1-1-2012

Validation of Bioluminescent Escherichia Coli O157:H7 for Use as a Pre-Harvest Food Safety Model

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VALIDATION OF BIOLUMINESCENT *ESCHERICHIA COLI* O157:H7 FOR USE AS A PRE-HARVEST FOOD SAFETY MODEL

By

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A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Agriculture and Life Sciences
in the Department of Animal and Dairy Sciences

Mississippi State, Mississippi

May 2012

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Title of Study: VALIDATION OF BIOLUMINESCENT *ESCHERICHIA COLI* O157:H7 FOR USE AS A PRE-HARVEST FOOD SAFETY MODEL

Pages in Study: 95

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Cattle are naturally colonized by enterohemorrhagic *Escherichia coli* within the gastrointestinal tract. The most notorious of the enterohemorrhagic *E. coli* is *E. coli* O157:H7, which can cause serious illness to humans if ingested. To ensure that the United States has a safe food supply, research is ongoing in pre-harvest food safety and pathogen intervention strategies. While advances in pre-harvest intervention strategies are encouraging, no method has proven to completely eliminate and/or control O157:H7. A key limitation to successful pathogen intervention strategies is the inability to track and monitor pathogens in a real-time fashion. Through the use of bioluminescent plasmids harboring the *lux*CDABE cassette, pathogen tracking could be a viable solution. Bioluminescent plasmids are capable of facilitating the tracking, pathogenesis and physical locations of pathogens, thus enabling researchers to have a better understanding of the pathogenic process.

DEDICATION

I would like to dedicate this thesis and all the work required to obtain my Masters degree to my family and especially to the most important person in my life, my fiancé Zack. Thank you Dad and Mom for always being supportive in anything I have ever done and thank you for always giving me that extra push and love I needed most of the time. My little brother Derek, thank you for always being that quiet but yet supportive brother. Thank you to my little sister Jessi for always giving me something to laugh at when I needed it and someone to talk to when I needed it! Grandma and Grandpa, thank you for always being there for me and attending every event I have ever had, whether it is a meeting, sports game, graduation or anything else. I couldn't have done it without any of you. I would also like to thank my Aunt Carmen and Teagan for all the support you have given me in my life, I love you! Finally, I owe a special thank you to my fiancé Zack for all the pain and torture I have put you through during my Masters degree. You have been the rock that has helped me get through this. I appreciate everything you have done for me and everything you have put up with! I love you and thank you for everything.

ACKNOWLEDGEMENTS

I have a lot of people to thank for all the help I have received throughout my Masters degree. During my time here at Mississippi State University I have had the great pleasure of working with some extremely great people. I would like to thank the graduate and undergraduate students who have helped me along the way. Thank you to Katie Pfeiffer, Badger, for always being there and being a great friend. Landon Marks, Cheese, for always giving me something to laugh at and shake my head at. Also, thank you for never letting any of us miss lunch! Rand Broadway, for all the help and insight you have given me on my research projects. My academic big brother Joe Buntyn and big sister Shollie Falkenberg, thank you for your honesty and always looking out for me.

I would also like to thank Dr. Carolyn Huntington for allowing me to be a teaching assistant for Introduction to Animal Science. It was a great opportunity and I wouldn't trade it for the world. Thank you to all my undergraduate students who kept me on my toes and always, some way or another, made me laugh.

To my committee, Dr. Janet Donaldson, Dr. Todd Callaway, Dr. Mike Martin and Dr. Ty Schmidt thank you for your advice and guidance along the way. My research couldn't have been possible without all of your help. Dr. Donaldson, thank you for all the time and effort you have put into understanding the Animal Science language and techniques. I am sad to say that the rumen and fecal fluid is now in your hands! You have been a great help to me and you were always there whenever I needed anything. Dr. Callaway, thank you for all the knowledge and insight you have given me over the past

two years on all my projects. Your sense of humor is always needed and is always a good sigh of relief. Dr. Martin, thank you for always giving great advice and a history lesson, you are one of the most knowledgeable people I know. Finally, my major professor Dr. Schmidt, thank you for always being there for me and everything you have ever done for me. You have been a great professor, researcher and mentor. Thank you for taking me into your lab and giving me a chance to figure out what this graduate school thing is all about. You have given me many opportunities and helped me gain many acquaintances along the way, and I am truly thankful for that.

