1999). Bacterial protein synthesis was decreased by infusing glycerol into the rumen of bulls (Kijora *et al.*, 1998). Overall, effects of glycerin on fiber and protein digestion within the rumen could be responsible for eliciting changes in nutrients flowing to the hindgut of animals, thus creating conditions that are less favorable for the pathogen. It could, as well, alter conditions within the gut to increase populations that effectively compete with *E. coli* O157. Many microorganisms are able to use glycerol as a substrate, but only few of them can do so under anaerobic conditions. In the rumen, *Selenomonas* is one of the key glycerol fermenters, producing propionate, lactate, succinate and acetate (Krehbiel, 2008). Increase of glycerol supply to the rumen could potentially benefit *Selenomonas* and have a negative effect on *Escherichia coli*. Additionally, decline in rumen pH observed *in vivo* and *in vitro* with increasing levels of glycerin (Kijora *et al.*, 1998, Lee *et al.*, 2011) could be another factor explaining the reduction in *E. coli* O157 shedding. Effects of glycerin on pH have not, however, been consistent, and some experiments have failed to illustrate an effect of glycerol on pH (Rico *et al.*, 2012) or, in

contrary, observed an increase in pH (Parsons and Drouillard, 2010). Discrepancies in results may be explained in part by differences in basal diets or glycerol levels used among studies. Therefore, the relationship between *E. coli* O157 prevalence reduction by glycerin and pH is not definite and would need more attention.

Despite the mechanisms involved in reduction of pathogen shedding, addition of glycerin efficiently reduced percentage of feces positive for *E. coli* O157 in animals fed the grain-based diet. We speculate that numerical differences observed in *E. coli* O157 prevalence in grain-based diets with and without glycerin might have been significant with higher rates of inclusion, as a linear effect of glycerin was observed in our first study. The absence of an effect of glycerin in cattle fed diets containing byproducts tempers the potential to use glycerin as a mitigating agent. Comparison of 2% glycerin grain-based diet and the 2% glycerin byproduct-based diet underscore potentially important differences in fiber content, 11.8 and 28.8% respectively, and fat content, 2.5 and 6.6%, respectively. It is conceivable that glycerin effects are different for microflora from an animal fed a typical corn-based diet compared to cattle fed diets with greater concentrations of fiber and fat. Finally, in our finishing experiment, we did not observe any difference in shedding of *E. coli* O157 in animal fed 0 or 15% WDGS with soybean hulls, which contradicts previous findings (Dewell *et al.*, 2005, Varel *et al.*, 2008, Jacob *et al.*, 2010). Even if the large majority of the studies did show a relationship between distiller's grains inclusion and pathogen prevalence, few others did not observed any effect of the byproduct inclusion on *E. coli* O157:H7 fecal prevalence (Jacob *et al.*, 2009, Klopfenstein *et al.*, 2009) which might be explained by the variability in nutrients content between various distiller's grains (Kleinschmit *et al.*, 2007). Glycerol content of the byproduct used in previous studies have rarely been reported, it is possible that the presence of 1.09% of glycerin in the initial byproduct-based diet used in

this experiment might have affected the prevalence of *E. coli* O157, inhibiting its increase. The subsequent addition of 2% glycerin in the diet may not have been sufficient to lead to a significant difference in *E. coli* O157 prevalence, but did induce a numerical decrease.

Taken together that glycerin is not the compound responsible for increased prevalence of *E. coli* O157 in animal fed distiller's grains and that WDGS did not have an effect on pathogen prevalence, it is probable that the compound allegedly benefiting *E. coli* O157 was absent or in too low concentration in the WDGS used in this study.

In conclusion, glycerin inclusion ranging from 2 to 8% decreased the shedding of *E. coli* O157 in feces of growing heifers, and tended to do so in finishing heifers. Based on our results, we can conclude that glycerin is unlikely to be the component of distiller's grains responsible for greater shedding of *E. coli* O157 in feedlot cattle. Glycerin could indeed be a means of mitigating prevalence of the pathogen in feces, though its utility for this purpose may be influenced by composition of the basal diet, and explanations for this difference are still lacking. Given the increasing availability of glycerin from biodiesel production and the need for preharvest intervention to control pathogen shedding in feedlot cattle, glycerin may prove useful as a potential candidate to alleviate pathogen shedding in cattle fed grain-based diets.

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Table 3-1: Composition of growing experimental diets (dry basis).

Ingredients, %	0% Glycerin	4% Glycerin	8% Glycerin
Corn silage	62.50	62.50	62.50
Wet corn gluten feed	32.90	28.30	23.90
Crude glycerin[1]	-	3.70	7.50
Soybean meal	-	1.60	3.10
Limestone	0.50	0.50	0.50
Urea	0.70	0.90	0.90
Vitamin/mineral premix[2]	1.60	1.40	-
Feed additive premix[3]	2.50	2.50	2.50
Nutrient Composition, %			
Dry matter	43.30	43.70	44.20
Crude protein	12.80	12.50	11.90
Neutral detergent fiber	36.20	34.60	33.00
Crude fat	2.57	2.45	2.34
Calcium	1.00	1.10	1.10
Phosphorus	0.66	0.61	0.56
Glycerol	0.06	3.41	6.86

[1]Contained 14.3% moisture, 6.68% ash, 2.58% Na, 0.04% N, and less than 0.01% methanol.

[2]Formulated to provide 0.1 mg Co, 10 mg of Cu, 0.6 mg of I, 60 mg of Mn, 0.25 mg Se, 60 mg Zn, and 2640 IU vitamin A per kg diet DM.

[3]Provided 300 mg of Rumensin (Elanco Animal Health, Greenfield, In) per animal daily in a ground corn carrier.

Table 3-2: Composition of finishing grain-based diets and byproduct-based diets with or without 2% crude glycerin (dry basis).

	Grain-based Diets		Byproduct-based Diets	
Ingredients, %	0% Glycerin	2% Glycerin	0% Glycerin	2% Glycerin
Corn	80.60	78.20	46.60	44.20
Soybean hulls	-	-	25.00	25.00
Wet distiller's grains	-	-	15.00	15.00
Corn silage	6.00	6.00	6.00	6.00
Soybean meal	4.40	4.80	-	0.40
Alfalfa hay	3.00	3.00	3.00	3.00
Crude glycerin[1]	-	2.00	-	2.00
Limestone	1.70	1.70	1.40	1.40
Urea	1.20	1.20	0.40	0.40
Vitamin/mineral premix[2]	0.90	0.90	0.40	0.40
Feed additive premix[3]	2.20	2.20	2.20	2.20
Nutrient Composition, %				
Dry matter	76.10	76.30	64.60	64.70
Crude protein	14.80	14.80	14.70	14.70
Neutral detergent fiber[4]	11.90	11.80	28.90	28.80
Crude fat[5]	2.50	2.50	6.60	6.60
Calcium	0.70	0.70	0.90	0.90
Phosphorus	0.30	0.30	0.30	0.30
Glycerol	0.01	1.82	1.09	2.90

[1]Contained 14.3% moisture, 6.68% ash, 2.58% Na, 0.04% N, and less than 0.01% methanol

[2]Formulated to provide 0.1 mg Co, 10 mg of Cu, 0.6 mg of I, 60 mg of Mn, 0.25 mg Se, 60 mg Zn, 2640 IU vitamin A, and 11 IU vitamin E per kg diet DM.

[3]Feed additive premix provided 300 mg of monensin (Elanco Animal Health, Greenfield, IN), 90 mg tylosin (Elanco), and 0.5 mg of melengestrol acetate (Pfizer Animal Health, Exton, PA) per animal daily in a ground corn carrier. Zilpaterol HCl (Intervet Inc., Millsboro, DE) was fed for 21 d before harvest at the rate of 8.33 mg/kg of diet DM, followed by a 3-d withdrawal period.

[4]NRC (2000) feed library NDF values for soybean meal were used in calculation of NDF content.

[5]NRC (2000) feed library fat values for soybean meal and soybean hulls were used in calculation of crude fat content.

Figure 3-1: *E. coli* O157 prevalence in feces of growing cattle at each sampling days. Letters on top of the bars represent the comparison between sampling days. Bars with different superscript are significantly different (*P*<0.05). Sampling day effect *P*<0.001. SEM = 0.01
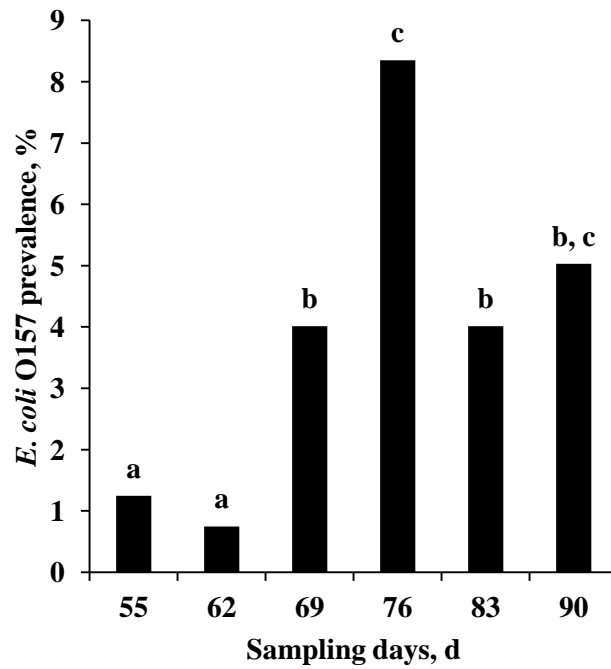
Figure 3-2: *E. coli* **O157 prevalence in feces of cattle fed growing diets containing 0, 4, and 8% crude glycerin. Letters above bars represent the comparison between treatments. Bars with different superscripts are significantly different (*P*<0.01). Treatment effect *P*=0.0052. linear effect of treatment *P*=0.0012. SEM = 0.008**
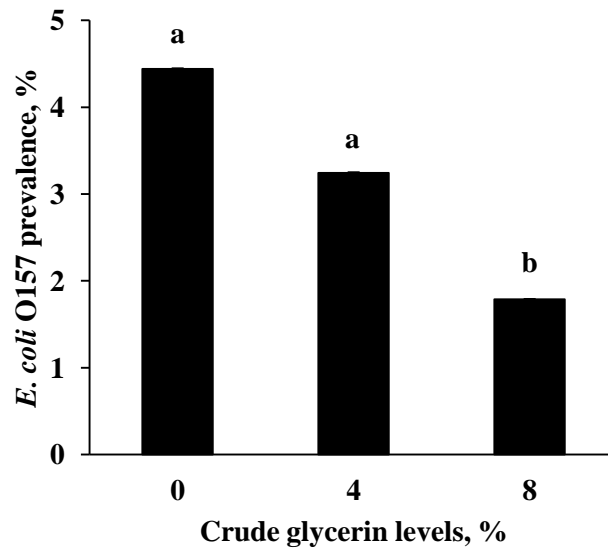
Figure 3-3: *E. coli* O157 prevalence in feces of finishing cattle at each sampling day. Letters above bars represent comparisons among means between sampling days. Bars with different superscript are significantly different (*P*<0.05). Sampling day effect *P*>0.1. SEM = 0.08.
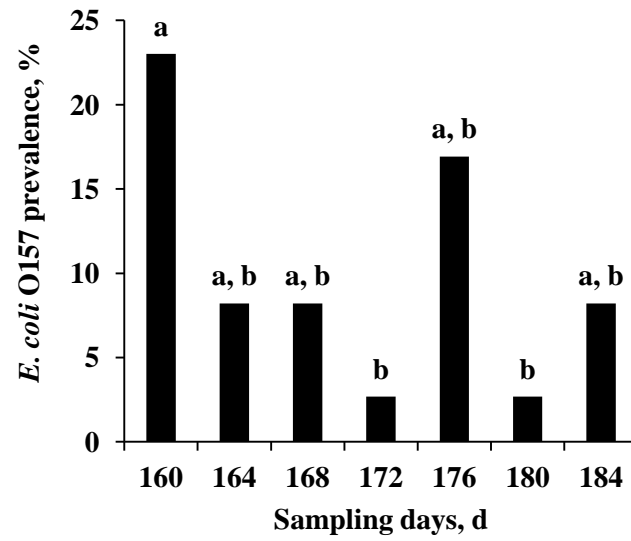
Figure 3-4: *E. coli* **O157 prevalence in feces of cattle fed a finishing diets based on grain or byproduct with or without 2% glycerin over the 24-day period. Letters above bars represent the comparison between treatments. Bars with different superscripts are significantly different (*P*<0.05). SEM = 0.041.**
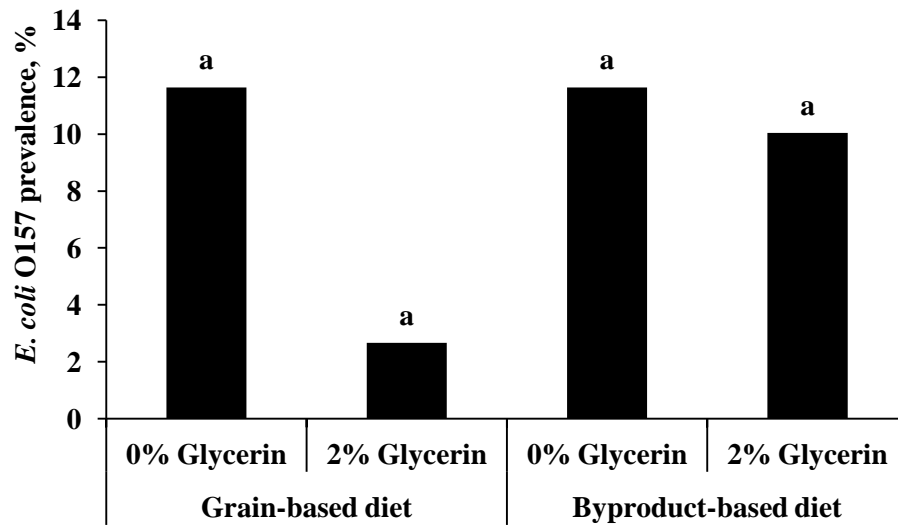
Chapter 4 Transit effects on fecal *Escherichia coli* O157 prevalence and coliform concentrations in feedlot cattle

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ABSTRACT

Our objective was to evaluate the effects of transportation and lairage on fecal shedding of *E. coli* in feedlot cattle. The study was a randomized complete block design with 20 steers per block (10/treatment), 3 blocks (replications), and 2 treatments: a non-transported group and transported steers that were placed into a trailer, hauled for 1 h, and subsequently allowed to rest. Fecal samples were taken pre-transport and after 4 and 28 h lairage. Fecal samples were collected from both groups at h 0, 5, and 29 from freshly voided fecal pats. One gram of feces was transferred to a phosphate buffer saline (PBS) tube, serially diluted, and plated onto Petrifilm for enumeration of total coliforms. Another sample (1 g) was added to gram-negative broth containing cefixime, cefsulodin, and vancomycin, and subjected to immunomagnetic separation. Resulting beads were plated onto MacConkey agar with sorbitol, cefixime, and tellurite. Non-sorbitol fermenting colonies were selected and tested for indole production and O157 antigen agglutination. Results were confirmed using an API 20E kit. Prevalence of *E. coli* O157 was transient across replications. *E. coli* O157 occurrence in the transported group was relatively constant across the three sampling times (10, 3.3, and 16.7%, for h0, 5, and 29, respectively; $P > 0.37$). Prevalence increased numerically in the control group at h 5 (33%) compared with h 0 (17%; $P = 0.37$) and 29 (13%; $P < 0.28$) but was not significant. Numbers of coliforms remained fairly constant across replication. Prevalence of *E. coli* O157 was not correlated with concentration of coliforms ($P > 0.35$). Coliform concentrations in control steers remained stable from 0 to 28 h post-transit. Transported animals had lower coliform concentrations at h5 (3.2 log cfu/gram; $P < 0.001$), but returned to pre-transport levels of 4.5 log cfu at h 29 ($P > 0.9$). Results suggest that shedding can vary greatly within a period of 29 h. Additional post-transit sampling times are needed to be able to conclude the effects of transport

stress on *E. coli* O157 prevalence and the changes undergone in pathogen shedding patterns after transportation.

Keywords: *E. coli* O157, transport

INTRODUCTION

Foodborne illness from *Escherichia coli* O157:H7 is a major concern for the food industry. Contamination of food products can occur at slaughter by contact with hide or feces; therefore, limiting *E. coli* O157:H7 shedding is important to prevent outbreaks. Previous studies have demonstrated a relationship between stress and levels of pathogens shed in feces (Freestone and Lyte, 2010). Transport to the abattoir represents a significant stressor for cattle (Grandin, 1997). When subjected to stress, cattle develop a stress response through the sympathetic adrenal medullary axis and the hypothalamic pituitary adrenal axis, releasing epinephrine, norepinephrine, and cortisol (Matteri et al., 2000) into the gastrointestinal tract and into general circulation.

Bacteria use hormone-like compounds, or autoinducers (AI; Hughes and Sperandio, 2008) to communicate with each other. This phenomenon is known as quorum sensing. Binding of AI to adrenergic receptors on the membrane of bacteria (Chen et al., 2006; Kendall and Sperandio, 2007) triggers a cascade of phosphorylation that activates the locus of enterocyte effacement (LEE) genes, flagella regulon (*Flh*DC) motility genes, and Shiga toxin genes (Hughes and Sperandio, 2008).

Recent studies have shown that communication through this system is not limited to signaling molecules produced by prokaryotic organisms. Eukaryote signaling molecules, norepinephrine and epinephrine, are able to substitute autoinducer 3 (AI-3; Clarke and Sperandio, 2005) and increase *E. coli* O157:H7 motility, adherence, and virulence. Furthermore, norepinephrine releases iron sequestered by lactoferrin and transferrin in the lumen of the gut, thus making iron available for bacterial growth (Freestone and Lyte, 2008; Freestone et al., 2007).

Based on these mechanisms, we hypothesized that transported animals will be at greater risk for *E. coli* O157 colonization than non-transported animals, and that the increase in pathogenic bacteria could also induce a shift in concentrations of total *E. coli* and coliforms.

MATERIALS AND METHODS

Study design

The study was a randomized complete block design with a split-plot design. There were 20 steers per block, 3 blocks (replications), and 2 treatments (10 steers/treatment): a non-transported group and a transported group. Transported animals were placed into a trailer, transported for 1 h, and, upon return to the research facility, unloaded and placed into concrete-surfaced pens (36 m^2) with an overhead shade covering approximately 50% of the pen and ad libitum access to water, thus mimicking pre-slaughter lairage. Neighbor pens were empty to prevent contact with other animals. Non-transported steers remained in their pens at all times throughout the experiment. The experiment was repeated 3 times (blocking factor) on consecutive days in August 2011. The summer time frame was chosen to increase the likelihood of high *E. coli* O157 prevalence in the cattle. Steers were randomly allocated to treatment. A new set of 10 steers was used for both groups for each replication to avoid the potential for adaptation to transport stress.

Animals

Crossbred steers (527 ± 110 kg initial BW) were used in this experiment. Animals were present at the feedlot prior to the experiment and were accustomed to their housing arrangements and penmates. Steers were fed a finishing diet based on dry-rolled corn, corn silage, and steep corn liquor (Table 4-1) once a day at 0830 h and had ad libitum access to water in their

respective pens. Steers from the transported group were loaded into a trailer at 0800 h, before feeding, on experiment day and were left to rest in a different pen. These steers only had access to food the next day at 0830 h.

Collection of fecal samples

Fecal samples were taken pre-transport (h 0), and at 4 and 28 h post-transport (h 5 and h 29) from freshly voided fecal pats (Figure 4-1). Samples were placed into plastic bags and kept on ice until they were transported to the Preharvest Food Safety Laboratory at Kansas State University.

Total Escherichia coli and coliform counts. One gram of feces was transferred to a tube containing PBS. The PBS tube was serially diluted, plated on Petrifilm, and incubated for 24 h at 37 °C for enumeration of total coliforms and *E. coli*. Most *E. coli* bacteria produce glucuronidase and will appear as blue colonies on Petrifilm, whereas the other coliforms will appear red. *Escherichia coli* O157 does not produce beta-glucuronidase, and thus is enumerated along with other coliforms.

Escherichia coli **O157 isolation**

One gram of feces was transferred to 9 mL Gram-negative broth (Difco-BD, Franklin Lakes, NJ) with 0.05 mg/L cefixime, 10 mg/L cefsulodin, and 8 mg/L vancomycin (GNccv). The GNccv tubes were incubated at 40 °C for 6 h. After incubation, tubes were subjected to immunomagnetic separation using serotype-specific beads for *E. coli* O157 (Invitrogen Dynal AS, Oslo, Norway). Beads were resuspended in 200 μL of phosphate buffer and plated onto two MacConkey sorbitol plates (CT-SMAC) containing cefixime (0.05 mg/L) and potassium tellurite (2.5 mg/L). Up to 6 non-sorbitol fermenting colonies from the CT-SMAC plate were selected

and inoculated into 5 mL Tryptic soy broth (TSB). Colonies were grown overnight at 37 °C and tested for indole production. Indole-positive colonies were plated onto SMAC and further tested for O157 antigen agglutination. Colonies positive for indole production and antigen agglutination were confirmed as *E. coli* O157 by Gram staining and API 20E (Biomerieux, Durham, NC).

Statistical analyses

In this experiment, the experimental unit was a group of 10 steers, and individual steers were considered a subsample. Replication was the blocking factor. Sampling times and treatments were the fixed effects. Sampling time was considered a split-plot factor (repeated measurement) after verifying that the covariance between experimental units at h0 and h5 was similar to the covariance between experimental units at h 5 and h 29. Total coliforms and *E. coli* data were log-transformed and analyzed as continuous variables using the MIXED procedure of SAS (SAS 9.2 Inst. Inc., Cary, NC). *Escherichia coli* O157 prevalence was expressed as the number of positive samples over the number of animals for the specific treatment and the specific sampling time and analyzed using a GLIMMIX procedure of SAS. To assess the effect of *E. coli* O157 on total coliforms and total *E. coli* a MIXED procedure of SAS was used with *E. coli* O157, treatments and sampling times as fixed effects and steers, replications, and interaction between replication x treatment and replication x treatment x sampling time x *E.coli* O157 as random effects.

RESULTS

Overall prevalence of *E. coli* O157 in fecal samples varied with replications (Figure 4-2). Transport did not affect the prevalence of the pathogen (Figure 4-3; $P > 0.09$). Additionally, we observed no sampling time effect ($P > 0.9$) and no interaction between sampling time and

treatment ($P > 0.17$). Prevalence of *E. coli* O157 in the transported group was fairly constant across the 3 sampling times (10, 3.3, and 16.7%, respectively; $P > 0.37$), with a slight decrease at h 5. Non-transported group also showed fairly constant prevalence across the 3 sampling times (17%, 33%, and 13%; $P > 0.37$), with a slight increase at h 5. Even if the effect of treatment was not significant, prevalence of the pathogen in feces of transported cattle was significantly lower than their counterpart at h 5 ($P < 0.05$).