TABLE OF CONTENTS

Page

LIST OF TABLES

TABLE Page

LIST OF FIGURES

FIGURE Page

CHAPTER I

INTRODUCTION

In 2011, *Escherichia coli* O157:H7 was responsible for the recalls of an estimated 1 million pounds of ground beef due to possible contamination (USDA-FSIS, 2011). *Escherichia coli* O157:H7 was first identified as a pathogen in 1982 (Wetzel and LeJeune 2006) and was linked to undercooked meat. The primary means for human contraction of *E. coli* O157:H7 is through the ingestion of raw or undercooked meat, water runoff from cattle environments (Armstrong et al., 1996) and/or animal-to-person contact (Wetzel and LeJeune, 2006).

Cattle are a reservoir for O157:H7 and contribute to the spread, as well as the epidemiology of human O157:H7 infection (Griffin and Tauxe, 1991; Sargeant et al., 2006). *Escherichia coli* O157:H7 has the ability to colonize within the gastrointestinal tract of cattle without inflicting clinical illness and subsequently enabling cattle to become a host for O157:H7 (Low et al., 2005). Cattle lack the globotriaosylceramide (Gb_3) receptor shiga toxins within the large intestine bind to, thus why cattle are able to harbor O157:H7 within the gastrointestinal tract but remain symptomatic (Hoey et al., 2002). *Escherichia coli* O157:H7 prevalence studies estimate that approximately 30% of cattle throughout the United States harbor this pathogen in their feces (Elder et al., 2000; Callaway et al., 2003).

Many pre-harvest strategies have been implemented by researchers to potentially reduce and/or eliminate O157:H7 within cattle such as vaccinations, direct-fed microbials

and diet manipulations. Although some intervention strategies have proven to reduce O157:H7 populations, none have completely eradicated O157:H7 or proven to be repeatable and consistent. One of the main limitations associated with pre-harvest intervention development is the inability to physically track O157:H7 in real-time.

A potential solution to the inability to monitor pathogens is through the implementation of bioluminescent plasmids. Bioluminescent plasmids that contain the *lux*CDABE operon encode enzymes responsible for synthesis of the luciferase (Meighen, 1993). These plasmids can then be introduced into pathogens, which can then be monitored through an intensified charged-couple device camera (Ryan et al., 2011). Employing bioluminescence *in vivo* can potentially facilitate tracking of pathogens (Contag et al., 1997), allowing for the indication of the physical location (Siragusa et al., 1999) and thus increasing researchers understanding of pathogenic processes (Contag et al., 1995) of O157:H7.

CHAPTER II

LITERATURE REVIEW

Escherichia coli O157:H7 and pre-harvest food safety

The bacterium *Escherichia coli* (*E. coli*) is one of the most commonly isolated bacteria in throughout the meat and food industries (Matic et al., 1997). *Escherichia coli* are gram-negative, facultative anaerobic, rod-shaped bacteria that are non-sporulating with an optimal growth at 37°C. There are over 700 known serotypes of *E. coli* (Ray and Schaffer, 2011). Serotyping as a means of identifying various serovars of *E. coli* is achieved by serotyping by antigens. In example *E.coli* O157:H7 indicates that the O antigen encompasses the cell wall outer membrane or somatic antigen number 157, while the H antigen indicates the flagella antigen 7. The O antigen is part of the lipopolysaccharide present in the outer membrane of bacteria (Sheng et al., 2008).

While most *E.coli* are non-pathogenic, certain serotypes of *E. coli* can be pathogenic; these pathogens account for devastating foodborne related diseases throughout the world (Doyle, 1991; Hilborn et al., 1999; Vogt and Dippold, 2005). Nonpathogenic *E. coli* are cultured in the normal flora of warm blooded animal's gastrointestinal (GI) tracts (Rembacken et al., 1999; Guarner and Malagelada, 2003). Nonpathogenic *E. coli* can also prevent pathogenic *E. coli* from colonizing within the intestine by providing a protective barrier (Hudault et al., 2000; Reid et al., 2001). Nonpathogenic bacteria can provide colonization resistance such as a protective barrier,

in which bacteria can uphold an ecological balance and prevent pathogenic bacteria from colonizing through competitive exclusion (van der Waauj et al., 1971; Rolfe, 2000).