As a secondary objective, we evaluated concentrations of *E. coli* (Figure 4-4) and other coliforms (Figure 4-5) in samples to determine if these populations were related to variations in *E. coli* O157 prevalence. We observed no effect of treatment ($P > 0.7$) or sampling time ($P > 0.09$) and no interaction between treatment and sampling time ($P > 0.08$) on total *E. coli*. Numbers of *E. coli* or other coliforms remained fairly constant across replications. The non-transported group had total enumerable *E. coli* numbers (log cfu/gram; Figure 4-4) of 6.10 at h 0, 5.84 at h 5, and 5.88 at h 29, which were not significantly different ($P > 0.18$). The transported group enumerable *E. coli* numbers were not different when comparing h 0 to h 5 (5.92 vs. 5.77 log cfu/gram; $P > 0.3$), or h 0 to h 29 (5.92 vs. 6.25 log cfu/gram; $P < 0.07$); however, *E. coli* counts increased significantly from h 5 to h 9 (5.77 vs. 6.25 log cfu/g; $P < 0.02$). Unlike total *E. coli*, coliform concentrations revealed a sampling time effect ($P < 0.004$) and a tendency for a treatment × sampling time interaction ($P = 0.059$), but no treatment effect ($P > 0.7$). Coliform counts for the non-transported group (Figure 4-5) remained relatively stable over the different sampling times (4.77, 4.18, and 4.13 log cfu/gram; $P > 0.14$). Transported cattle had decreased fecal coliform concentrations at h 5 (3.2 log cfu/gram; $P < 0.001$) compared with h 0 (4.57 log cfu/gram) but returned to pre-transport levels of 4.54 log cfu at h 29 ($P > 0.9$). No significant

correlation was observed between the prevalence of *E. coli* O157 and concentrations of total

fecal coliforms (P > 0.35) or total *E. coli* (P > 0.95; Figure 4-6).

DISCUSSION

We had hypothesized that transported cattle would be at greater risk for *E. coli* O157

colonization than non-transported animals and that increase in pathogenic bacteria might also

induce shifts in total *E. coli* and coliform concentrations. We observed that concentrations of

total *E. coli* and total coliform were fairly constant across treatments and replications. Coliform

and *E. coli* concentrations decreased numerically at h 5 regardless of treatment. The similarity in

patterns in transported and non-transported groups suggests that transport was not the causative

factor in this change. Variation in population could be attributed to a circadian rhythm; however,

h 5 and h 29 samplings occurred at the same time during the day with a 24-h interval.

Differences observed between these two sampling times questioned the hypothesis of a circadian

rhythm; moreover, animals from the transported group did not have access to feed on the first

day of sampling, which could be responsible for the variation in concentration of coliform and

total *E. coli* in that group. Data collected in this study were not sufficient to draw a conclusion

about this change.

Contrary to our expectations, we found no correlations between prevalence of *E. coli*

O157 in feces and concentrations of total *E. coli* or coliform. *Escherichia coli* O157 lacks beta-

glucuronidase, and thus is confounded with total coliform counts (Thompson et al., 1990),

potentially explaining why were unable to detect correlations between increases in pathogens and

total coliforms. The binomial nature of pathogen prevalence also may not be sufficiently robust

to detect this relationship.

Prevalence of *E. coli* O157 4 and 28 h after transport revealed an important variation of *E. coli* O157 shedding from one replication to the other. The transient nature of *E. coli* O157 is well known, and although we conducted the experiment during the summer when expected pathogen prevalence is greatest (Chapman et al., 1997; Hancock et al., 1997), variation between replicates was not unexpected. The absence of a statistically significant interaction between sampling time and treatment, as well as a sampling time or treatment effect, suggests that transport may not have influenced fecal pathogen shedding in this experiment. Nevertheless, it is important to note that non-transported animals had increased shedding of the pathogen at h 5, whereas transported animals showed a slight but insignificant decrease at that time. Such change in shedding patterns of transported cattle relative to their non-transported counterparts could be the consequence of transport-related stress. Animals under stress tend to defecate and urinate more often than non-stressed animals (Friend, 1991). Increased defecation due to transport stress could induce a rapid washout of the pathogen, depleting numbers by h 5. Another hypothesis is that under stressful conditions, *E. coli* O157:H7 virulence and attachment is amplified by action of catecholamine on LEE and motility genes (Hughes and Sperandio, 2008), delaying excretion of the pathogen in feces. The locus of enterocyte effacement (LEE) encodes a type III secretion system, which introduces virulence associated proteins into host epithelial cells via a hollow filamentous extension of the needle complex encoded by the espA gene (Roe et al., 2003) and causes attachment effacement of the EHEC. Likewise, flagellar regulon (*Fl*hDC) encodes for a flagella-mediated motility and has been shown to play a role in the adherence of the pathogen to the epithelium cells (Giron et al., 2002). Greater attachment of the pathogen to the gastrointestinal tract would likely make the bacteria more resistant to washing off. Independently from the explanation of the change in timing of fecal shedding, it is important to note that the

pathogen prevalence was highly transient within a period of only 29 h, which implies that pathogen populations can amplify and decay relatively quickly. Such observations underscore the importance of the choice of sampling time to assess the effects of stress on *E. coli* O157 shedding patterns.

In our design, fecal samples were obtained at only two time points post-transport, h 5 and h 29. The 4-h lairage period was chosen arbitrarily based on the time animals commonly spend in the lairage area at abattoirs before harvest. Considering the rapid fluctuation in pathogen populations within feces, additional sampling times appear to be needed to better qualify the changes in *E. coli* O157 shedding under transport stress. In future studies, all feces produced by the animals, including in the trailer, should be collected to portray *E. coli* O157 prevalence patterns following transport.

The present *E. coli* O157 analysis was qualitative and not quantitative; in such conditions, the potential for presence of supershedder animal(s) in the non-transported group cannot be excluded. Supershedders are defined as cattle shedding more than 10^3 to 10^4 cfu/g of feces, and their incidence in a pen has been shown to increase the prevalence of *E. coli* O157 for the whole pen (Cobbold et al., 2007; Stephens et al., 2009). To overcome this limitation, quantifying *E. coli* O157 present in feces of individual animals would be useful.

Another measurement that would be beneficial to our design is the pathogen prevalence on the hides of cattle. Hides have been shown to be a main vector for carcass contamination (Arthur et al., 2009; McGee et al., 2004). Animal hides, with transport space limitations, are likely to be contaminated with feces from others and disseminate the pathogen.

We hypothesized that handling, loading, transport, unloading, and confronting cattle with unfamiliar housing conditions (i.e., new pens) would induce stress responses in the cattle used in

our experiment. We made no attempt to quantify stress response to confirm this, and thus cannot exclude the possibility that our model failed to induce the desired stress response. Assessment of stress response typically requires blood sampling and subsequent characterization of the secretion of cortisol, epinephrine, norepinephrine, or other stress-related compounds. Handling of animals to obtain these samples arguably would induce some degree of stress, potentially masking effects of our desired treatments. Analysis of cortisol levels in feces has been reported as a reliable indicator of stress (Mostl and Palme, 2002), and may have been a useful addition to the present experiment. Cortisol released into circulation in response to stressful events is metabolized and excreted in urine and feces (Mostl and Palme, 2002). On average, the metabolite can be detected in feces about 12 h following a stress event, and concentrations in feces parallel those of circulating cortisol immediately after induction of stress (Palme et al., 2000). Our intent was to minimize exposure to stress in the non-transported group, but we cannot exclude the possibility that these animals were stressed by the mere presence of humans in their pens waiting to collect fresh fecal pats.

Previous studies have been performed to assess the impact of transportation on prevalence of *E. coli* O157 in cattle, but none of these studies compared shedding patterns of the pathogen in transported animals vs. non-transported animals. These studies evaluated *E. coli* O157 prevalence in fecal samples collected at the feedyard and after transport in the lairage pens or right after slaughter. Some authors observed an increase in *E. coli* O157 following transportation (Arthur et al., 2007; Bach et al., 2004; Dewell et al., 2005; Dewell et al., 2008), whereas others observed no significant effect of transport (Barham et al., 2002; Fegan et al., 2009; Minihan et al., 2003) on pathogen shedding.

Differences in detection techniques used can account for part of the discrepancy in the results. Animal genetics, age, gender, and management history have been shown to influence stress perception (Stanger et al., 2005). These criteria should be taken into consideration when comparing results from studies, because they confer a unique capacity on each animal to cope with stress. Multiple factors are involved in transport stress (Nielsen et al., 2011), and variation in each of these factors could affect findings. Over the course of their journey, cattle are subjected to feed and water depravation (Warriss et al., 1995); psychological stressors, such as handling, novelty, and disruption of their social organization (Grandin, 1997); repression of their basic behaviors, such as lying down (Munksgaard et al., 2005); and they may be exposed to large bacterial loads (Avery et al., 2004) from other animals and the surrounding environment. Variations in the effects of transport on *E. coli* O157 prevalence in fecal samples are thus to be expected.

In conclusion, our hypothesis was that stress from transport would alter fecal shedding of *E. coli* O157. Results suggest that shedding patterns for pathogens can vary greatly within a period of 29 h. Additional post-transit sampling times with pathogen quantification and collection of stress-related measurements are needed to conclude the effect of transport stress on *E. coli* O157 prevalence and the changes undergone in pathogen shedding patterns after transportation. Change in shedding patterns could have important ramifications for beef safety.

ACKNOWLEDGMENTS

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Table 4-1. Diet composition (dry basis).

Ingredients, %	Finishing diet
Dry-rolled corn	74.20
Corn silage	12.00
Steep corn liquor	8.00
Vitamin/mineral premix[1]	3.63
Feed additive premix[2]	2.16
Nutrient composition, %	
DM	72.23
CP	14.00
NDF	11.36
Crude fat	0.007
Ca	0.70
P	0.45

[1]Formulated to provide 0.1 mg Co, 10 mg Cu, 0.6 mg I, 60 mg Mn, 0.25 mg Se, 60 mg Zn, and 2640 IU vitamin A and 11 IU vitamin E per kilogram of diet DM.

[2]Provided 300 mg of Rumensin (Elanco Animal Health, Greenfield, IN), 90 mg tylosin (Elanco) per animal daily in a ground corn carrier.

Figure 4-1. Experimental design.
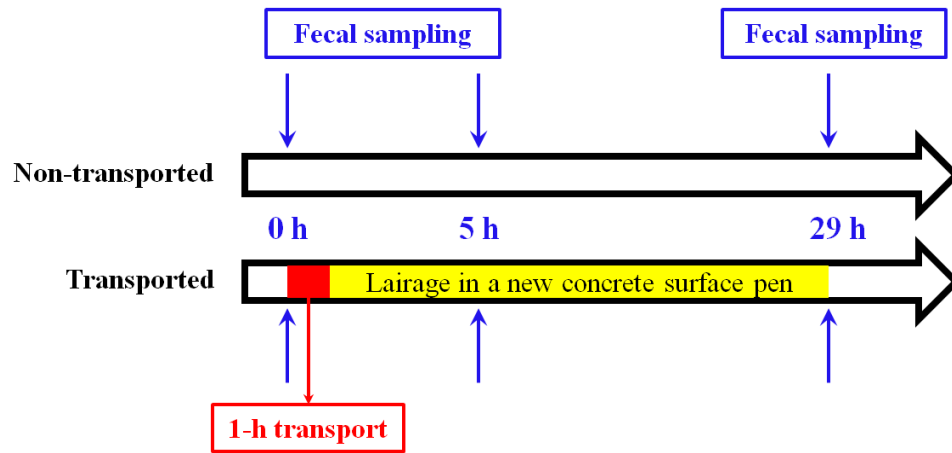
Figure 4-2. Total prevalence of *Escherichia coli* O157 within each replication.
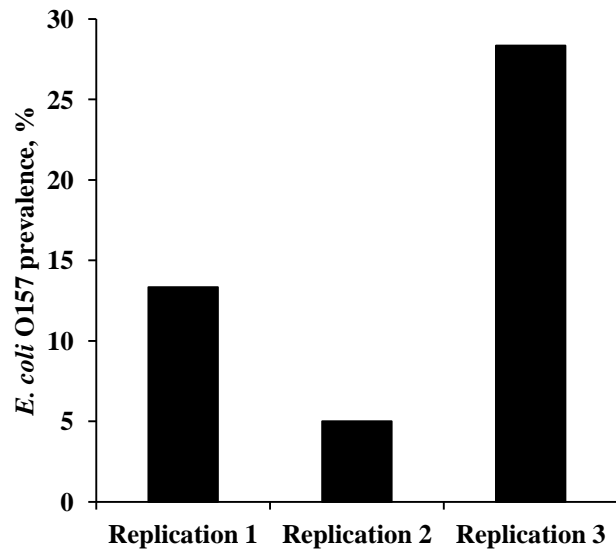
Figure 4-3. Prevalence of *Escherichia coli* O157 in feces collected at h 0, 5, and 29 from cattle subjected (□), or not (■), to 1-h transport. Letters above bars represent comparisons among means between and within treatments. Bars with different superscript are significantly different (P < 0.05). Treatment effect P = 0.0919; sampling day effect P = 0.9369; treatment × sampling time interaction P = 0.1786. SEM = 0.063.
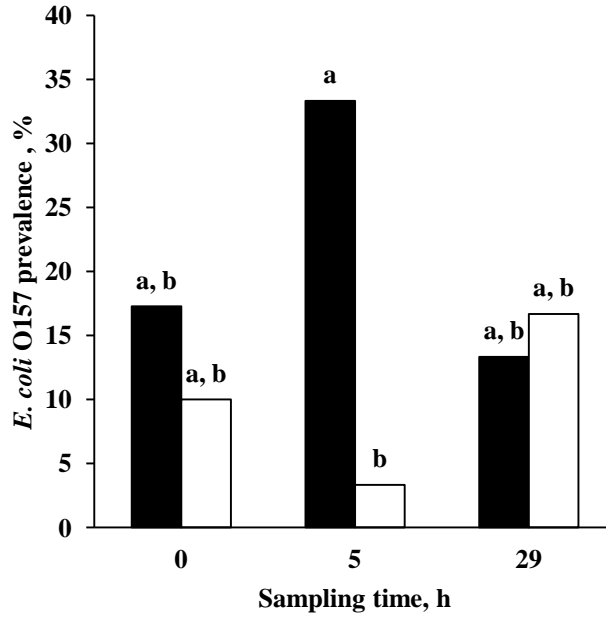
Figure 4-4. Fecal concentrations of *Escherichia coli* in feces collected at h 0, 5, and 29 from cattle subjected (□), or not (■), to 1-h transport. Letters above bars represent comparisons among interaction means between and within treatments. Bars with different superscript are significantly different (P < 0.05). Treatment effect P = 0.7715; sampling day effect P = 0.0919; treatment × sampling time interaction P = 0.0874. SEM = 0.094.
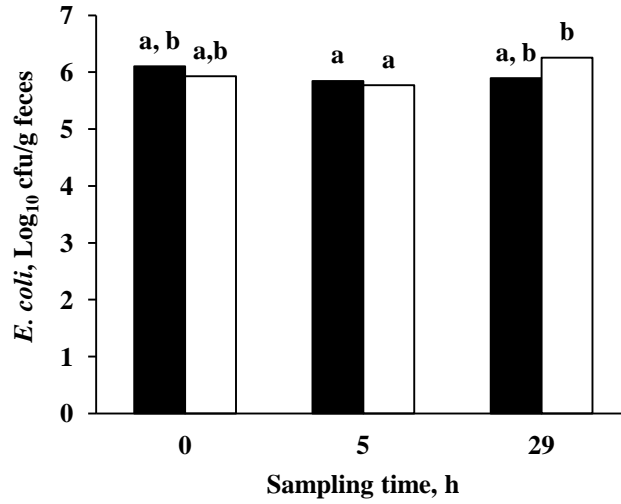
Figure 4-5. Fecal concentrations of coliforms other than *Escherichia coli* in feces collected at h 0, 5, and 29 from cattle subjected (□), or not (■), to 1-h transport. Letters above bars represent comparisons among interaction means between and within treatments. Bars with different superscript are significantly different (P < 0.05). Treatment effect P = 0.7171; sampling day effect P = 0.0044; treatment × sampling time interaction P = 0.0597. SEM = 0.43.
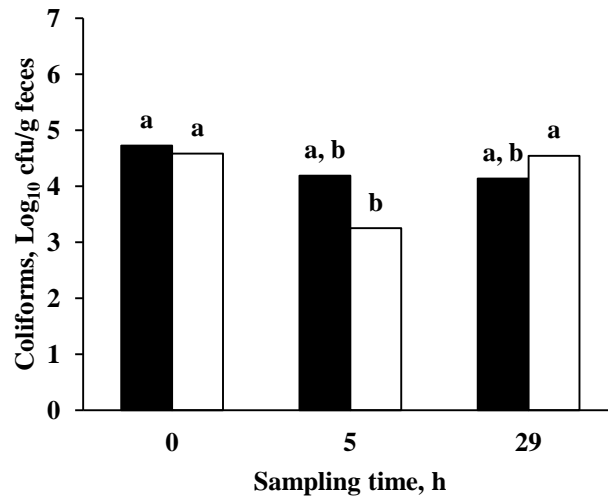
Figure 4-6. Fecal concentrations of coliforms (■) and *Escherichia coli* (□) in relation to *E. coli* O157 prevalence (▦) in feces collected at h 0, 5, and 29 from cattle subjected, or not, to 1-h transport. *E. coli* O157 prevalence effect on total coliform P > 0.35; *E. coli* O157 prevalence effect on total *E. coli* P > 0.95.
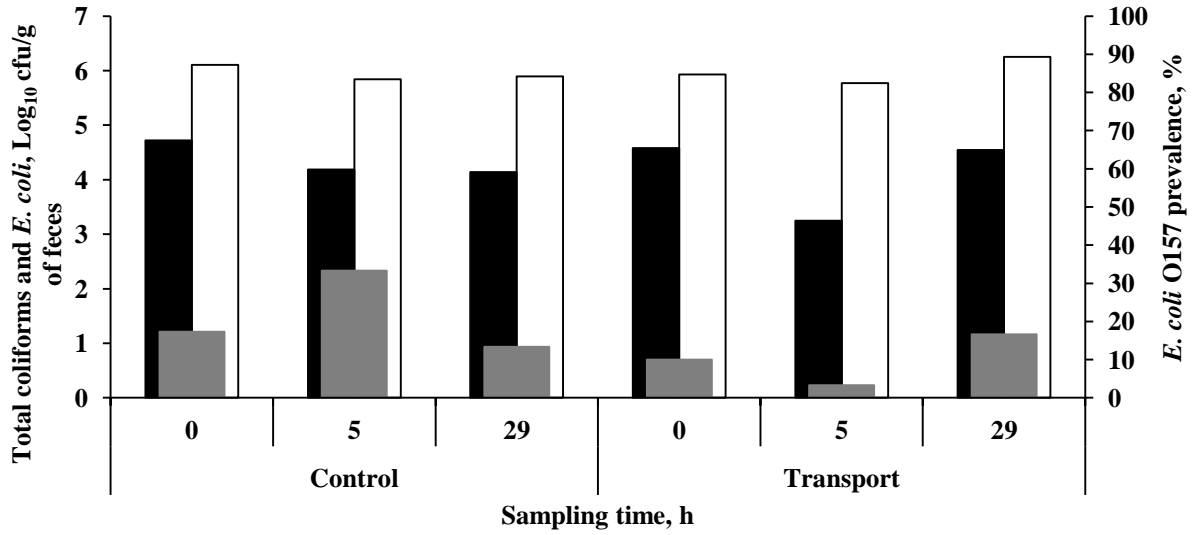
Chapter 5 Long-chain fatty acids profile of cattle fecal samples as an indicator for the shedding of *E. coli* O157

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Running Title: Indicator of *E. coli* O157 presence in cattle feces

ABSTRACT:

We analyzed long-chain fatty acid profiles of fecal samples to determine if these metabolites could be useful indicators of the presence or absence of *E. coli* O157. Of the 39 methyl esters of long-chain fatty acids evaluated, only eicosapentaenoic acid concentration was associated with the presence of the pathogen.

INTRODUCTION

E. coli O157 is well known for its pathogenic action in humans, and cattle are recognized as an important reservoir for the pathogen (1). Studies designed to increase knowledge of factors influencing *E. coli* O157 shedding in ruminants are challenging due to the transient nature of the bacteria (2). To overcome this problem, experiments often require a large number of replicates and multiple sampling times. Detection methods used are labor-intensive, costly, and often lack sensitivity to detect an infectious dose of *E. coli* O157, which can be as low as 1 to 100 CFU (3). Progress in identifying effective preharvest interventions within commercial feedlot settings would be facilitated by the development of detection methods that could be implemented on a large scale, at relatively low cost, and that would be suitable for high-volume screening. We investigated the potential for exploiting long-chain fatty acid metabolites produced by bacteria as an indicator of the presence of *E. coli* O157 in feces. We hypothesized that fecal samples positive for *E. coli* O157 would have fatty acid profiles that could differentiate them from negative samples. Our objective was to identify one or more long-chain fatty acids, or combinations thereof, which could serve as a "signature" for *E. coli* O157 presence in feces.

THE STUDY

Fecal samples were obtained from the weekly sampling of 368 crossbred heifers (234 ± 3.2 kg BW) over a 6-week period. Heifers were fed a growing diet containing 2 levels of crude glycerin (0 or 8% of diet dry matter), dry-rolled corn, corn silage, alfalfa hay, and corn steep liquor. All diets were formulated to be isonitrogenous (Table 5-1).

Animals were held briefly within a hydraulic restraining device to facilitate sampling of feces via rectal palpation. Fresh fecal samples were immediately placed on ice and transported 3

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km to the Preharvest Food Safety Laboratory (Kansas State University, Manhattan, KS) for analysis. Approximately 1g of feces was weighed and placed in 9 mL gram-negative broth (Difco-BD, Franklin Lakes, NJ) with cefixime (0.05 mg/L), cefsulodin (10 mg/L), and vancomycin (8 mg/L; GNccv) for a 6-hour incubation at 40°C. The remaining portion of each fecal sample was placed into a -20°C freezer. One milliliter of the GNccv broth was then added to a sterile tube containing 20 µL of *E. coli* O157 specific beads and subjected to immunomagnetic separation. Resulting *E. coli* O157 beads were resuspended in 100 µL of phosphate buffer and plated onto a selective agar for *E. coli* O157, sorbitol-MacConkey agar with cefixime (0.5 mg/L), and potassium tellurite (2.5 mg/L), for an overnight incubation at 37°C. Following incubation, 6 non-sorbitol fermenting colonies were selected and tested for indole production and O157 antigen agglutination. Colonies positive for both agglutination and indole production were considered *E. coli* O157. Results were confirmed by Gram staining and API 20E kit (Biomerieux, Durham, NC).