Growth and reproduction of *Escherichia coli*

Typical growth patterns of *E. coli* and other bacteria are measured in four different phases: lag phase, log phase, stationary phase and death phase (Roszak and Colwell, 1987). When bacteria are introduced to medium, adjustment to the surrounding environment and growth conditions will occur, thus representing lag phase (Baranyi, 2002). The log phase or also known as exponential phase is characterized by cell growth, where bacteria exhibit continual doubling bacterial populations (Jensen and Hammer, 1993) until the nutrients of the medium utilized are depleted and cellular waste products accumulate (Kolter et al., 1993). When the growth rate begins to decrease due to lack of nutrients, bacteria will enter stationary phase. Stationary phase is characterized by a stable and constant rate of bacterial growth that is equivalent to the rate of bacterial death (Delpy et al., 1956). After all nutrients have been depleted death phase emerges and bacteria will begin to die.

Bacteria such as *E. coli* reproduce by means of binary fission. The single bacterium will elongate during the growth phase, reaching twice its length, and binary fission will occur (Angert, 2005). The single cell bacterium's DNA replicates and segregates into two identical chromosomes (Donachie, 2001). After the chromosomal segregation within the bacterium the individual chromosomes will move to opposite ends of the bacterium (Donachie, 2001). This allows for cytokinesis, which involves the actual splitting of the bacterium into two identical daughter cells (Angert, 2005).

Escherichia coli virulence groups

Pathogenic *E. coli* can be broken down into five groups, based on virulence attributes (Johnson, 1991). These five groups are enterotoxigenic (ETEC), enteroaggregative (EAggEC), enteropathogenic (EPEC), enteroinvasive (EIEC) and enterohemorrhagic (EHEC; Levine, 1987). The ETEC utilize fimbriae, which are hair-like structures on the outer membrane and are shorter than flagella. The ETEC use fimbriae to attach to the specific receptors on enterocytes within the intestinal lumen (Levine and Edelman, 1984). The ETEC virulence group can produce heat-stable and/or heat liable enterotoxins (Moseley et al., 1980). This virulence group is the primary cause of traveler's diarrhea which results from an accumulation of fluid and electrolytes within the GI tract lumen (Levine, 1987). Common ETEC serogroups include O8, O139, O141, O147 and O149 (Nagy and Fekete, 1999).

Enteroaggregative *E. coli* (EAggEC) or also known as enteroadherent, are one of the newest *E. coli* virulence groups. The EAggEC have fimbriae similar to ETEC that attach to the intestinal epithelial cells and some strains are capable of producing a heat-stable enterotoxin (Kaper et al., 2004). The result from an EAggEC infection results in watery diarrhea, and is usually observed in children in developing countries (Nataro et al., 1995). In comparison to the other virulence groups, EAggEC has a longer incubation period within humans, which has been estimated to be 20 to 48 hours (Steiner et al., 1998). Commonly isolated EAggEC serogroups include O44 and O3 (Steiner et al., 1998).

The enteropathogenic *E. coli* (EPEC) possess the *eae* gene, responsible for the attachment and effacement (A/E) lesions that occur on the intestinal lining of the host (Kaper et al., 2004). The EPEC group does not generally produce enterotoxins, but can

adhere to the intestinal mucosa (Levine et al., 1978). The EPEC are commonly observed in infant's diarrhea (Levine and Edelman, 1984). Common serogroups within the EPEC virulence group are O111 and O55 (Levine et al., 1978).

Enteroinvasive *E. coli* (EIEC) are extremely invasive serotypes of *E. coli* that utilizes adhesion proteins to bind and enter the intestinal cells (Resta-Lenert and Barrett, 2003). Although EIEC do not produce any toxins, the intestinal wall is severely damaged by the rapid colonization and implementation of adhesion proteins within the intestinal epithelial cells (Resta-Lenert and Barrett, 2001). Patients infected with EIEC usually have symptoms that include bloody voluminous diarrhea, blood and mucus in the stool, abdominal cramps, vomiting, fever, chills and malaise (Simonovic et al., 2000). Common serogroups of EIEC include O124, O28 and O112 (Pupo et al., 1997).

Enterohemorrhagic *E. coli* (EHEC) are the most widely known virulence group. This group of *E. coli* possesses the *eae* gene that produces the A/E lesions responsible for producing shiga-like toxins. The EHEC virulence group utilizes the protein, *intimin*. *Intimin* aids in the adhesion of A/E lesions to the microvillus of the intestinal wall by intimately attached to the translocated intimin receptor (*tir*). Susceptible cells within the large intestine have shiga toxin receptors that allow binding of the shiga toxins. These receptors are the globotriaosylceramide (Gb_3) receptors, part of the glycolipid globotriaosylceramide family. Monogastrics, including humans, have Gb_3 receptors whereas cattle lack the Gb_3 receptor (Pruimboom-Brees et al., 2000).