Fatty acids analyses were performed on fecal samples obtained during weeks 3, 4, and 5 of the experiment. Animals were classified as *E. coli* O157 positive if at least 1 sample over the 3-week period tested positive for the pathogen. Animals were classified as *E. coli* O157 negative only if all samples during the 6-week experiment tested negative for *E coli* O157. A subset of 21 positive and 61 negative samples were obtained. For analysis of long-chain fatty acids, samples were thawed, ground, derivatized with methanolic-HCl (4), and subsequently analyzed for concentrations of 38 fatty acids (from C6:0 to C24:1) by gas chromatography using a Supelco SP-2560 capillary column.

Fatty acid concentrations were expressed as a percentage of the sample weight. Statistical analyses of the data were performed using stepwise regression (SAS 9.2, Cary, NC). First, the

relationship between individual fatty acid concentrations in the fecal samples and *E. coli* O157 status was evaluated using a Pearson correlation and Kendall's tau coefficient. Fatty acids with a *P*-value less than 0.200 were retained in the model as possible indicators (Table 5-2), and included lauric acid (12:0), stearic acid (18:0), elaidic acid (18:1 *trans*-9), *cis*-vaccenic acid (18:1 *n*-11), oleic acid (18:1 *cis*-9), linoleic acid (18:2 *trans*-6), conjugated linoleic acid *cis*-9, *trans*-11 and *trans*-10, *cis*-12 (18:2 *cis*-9, *trans*-11 and 18:2 *trans*-10, *cis*-12), 20:1 fatty acid, eicosapentaenoic acid (EPA; 20:5 *n*-3), and docosapentaenoic (DPA; 22:5 *n*-3). Analysis of variance were then performed using the Generalized Linear Models procedure (SAS 9.2, Cary, NC) to determine if week of sampling (week 3, week 4, and week 5) and dietary glycerin level (0% or 8%) affected the fatty acid content of the sample and the presence of *E. coli* O157 . Neither sampling time (p = 0.4114), nor glycerin inclusion (p = 0.3851) influenced fatty acid composition of samples. However both sampling time (p = 0.0321) and glycerin inclusion (p = 0.0207) affected *E. coli* O157 prevalence in the samples. Consequently both sampling and dietary glycerin level were conserved in the model. A logistic regression was then performed on the model to further select variables that would be useful indicators of *E. coli* O157 status of the sample. Out of the 14 variables present in the model, EPA was the only fatty acid determined to be related to presence of the pathogen in feces (p < 0.0153).

CONCLUSIONS

Total fatty acid content of the fecal samples was not influenced by the inclusion of glycerin in the diet, sampling date, or the presence or absence of *E. coli* O157. Stearic acid, palmitic acid, oleic acid, elaidic acid, and linoleic acid were the predominant fatty acids in both O157-positive and -negative fecal samples. This observation was supported by the fact that concentrate diets usually contain 15 to 20% palmitic acid, 1–5% stearic acid, and 25–35% oleic

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acid (5); moreover, linoleic acid is the primary fatty acid found in grain-based diets (30–60%; (6)), and is converted to cis-9, trans-11 conjugated linoleic acid by linoleic acid isomerase in the rumen (7) to be hydrogenated to vaccenic acid (8). The content of these major fatty acids was not significantly affected by the presence of the pathogen; in fact, only the EPA content was notably increased in fecal samples that tested positive for *E. coli* O157 (0.0044 vs. 0.0027% for positive and negative samples, respectively). Eicosapentaenoic acid is commonly found in the bacterial genera *Shewanella* (9) and *Colwellia* (10), which are mainly deep-sea environment microorganisms. In contrast, *E. coli* are not known to produce polyunsaturated fatty acid (11), and the synthesis of EPA from α-linolenic acid in the rumen is considered to be limited. In such conditions, the increased concentration in *E. coli* O157 positive sample cannot be attributed to a direct production of EPA by the pathogen. It is possible, however, that composition of the overall microbial population that exists in the presence of *E. coli* O157 is less capable of hydrogenating long-chain fatty acids, or that the environment favors EPA producing microorganisms. Nevertheless, based on our analysis, EPA appears to be the only long-chain fatty acid linked to the *E. coli* O157 status of the sample. The lack of differences between the fatty acid profiles of positive and negative samples may be explained by the technique used to initially classify the samples. IMS has a detection limit for *E. coli* O157 of 10^2 CFU/mL. Samples classified as negative could contain low concentrations of the pathogen, potentially yielding smaller amounts of the fatty acids of interest, thus impeding our ability to establish clear relationships between pathogen presence and fatty acid concentrations. Utilizing quantitative data for *E. coli* O157 concentrations in feces could help alleviate this bias, potentially allowing for the identification of a "signature" metabolite for the pathogen.

ACKNOWLEDGEMENTS

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Table 5-1: Composition of experimental diets (dry basis)

Ingredients, %	0% Glycerin	8% Glycerin
Corn silage	60	60
Wet corn gluten feed	35	25.4
Crude glycerin	-	8
Soybean meal	-	1.6
Limestone	1.6	1.6
Urea	0.4	0.4
Vitamin/mineral premix[*]	0.3	0.3
Feed additive premix[†]	2.7	2.7
Nutrient composition		
Dry matter, %	43.3	44.2
Crude protein, %	13.0	12.1
Neutral detergent fiber, %	36.2	33.2
Calcium, %	0.75	0.75
Phosphorus, %	0.51	0.42

[*]Formulated to provide (dry basis) 0.1 mg cobalt, 10 mg copper, 0.6 mg iodine, 60 mg manganese, 0.25 mg selenium, 60 mg zinc, 0.3% salt, and 2200 IU vitamin A per kg of diet dry matter.

[†]Provided 300 mg of monensin (Elanco Animal Health; Greenfield, IN) per heifer daily in a ground corn carrier.

Table 5-2. Pearson correlation and Kendall's tau coefficient analysis between the concentration of single long-chain fatty acids and the *E. coli* O157 status of the fecal samples

LCFA, % of sample weight	*E. coli* O157-negative		*E. coli* O157-positive		Pearson		Kendall	
	Mean	SD	Mean	SD	r	*p* value	r	*p* value
12:0*	0.0166	0.0044	0.0182	0.0045	0.1574	0.158	0.1396	0.127
14:0	0.0347	0.0110	0.0382	0.0136	0.1313	0.240	0.1054	0.249
14:1	0.0003	0.0006	0.0002	0.0005	-0.1124	0.315	-0.1210	0.243
15:0	0.0264	0.0077	0.0252	0.0071	-0.0714	0.524	-0.0520	0.570
15:1	0.0216	0.0063	0.0212	0.0063	-0.0286	0.798	-0.0117	0.899
16:0	0.2821	0.0983	0.2545	0.0789	-0.1286	0.249	-0.0887	0.331
16:1	0.0426	0.0086	0.0425	0.0086	-0.0020	0.986	0.0019	0.983
17:0	0.0438	0.0146	0.0401	0.0094	-0.1178	0.292	-0.0602	0.510
17:1	0.0027	0.0016	0.0026	0.0015	-0.0243	0.829	-0.0171	0.853
18:0*	0.7859	0.4209	0.6595	0.3700	-0.1354	0.225	-0.1178	0.197
18:1 *trans*-9*	0.0764	0.0366	0.0642	0.0318	-0.1502	0.178	-0.1349	0.140
18:1 *n*-11*	0.0129	0.0072	0.0101	0.0047	-0.1836	0.099	-0.1230	0.179
18:1 *cis*-9*	0.0556	0.0194	0.0503	0.0197	-0.1181	0.291	-0.1233	0.177
18:1 *n*-7	0.0085	0.0032	0.0090	0.0049	0.0703	0.531	-0.0068	0.941
18:2 *trans*-6*	0.0014	0.0012	0.0022	0.0022	0.2163	0.051	0.1238	0.183
18:2 *cis*-6	0.0454	0.0204	0.0422	0.0138	-0.0741	0.508	-0.0209	0.819
18:2 *cis*-9, *trans*-11*	0.0024	0.0026	0.0015	0.0027	-0.1473	0.187	-0.1791	0.063
18:2 *trans*-10, *cis*-12*	0.0044	0.0014	0.0042	0.0025	-0.0442	0.693	-0.1550	0.093
18:2 *cis*-9, *cis*-11	0.0008	0.0007	0.0009	0.0006	0.0120	0.915	0.0225	0.811
18:2 *trans*-9, *trans*-11	0.0037	0.0013	0.0038	0.0013	0.0320	0.775	0.0304	0.742
18:3 *n*-6	0.0030	0.0047	0.0026	0.0033	-0.0435	0.698	0.0060	0.949
18:3 *n*-3	0.0111	0.0032	0.0108	0.0026	-0.0462	0.680	-0.0298	0.746
20:0	0.0193	0.0065	0.0178	0.0065	-0.1003	0.370	-0.1084	0.236
20:1*	0.0028	0.0022	0.0025	0.0032	-0.0571	0.610	-0.1361	0.140
20:2	0.0028	0.0015	0.0029	0.0016	0.0497	0.658	0.0250	0.786
20:3 *n*-6	0.0021	0.0018	0.0022	0.0017	0.0203	0.857	-0.0029	0.975
20:4 *n*-6	0.0038	0.0008	0.0038	0.0009	-0.0143	0.899	-0.0424	0.647
20:5 *n*-3*	0.0027	0.0022	0.0044	0.0036	0.2846	0.010	0.1615	0.081
21:0	0.0084	0.0023	0.0082	0.0022	-0.0487	0.664	-0.0361	0.694
22:0	0.0217	0.0042	0.0216	0.0044	-0.0169	0.880	-0.0122	0.894
22:5 *n*-3*	0.0044	0.0019	0.0048	0.0022	0.0989	0.377	0.1228	0.182
22:6 *n*-3	0.0023	0.0015	0.0019	0.0011	-0.1010	0.367	-0.0796	0.389
24:0	0.0250	0.0048	0.0242	0.0034	-0.0745	0.506	-0.0404	0.659
24:1	0.0055	0.0026	0.0062	0.0030	0.1094	0.328	0.0396	0.667
Total LCFA†	1.5898	0.6157	1.4115	0.5282	-0.1312	0.240	-0.1023	0.262

*Statistical correlation (p<0.200) between the concentration of the long-chain fatty acid and the *E. coli* status of the sample.

†Total LCFA represent the percentage of fatty acids (C6:0 to C24:1) in the fecal sample.

Chapter 6 Effects of menthol supplementation in feedlot diets on the prevalence of antimicrobial resistant *Escherichia coli* in cattle feces

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Running title: Menthol effect on antimicrobial resistance in *Escherichia coli*

ABSTRACT

The important pool of antibiotic resistance found in our environment and, more precisely, in ruminant microflora is becoming a growing concern, as these bacteria can transfer drug resistance determinants to pathogenic bacteria that affect humans and endanger the future of antimicrobial therapy. Moreover, the occurrence of multiple drug resistance genes on the same mobile genetic elements, such as plasmids, facilitates spread of multidrug resistance. Recent work uncovered anti-plasmid capacities of menthol *in vitro*, and the present study aimed to determine if this characteristic could be exploited *in vivo* to decrease the prevalence of multidrug resistant bacteria in feedlot cattle by supplementing their diet with menthol. Menthol crystals were crushed and added to the diets of steers at 0.3% of DM content. Fecal samples were then collected once each week for 4 weeks and analyzed for total *E. coli* population, as well as minimum inhibitory concentration of *E. coli* isolates and *tet* gene determinants. Results revealed no effect of menthol supplementation on total *E. coli* population or resistance profile to azithromycin, ceftriaxone, ciprofloxacin, gentamicin, nalidixic acid, cefoxitin, amoxicillin, ceftiofur, sulfamethoxazole, kanamycin, streptomycin, sulfisoxazole, chloramphenicol, and ampicillin; however, 30-day of menthol addition to steer diets increased prevalence of tetracycline resistant *E. coli* ($P < 0.006$) and *tet*B detection. Though it still is unclear how menthol exerts its effects, results of our study corroborate earlier *in vitro* observations, suggesting that exposure to menthol may impact the expression of antimicrobial resistance in gut bacteria.

Keywords: *Escherichia coli*, antibiotic resistance, menthol

INTRODUCTION

The rise of antibiotic resistant bacteria is observed worldwide (31) and has become a growing concern due to its potential for endangering the future of antimicrobial drug therapy. Excessive use of therapeutic and non-therapeutic antimicrobials in human and animal health is held responsible for the dissemination of antimicrobial resistance (5, 15). Livestock represent a large reservoir for antibiotic resistant bacteria and resistance gene determinants that can spread to environmental bacteria and human microflora (29) by horizontal gene transfer. This dissemination is alarming insofar as genes encoding for multidrug resistance (MDR) are emerging on mobile genetic elements, such as plasmids, transposons and integrons (6, 18), and can easily propagate from commensal to pathogenic bacteria and from livestock to human microflora. Consequently, compounds capable of limiting or preventing plasmid activity and dissemination are of interest for their potential role in restricting the occurrence of multidrug resistant bacteria.

Menthol is a monoterpene alcohol known for its cooling property, anesthetic quality, anti-pruritic activity, and antibacterial and antifungal activities (23). In addition to these many attributes, menthol was recently shown to have some anti-plasmid activity. Schelz et al. (25) investigated the effect of peppermint oil and menthol *in vitro* on bacteria and their plasmids and demonstrated that menthol and peppermint oil have anti-plasmid activity similar to sodium dodecyl sulfate. SDS is used for plasmid bacterial curing (8), disrupting the membrane sites of plasmid attachment. Anti-plasmid activity of menthol *in vivo* has not been verified, but we speculated that inclusion of menthol in cattle diets could lead to a reduction in plasmid prevalence in bacterial populations and therefore to a reduction in multidrug resistant bacteria in the gut.

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Our objective was to investigate the effect of menthol addition in the diet of feedlot cattle on fecal *Escherichia coli* population, minimum inhibitory concentration and tetracycline gene resistance.

MATERIAL AND METHODS

Animals

Twenty six Holsteins steers (568.8 ± 55 kg) were blocked by body weight and allocated to individual feeding pens within 3 barns containing, respectively, 10, 10, and 6 steers. Each concrete-surfaced pen measured 1.5 m × 6 m, was covered with corrugated roofing and equipped with individual feed bunks. Water fountains were shared between two adjacent pens. Two treatments were randomly assigned to animals and were equally represented in each of the barns. Treatments consisted of a control group receiving a basal diet with no menthol, and a menthol group receiving 0.3% menthol in their diet on a dry matter basis. Crushed menthol (99.71%, Prinova USA LLC, Carol Stream, IL) was included in the basal diet consisting of steam flaked corn, corn gluten feed, and corn silage (Table 6-1). Animals were fed *ad libitum* and had free access to water.

Sample collection and processing

Fresh fecal samples were obtained from each animal, by rectal palpation, before feeding on days 0, 16, 23 and 30. Samples were immediately placed on ice and transported 3 km to the Preharvest Food Safety Laboratory of Kansas State University. Fecal samples were homogenized in a stomacher, and 1 g of each sample was added to a 9 mL phosphate buffered saline (PBS) in

a falcon tube and thoroughly vortexed. The remaining portions of feces and PBS diluted feces were stored for later analyses at -80°C.

Escherichia coli isolation

A 100-µL volume of fecal suspension was spread-plated on MacConkey agar (BD Diagnostic Systems, Franklin Lakes, NJ) and incubated overnight at 37°C. Three lactose-fermenting single colonies were selected on each plate and re-plated onto tryptic soy agar (TSA; Thermo Fisher Scientific, Lenexa, KS). After an overnight incubation at 37°C, colonies were tested for indole production by a spot indole test. Positive colonies were picked and stored on cryobeads (Cryocare[tm]; Key Scientific Products, Stamford, TX) at -80°C until further analysis.

Total *Escherichia coli* counts

Fecal samples stored at -80°C were thawed and 1 g of feces was resuspended in 9 mL PBS. Tubes were thoroughly vortexed and further diluted in PBS if needed. Tubes were vortexed again and a 4-mL aliquot was pipetted into a micro beaker. Debris was allowed to settle for 3 min. A 50-µL volume of the suspension was then plated in duplicate onto MacConkey agar using an Eddy Jet spiral plater (IUL instruments, Barcelona, Spain), and incubated overnight at 37°C. After incubation, lactose-fermenting colonies were counted following spiral plating guidelines and total *Escherichia coli* concentrations were established.

Minimum inhibitory concentration (MIC) determination

Isolates from control and high menthol groups were selected to test MIC for cefoxitin, azithromycin, chloramphenicol, tetracycline, ceftriaxone, amoxicillin, ciprofloxacin, gentamicin, nalidixic acid, ceftiofur, sulfisoxazole, trimethropim/sulfamethoxazole, kanamycin, ampicillin,

and streptomycin using the broth microdilution method. Isolates were revived on blood agar plates (Thermo Fisher Scientific Remel Products, Lenexa, KS) by an overnight incubation at 37°C. Resulting colonies were mixed with demineralized water (Trek Diagnostics Systems, Cleveland, OH) to obtain a 0.5 McFarland turbidy. A 50-μL aliquot of the mix was added to cation adjusted Mueller-Hinton broth (Trek Diagnostics Systems, Cleveland, OH). Tubes were vortexed and placed in the Sensititre® automated inoculation delivery system (Trek Diagnostics Systems, Cleveland, OH) to inoculate the Gram-negative NARMS panel plates (CMV2AGNF, Trek Diagnostics Systems, Cleveland, OH) with 100 μL of Mueller-Hinton broth. Plates were incubated for 18 h at 37°C and read manually using the Sensititre® manual viewer (Sensitouch®). Resistance or sensitivity of the bacteria were determined based on CLSI guidelines (7).

Tetracycline resistance gene analysis by polymerase chain reaction

DNA extraction on every isolate tested for MIC was performed as follows: colonies were revived on blood agar plates, and after an overnight incubation at 37°C one colony was mixed with 500 μL of deionized water in a 1.5 mL microcentrifuge tube and boiled for 10 min at 100°C. Tubes were then centrifuged at 10,000 x g for 5 min and refrigerated until use. Polymerase chain reaction assays for resistance genes against tetracycline, *tet*A and *tet*B were performed in duplex (12). Primers for *tet*A and *tet*B (Integrated DNA Technologies, Coralville, IA; Table 6-2) were rehydrated with DNase free water (QIAgen, Valencia, CA) to obtain a stock solution of 100 μM. Ten microliters of each primers were added to 460 μL of DNase free water to obtain a primer mix at 2 μM. Final reaction mix to be loaded in the QIAgility (Qiagen, Valencia, CA) was prepared by adding 1734 μL of DNase free water, 2550 μL PCR master mix

(Promega Corp., Madison, WI), and 510 µL Primer mix. QIAgility was programmed to dispense 47 µL of the final reaction mix and 3 µL DNA template into each well. A 1:1 mixture (Agilent Tech., Santa Clara, CA) of the DNA obtained from *E. coli* ATCC 47042 (*tet*B control) and XL1-Blue *E. coli* strain (*tet*A control) was used as positive control (10 ng/µL).

The 96-well plates were amplified using an Eppendorf Mastercycler® gradient thermal cycler (USA Scientific, Inc., Ocala, FL). The thermal profile used was 95°C for 15 minutes, 31 cycles of 94°C for 30 s, 60°C for 90 s, and 72°C for 90 s, followed by a single final extension step of 72°C for 10 min. Ninety six-well plates containing PCR products were then transferred to the Automated QIAxcel System (QIAgen, Valencia, CA). Microcapillary electrophoresis was performed using a QIAxcel DNA screening cartridge (QIAgen, Valencia, CA), a QX alignment marker (15bp/1 kb; QIAgen, Valencia, CA) and a 50 to 800 bp QX size marker (QIAgen, Valencia, CA). The electrophoresis was documented and analyzed for the presence of specific bands.

Statistical Analysis

Total *Escherichia coli* CFU were $\log_{10}$ transformed and normality of the results was verified. Results were then analyzed using the GLIMMIX procedure of SAS (9.2, Cary, NC). Treatment and sampling day were included in the model as fixed effects, while *Escherichia coli* isolates were considered a random effect. Frequency analyses of resistant *Escherichia coli* isolates to the multiple antibiotics tested were performed using the FREQ procedure of SAS. Chloramphenicol, streptomycin, sulfisoxazole, ampicillin, and tetracycline resistance data were further analyzed using the GLIMMIX procedure of SAS, where treatment and sampling day were included as fixed effects and *Escherichia coli* isolates were included as random effect. Low prevalence of

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resistant or susceptible isolates, for chloramphenicol, streptomycin, and sulfisoxazole, precluded us from including in the model the interaction between sampling day and treatment. *Escherichia coli* isolates were considered multidrug resistant in this study when presenting resistance to 5 or more of the antibiotics tested. Multi-drug resistant phenotypes, *tet*A, *tet*B, and the number of isolates resistant to tetracycline but not carrying *tet*A or *tet*B gene, were analyzed using the previously described Glimmix model.

RESULTS

Total *Escherichia coli* population in fecal samples was not influenced by the presence or absence of 0.3% menthol in the diet of cattle ($P = 0.84$; Figure 6-1) or by the day of sampling ($P = 0.23$). Minimum inhibitory concentrations of *Escherichia coli* isolates from steers fed diets with or without 0.3% menthol are presented in Table 6-3. All isolates, regardless of treatment, were sensitive to azithromycin, ceftriaxone, ciprofloxacin, gentamicin and nalidixic acid. In addition, cefoxitin, amoxicillin, ceftiofur, sulfamethoxazole, and kanamycin had an overall prevalence of resistant isolates equal to or lower than 3.3%, and there were no differences in frequencies between isolates originating from animals fed diets with and without menthol ($P > 0.05$). Chloramphenicol and streptomycin resistant isolates were found in 2.6 and 10.3% of the control group and 4.6 and 6.5% of the high menthol group; however, there were no significant effects due to treatment ($P > 0.5$) or sampling day ($P > 0.2$). Ninety one percent of *E. coli* isolates from steers receiving control diets and 92.2% of *E. coli* isolates from steers receiving diets with 0.3% menthol were resistant to sulfisoxazole (treatments not different; $P > 0.9$). Resistance to sulfisoxazole was influenced by the day of sampling, with a significant decrease in resistance for both treatment groups on day 16 compared to days 0, 23, and 30 ($P = 0.04$). There

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was no interaction effect between sampling day and treatment ($P > 0.29$) on ampicillin resistance, and no treatment ($P > 0.66$) or sampling day ($P > 0.66$) effects. After 30 days of menthol supplementation, 20.5% of *E. coli* isolates from the menthol group were resistant to ampicillin, which tended to be greater than that of the control group, for which only 5.1% of isolates expressed resistance (effect of treatment, $P = 0.06$). There was no apparent interaction between sampling day and treatment ($P > 0.2$) on tetracycline resistance, as well as, no sampling day effect ($P > 0.05$), but a treatment effect was observed ($P < 0.01$). Percent of isolates resistant to tetracycline tended to increase between day 0 and day 30 in the menthol group (51.3% to 71.8%; $P < 0.07$), whereas there was no significant change in the control group (41% to 38.5%; $P > 0.8$). After 30 days of menthol supplementation, isolates from the menthol group were 33% more likely to be resistant to tetracycline than isolates from the control group ($P < 0.004$). Table 6-4 presents the percentage of multidrug resistant (MDR) isolates in each treatment group. Isolates were considered MDR if resistant to 5 or more antibiotics. Frequency analysis showed that overall prevalence of MDR isolates in the control group (1.3%) was lower than in the isolates obtained from steers receiving 0.3% menthol in their diets (6.5%; $P = 0.02$). There was no difference in MDR frequency between treatments on day 0, 16, and 23 ($P > 0.2$). However after 30 days of treatment, isolates from steers receiving 0.3% of menthol exhibited significantly higher levels of MDR isolates when compared to isolates from the control group ($P = 0.03$). Table 6-5 and table 6-6 summarize the prevalence of, respectively, *tet*A- and *tet*B-positive fecal *Escherichia coli* in steers fed diets with or without 0.3% menthol. No interaction between sampling day and treatment ($P > 0.2$) was observed on the presence of *tet*A gene in *E. coli* isolates. Sampling day ($P > 0.1$) and menthol treatment ($P > 0.2$) did not affect tetA gene detection, and there was overall 23 isolates from the control group and 19 isolates from the

menthol group carried *tet*A. Detection of t*et*B gene was not significantly influenced by the interaction between sampling day and treatment ($P > 0.18$), but it was influenced by sampling day ($P < 0.02$) and treatment ($P < 0.02$). Isolates from animals fed menthol had higher *tet*B prevalence than the control group on day 0, (41% and 12.8%, respectively; $P < 0.01$), and on day 30, (38.5% and 15.4%, respectively; $P < 0.03$). All isolates expressing *tet*A or *tet*B were found to be phenotypically resistant to tetracycline, except two isolates, one of which was classified as intermediate (MIC = 8 µg/mL) and the other one was considered susceptible (MIC $\leq$ 8 µg/mL). Conversely, 17 and 21 isolates from the control and high menthol groups, respectively, were found to be resistant to tetracycline but did not express *tet*A or *tet*B (Figure 6-2). No treatment effect ($P > 0.7$) or day effect ($P > 0.1$) was observed. On day 30, 25.6% isolates in the menthol group and 12.8% isolates in the control group were resistant to tetracycline and did not carry either *tet*A or *tet*B, but treatments were not different ($P > 0.1$). Isolates from the menthol group resistant to tetracycline but not carrying *tet*A or *tet*B, however, appeared to increase between day 0 (7.7%) and day 30 (25.6%; $P < 0.05$). This difference was not observed in the control group. Table 6-7 illustrates the various antibiotic resistance phenotypes found among the *E. coli* isolates tested. Only 5.2% of the total isolates were found to have no resistance to any of the antibiotics tested. Of the *E. coli* isolates, 50.2% were found to be resistant to sulfisoxazole only, and 28.2% were resistant to both sulfisoxazole and tetracycline. Most of the bacteria resistant to 5 antibiotics or more, as previously observed, came from the menthol group, and one of isolates from this treatment was found to be resistant to 8 antibiotics.

DISCUSSION

The microflora of ruminants constitute an important pool for antimicrobial-resistant bacteria and antimicrobial resistance gene determinants (1). *Escherichia coli* are prevalent in feces of cattle (21) and are often used to study drug resistance patterns. These bacteria, indeed, carry antibiotic resistance genes which can be intrinsically transferred to their progeny or disseminated by horizontal gene transfer (HGT; (17)). The occurrence of many of the antibiotic resistance genes on highly mobile genetic elements such as plasmids, transposons, or integrons (22) thus supports the potential for dissemination. Horizontal gene transfer occurs through 3 mechanisms: transformation by uptake of free DNA from environment, transduction by insertion of DNA in the cell by a bacteriophage, or conjugation by contact-transfer of genetic material from one bacterium to the other (27). Antibiotic resistances can therefore be transmitted between commensal and pathogenic bacteria as well as between livestock and human microflora, thereby presenting drug resistant organisms which may endanger drug therapy for humans and animals. Multiple drug resistance determinants have been found to be located on the same plasmid, further facilitating their propagation and co-selection. For instance, the multidrug resistance plasmid IncA/C found in enteric bacteria, such as *Salmonella enterica* and *Escherichia coli*, very often encodes for resistance to tetracycline (*tetA*), chloramphenicol/florfenicol (*floR*), streptomycin/spectinomycin (*aadA2*), sulfonamides (*sul1* and *sul2*) and extended spectrum β-lactamases (*bla$_{CMY-2}$*; (9)) and its spread to pathogenic bacteria may limit antibacterial means to fight infections caused by these bacteria. Therefore, all compounds eliminating or inactivating mobile genetic elements may be of use to control antibiotic resistance dissemination and preserve antimicrobial efficacy. We had hypothesized that previously demonstrated *in vitro* anti-

plasmid activity of menthol would affect the prevalence of drug resistance in commensal *E. coli* in cattle fecal samples.

The first objective of this work was to investigate the effect of menthol addition to the diets of feedlot cattle on fecal *E. coli* populations. Menthol is known to have antimicrobial activity against *E. coli*. Previous studies have demonstrated inhibitory effects with concentrations of 75 mM for *E. coli* O157:H7 (14) and as low as 16 mM with *E. coli* ATCC15221 (28). Menthol metabolism in the rumen is poorly understood, leading us to investigate the impact of 0.3% dietary menthol on *E. coli* populations in feedlot cattle. The lack of difference between total fecal *E. coli* count in the control group and menthol supplemented groups suggests either that the level of menthol reaching the hindgut was insufficient to inhibit the microflora or that bacteria were able to adapt to menthol presence in the GIT. Landau and Shapira (2012) recently showed entherohemorrhagic *E. coli* (EHEC) to have the ability to adapt to increasing levels of subinhibitory concentration of menthol (14) and a similar adaptation process could be anticipated for commensal *E. coli*.

Our second and main objective was to investigate if menthol inclusion in feedlot diets would affect *E. coli* resistance to antibiotics and prevalence of MDR organisms. Although total *E. coli* populations were not affected by 30 days of menthol supplementation, MDR *E. coli* increased by 13% in the menthol treated group. These findings were in contrast to our expectations, but clearly indicate an effect of menthol supplementation on multidrug resistance in bacteria. To further investigate this effect, we analyzed individual minimum inhibitory concentration (MIC) of fecal *Escherichia coli* isolates from the control group and the group receiving 0.3% of menthol daily after 0, 16, 23, and 30 days of exposure to treatments. Results of MIC evaluations revealed that all isolates were susceptible to azithromycin, ceftriaxone,

ciprofloxacin, gentamicin and nalidixic acid, and only a small percentage of isolates were resistant to cefoxitin, amoxicillin, ceftiofur, sulfamethoxazole, and kanamycin, regardless of treatment received by the animals and of sampling day. Similar observations were made by Mirzaagha et al., who found all 531 *E. coli* isolates collected from feedlot cattle fed diets with and without chlortetracycline and/or sulfamethazine were susceptible to ceftriaxone, cefoxitin, gentamicin and nalidixic acid (20). Gow et al. also failed to detect any fecal *E. coli* resistance to ceftriaxone, ciprofloxacin, or nalidixic acid among the 207 isolates collected from cow calf herds in western Canada, and observed that only 1% were resistant to gentamicin, 1.5% to ceftiofur, and 4.8% to amoxicillin and cefoxitin. They did, however, observe higher resistance rates for kanamycin (15%) and sulfamethoxazole (55.1%) compared to our study. Chloramphenicol resistance, like previous antibiotics, was not affected by treatment in our experiment. The presence of resistant isolates (3.6% overall) was somewhat surprising, as chloramphenicol use in animal production systems was banned more than 30 years ago (10). Our observations are, however, not isolated, as other authors have reported even higher prevalence in commensal *E. coli* from cattle that range from 14.5 to 31% (11, 16, 24). Persistence of chloramphenicol resistance in the environment is thought to be due to the use of closely related antibiotics, such as florfenicol, or to a co-selection phenomenon (30). Unfortunately, low prevalence of chloramphenicol resistant isolates in this study did not allow us to reveal any resistance pattern associated with the presence of chloramphenicol resistance. Like chloramphenicol, prevalence of isolates resistant to streptomycin was not affected by treatment or sampling day. Overall, 16.8% of *E. coli* isolates tested in this experiment were resistant to streptomycin. Gow et al. reported 41.6% *E. coli* resistant isolates from cow calf (11), and Ma et al. found that 89.1% of *E. coli* isolates from dairy cows were resistant (16). Differences in animal production system practices

could explain the lower prevalence observed in our study, as animal exposure to antimicrobials are likely to be different. Resistance to sulfisoxazole was found in 91.6% of the *E. coli* isolates tested in our study, and was not influenced by menthol inclusion in the diet. A large scale study conducted in a feedlot in Texas also reported high resistance rate, with 65% of the 7,097 *E. coli* isolates tested being resistant to sulfisoxazole. Prevalence in the Texas study was not influenced by the type of growth promotants received by the animals (3), and further underscores the widespread nature of sulfisoxazole resistance determinants in commensal bacteria. The 12% decrease in resistance prevalence in the second week of our study could not be explained, but previous studies have also found drug resistance to be transient in repeated sampling (20). Overall ampicillin resistance, 8.7%, was not affected by the inclusion of menthol in the diets. This prevalence was lower than previously observed prevalence in *E.* coli from cattle, which have ranged from 18 to 48% (11, 19, 24). Despite the absence of a significant effect of menthol, the 15.4% increase in the number of *E. coli* isolates resistant to ampicillin in the menthol group compared to the control group after 30 days of treatment is worthy to mention. These observations could be indicative of an effect of menthol on ampicillin resistance, but our experimental design may not have been sufficiently robust to detect this effect. Conversely, menthol supplementation did have a significant effect on tetracycline resistance. After 30 days of treatment, 71% of the isolates from the menthol group tested resistant to tetracycline compared to only 38% in the control group. Moreover, *E. coli* isolates resistant to tetracycline within the menthol group increased by 20% between day 0 and day 30. These observations underline a clear effect of menthol on tetracycline resistance phenotypes, suggesting that further investigation of genotype profiles of these isolates may be warranted.

As of today, there are 40 known *tet* resistance determinants, most of which are found on mobile genetic elements that encode for efflux pump (26). *Tet*A and *tet*B genes are most prevalent in tetracycline resistant *E. coli* (4), which is why we choose to focus on these two. *Tet*A and B encode for an efflux pump in the lipid bilayer of the bacteria, which removes the tetracycline/cation complex from the cell by exchanging a proton (32). *Tet*B is usually more predominant than *tet*A and is linked to higher minimum inhibitory concentration (2). Out of the 309 isolates investigated in our experiment, 18.7% were found to carry *tet*B and 13.6% to carry *tet*A. Moreover, no isolates were found to carry both determinants, which corroborates previous findings (2, 13). *Tet*A and *tet*B are both believed to be most often located on plasmids, but from different incompatibility groups (13), potentially explaining why they are, rarely, detected simultaneously in bacteria. The absence of effect of menthol inclusion on the *tet*A expression seems to exclude the implication of *tet*A in the difference observed in tetracycline phenotypes in *E. coli* isolates from steers fed diets with and without menthol. *Tet*B was, however, affected by menthol inclusion, as *tet*B detection was greater among *E. coli* isolates originating from the steers fed menthol. One could speculate that menthol was selecting for bacteria carrying *tet*B gene, thus increasing the number of tetracycline isolates after 30 days of treatment. We hypothesized that menthol anti-plasmid effects shown *in vitro* could also be observed *in vivo*, and that menthol would in that case decrease drug resistance by inhibiting plasmid transfer. The increase in phenotypic tetracycline resistance and *tet*B gene presence could, in fact, result from an anti-plasmid effect of menthol. Research by Sawant et al. showed that *tet*B may be located on genomic DNA rather than on a mobile genetic element as previously thought (24). Considering our initial hypothesis and the findings of Sawant, it is thus reasonable to think that menthol may displace plasmids contained in bacteria and give a selective advantage to bacteria carrying

tetracycline resistance determinants on their DNA. The presence of higher *tet*B gene presence in isolates from the menthol group on the first sampling is, however, difficult to explain, and could temper this hypothesis. Increased detection cannot be associated to menthol inclusion as at that time menthol was not yet received by any animals. The difference between groups can only be explained by the transient nature of antibiotic resistance and the small number of experimental units.

Higher prevalence of tetracycline resistance in the menthol group was also accompanied by greater frequency of *E. coli* isolates phenotypically resistant to tetracycline but not expressing either *tet*A or *tet*B. Indeed, these isolates were three times more likely to be found resistant to tetracycline, but not to express the determinant tested after 30 days of menthol supplementation. Presence of other *tet* resistance determinants in *E. coli* isolates from steers receiving 0.3% menthol could be responsible for the increase in tetracycline resistance observed, but other determinants were not investigated in this study.

In conclusion, menthol supplementation of feedlot diets at a 0.3% rate for 30 days did not alter the total *E. coli* population in fecal samples and did not affect prevalence of resistance to azithromycin, ceftriaxone, ciprofloxacin, gentamicin, nalidixic acid, cefoxitin, amoxicillin, ceftiofur, sulfamethoxazole, kanamycin, streptomycin, sulfisoxazole, chloramphenicol, and ampicillin. Menthol supplementation did, however, increase the number of MDR bacteria, the prevalence of tetracycline resistant *E. coli* isolates, and the detection of *tet*B gene. The underlying mechanism associated with this increase could not be explained based on the present findings; nevertheless this study demonstrates a clear effect of menthol on bacterial drug resistance.

ACKNOWLEDGMENT

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Table 6-1. Diet information

Ingredients (% DM)	Menthol (%)	
	0	**0.3**
Steam flaked corn	50.39	49.89
Corn gluten feed	33.63	33.83
Corn silage	10.00	10.00
Vitamin/mineral premix[1]	2.16	2.16
Feed additive premix[2]	1.82	1.82
Menthol	0	0.3
Analyzed composition (%)		
Dry matter	65.20	65.19
Crude protein	14.00	14.00
Calcium	0.62	0.62
Phosphorus	0.51	0.51

[1]Formulated to provide 0.1 mg Co, 10 mg of Cu, 0.6 mg of I, 60 mg of Mn, 0.25 mg Se, 60 mg Zn, 2,205 IU vitamin A, and 22 IU vitamin E per kilogram of diet DM.

[2]Feed additive premix provided 300 mg of monensin (Elanco Animal Health, Greenfield, IN), 90 mg tylosin (Elanco Animal Health, Greenfield, IN), and 0.4 mg melengestrol acetate (MGA; Pfizer Animal Health, Exton, PA) per animal daily in a ground corn carrier.

Table 6-2. *tet*A and *tet*B primer sequences

Gene	Sequence (5'-3')	Tm°C	Product Size (bp)	Accession no.
*tet*A	F: GCTACATCCTGCTTGCCTTC	56	210 [c]	X61367
	R: CATAGATCGCCGTGAAGAGG	57		
*tet*B	F: TTGGTTAGGGGCAAGTTTTG	56	659 [c]	J01830
	R: GTAATGGGCCAATAACACCG	56		

Figure 6-1 Total fecal *Escherichia coli* counts ($\log_{10}$) from fecal samples of animals fed diets with (■) and without (□) 0.3% of menthol. SEM = 0.3632.
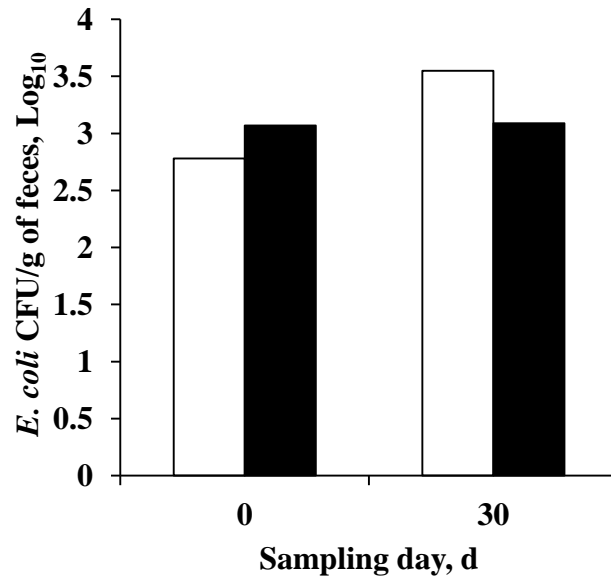
Table 6-3. Antimicrobial susceptibilities of fecal *Escherichia coli* isolates from steers fed diet supplemented with or without 0.3% menthol

	Number of resistant isolates/total isolates tested									
	Day 0		Day 16		Day 23		Day 30		Total (%)	
Menthol levels, %	0	0.3	0	0.3	0	0.3	0	0.3	0	0.3
Cefoxitin	0/39	0/39	0/39	1/36	0/39	1/39	0/39	3/39	0/156 (0)	5/153 (3.3)
Azithromycin	0/39	0/39	0/39	0/36	0/39	0/39	0/39	0/39	0/156 (0)	0/153 (0)
Chloramphenicol	2/39	0/39	1/39	0/36	1/39	5/39	0/39	2/39	4/156 (2.6)	7/153 (4.6)
Tetracycline	16/39	20/39	13/39	12/36	12/39	20/39	15/39[a]	28/39[b]	56/156 (35.9)[a]	80/153 (52.3)[b]
Ceftriaxone	0/39	0/39	0/39	0/36	0/39	0/39	0/39	0/39	0/156 (0)	0/153 (0)
Amoxicillin[†]	0/39	0/39	0/39	1/36	0/39	1/39	0/39	3/39	0/156 (0)	5/153 (3.3)
Ciprofloxacin	0/39	0/39	0/39	0/36	0/39	0/39	0/39	0/39	0/156 (0)	0/153 (0)
Gentamicin	0/39	0/39	0/39	0/36	0/39	0/39	0/39	0/39	0/156 (0)	0/153 (0)
Nalidixic acid	0/39	0/39	0/39	0/36	0/39	0/39	0/39	0/39	0/156 (0)	0/153 (0)
Ceftiofur	0/39	0/39	0/39	1/36	0/39	1/39	0/39	2/39	0/156 (0)	4/153 (2.6)
Sulfisoxazole	36/39	39/39	33/39	30/36	35/39	37/39	38/39	35/39	142/156 (91.0)	141/153 (92.2)
Sulfamethoxazole*	1/39	0/39	0/39	0/36	0/39	0/39	0/39	2/39	1/156 (0.6)	2/153 (1.3)
Kanamycin	1/39	0/39	0/39	0/36	0/39	0/39	0/39	0/39	1/156 (0.6)	0/153 (0)
Ampicillin	3/39	3/39	3/39	1/36	3/39	4/39	2/39	8/39	11/156 (7.0)	16/153 (10.5)
Streptomycin	3/39	0/39	6/39	3/36	3/39	4/39	4/39	3/39	16/156 (10.3)	10/153 (6.5)

* with trimethoprim
[†] with clavunic acid
[a, b] Data with different superscript letters are significantly different, $P < 0.01$

Table 6-4. Multidrug resistance (≥ 5 antimicrobials) prevalence (%) in fecal *Escherichia coli* from steers fed diet supplemented with or without 0.3% menthol

|	Number of MDR isolates/total isolates tested				
	Day 0	**Day16**	**Day23**	**Day 30**	**Total (%)**
Control	0/39	1/39	1/39	0/39	2/156 (1.3)
0.3% Menthol	0/39	1/36	4/39	5/39	10/153 (6.5)

Table 6-5: Prevalence of *tet*A-positive fecal *Escherichia coli* in steers fed diets supplemented with or without 0.3% menthol

	Number of *tet*A-positives isolates/total isolates tested				
	Day0	Day16	Day23	Day30	Total (%)
Control	7/39	6/39	6/39	4/39	23/156 (14.7)
0.3% Menthol	1/39	7/36	8/39	3/39	19/153 (12.4)

Table 6-6: Prevalence of *tet*B-positive fecal *Escherichia coli* in steers fed diets supplemented with or without 0.3% menthol

	Number of *tet*B-positive isolates/total isolates tested				
	Day0	Day16	Day23	Day30	Total (%)
Control	5/39	5/39	2/39	6/39	18/156 (11.5)
0.3% Menthol	16/39	3/36	6/39	15/39	40/153 (26.1)

Figure 6-2 Fecal *Escherichia coli* isolates phenotypically resistant to tetracycline but not carrying either *tet*A or *tet*B gene, from animal receiving diets with (■) or without (□) 0.3% menthol. SEM = 0.07.
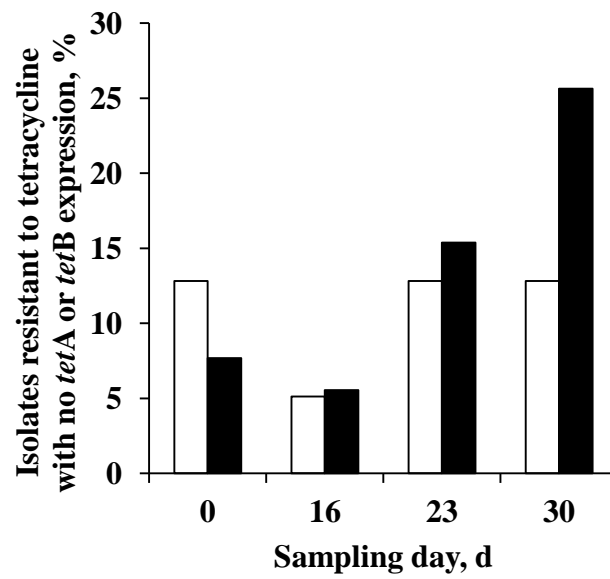
Table 6-7: Overall phenotype prevalence of antibiotic resistant in *Escherichia coli* isolates from cattle receiving diet with or without 0.3% of menthol.

Phenotypes	0% Menthol	0.3% Menthol	Total (%)
_FIS	89	66	155 (50.2)
_FIS_TET	35	52	87 (28.2)
No resistance	10	6	16 (5.2)
_STR_FIS_TET	5	6	11 (3.6)
_TET	4	6	10 (3.2)
_AMP_STR_FIS_TET	9	0	9 (2.9)
_AMP_FIS_TET	0	6	6 (1.9)
_AMP_CHL_STR_FIS_TET	2	3	5 (1.6)
_AUG_AMP_FOX_XNL_FIS_TET	0	3	3 (1.0)
_AMP_CHL_FIS_TET_SXT	0	2	2 (0.6)
_AUG_AMP_FOX_FIS_TET	0	1	1(0.3)
_AUG_AMP_FOX_XNL_CHL_STR_FIS_TET	0	1	1 (0.3)
_CHL_FIS	0	1	1 (0.3)
_CHL_FIS_SXT	1	0	1 (0.3)
_CHL_KAN_FIS_TET	1	0	1 (0.3)

Amoxicillin-clavulanic acid, AUG; ampicillin, AMP; azithromycin, AZI; ciprofloxacin, CIP; cefoxitin, FOX; ceftiofur, XNL; ceftriaxone, AXO; chloramphenicol, CHL; gentamicin, GEN; kanamycin, KAN; nalidixic acid, NAL; streptomycin, STR; sulfamethoxazole, SXT; sulfisoxazole, FIS; tetracycline, TET.