The Gb_3 receptors are established within the entire GI tract (duodenum, ileum, jejunum, colon and cecum) and within the kidney (Hoey et al., 2002). When shiga toxins bind to the Gb_3 receptor, the shiga toxin is then able to enter the cell and inhibit protein synthesis (Ling et al., 1998). The adherence of the shiga toxin to the intestinal mucosa

causes a rearrangement of actin in the host cell, causing significant deformation and death of the host cells (Strockbine et al., 1986). The deformed cells are sloughed off the intestinal lining into the intestinal tract which leads to accumulation of fluids subsequently causing diarrhea (Griffin and Tauxe, 1991). The damage to the intestinal lining of the host may lead to hemorrhaging, evident by bloody diarrhea (Riley et al., 1983). In severe cases, EHEC may enter the bloodstream causing septicemia, renal failure, hemolytic uremic syndrome (HUS) or thrombotic thrombocytopenic purpura (Griffin and Tauxe, 1991). Common serogroups of EHEC are O157, O26, O145, O103 and O111 (Fagen et al., 1999).

Escherichia coli O157:H7

The EHEC serotype *Escherichia coli* O157:H7 was first associated with a major foodborne outbreak in 1982 and has since been commonly linked to foodborne cases associated with the consumption of contaminated, undercooked meat (Wetzel and LeJeune, 2006). In 2011, there was an estimated 1 million pounds of ground beef recalled due to possible O157:H7 contamination (USDA-FSIS, 2011). In 2010, Scharff reported O157:H7 cost United States residents approximately $1 billion in premature deaths, lost wages and hospital bills, totaling approximately $15,000 per case of O157:H7 illness (Scharff, 2010). Severity of illness can affect cost as well, ranging from $26 for an individual who did not receive medical care to $6.2 million for a death of a patient that suffered from HUS (Frenzen et al., 2005).

According to the Center for Disease Control (CDC), 265,000 shiga toxin-producing *Escherichia coli* infections were documented in 2010, of which O157:H7 comprises 33% of these infections (CDC, 2011). The O157:H7 serotype is a virulent

serotype with an infectious dose of ≤ 50 cells (Besser et al., 1999). Children and the elderly are the most susceptible to infections due to their weakened immature immunological state (Fox et al., 2009). It is because of the severity associated with consumption of contaminated food products that the food and meat industries have invested more than $2 billion in intervention strategies within the processing plant (Kay, 2003; Koohmaraie et al., 2005).

Infection due to O157:H7 generally occurs through ingestion of contaminated products. Although the most common route of human O157:H7 infection is through the consumption of raw or undercooked meat, infections can result from other sources as well. For instance water runoff from ruminant environments (Armstrong et al., 1996) contaminated with feces used to irrigate crops (i.e. spinach and lettuce), the products can potentially become contaminated. Direct human interactions within various environments, such as cattle or other livestock facilities (Wetzel and LeJeune, 2006), petting zoos and swimming pools have also been suggested as potential environments where inoculation of O157:H7 can occur (Rangel et al., 2005).

Shiga toxin-producing *Escherichia coli* (STEC)

While most research of enterohemorrhagic *E. coli* (EHEC) has traditionally focused on O157:H7, there has been an increase in outbreaks of non-O157 strains of shiga toxin-producing *E. coli* (STEC). In fact, the CDC estimates that nearly 77% of all *E.coli* associated infections are due to non-O157 serogroups. The six additional STEC serogroups that the food and meat industry are concerned with include: O26, O45, O103, O111, O121 and O145 (Paddock et al., 2011). It has been traditionally believed that these non-O157 serogroups grow similarly as O157:H7 (Beutin et al., 1994). However, a

study recently conducted within our laboratory group suggests that the responses of these non-O157 serogroups are not universally the same as O157:H7.

The characterization of STEC involves each serogroup having capabilities to produce shiga toxins (Fratmico et al., 2004). Shiga toxins are compound toxins consisting of one A subunit and five B subunits which are linked by a disulfide bond (Paton and Paton, 1998). The A subunit inhibits protein synthesis by removing an adenine from the ribosome (O'Brien et al., 1987). The B subunits bind to the glycolipid receptors on the surface of the target cell and aids in endocytosis (O'Brien et al., 1987). Once the shiga toxin crosses the epithelial barrier within its host, it will then enter the bloodstream and target the Gb_3 receptors, thus inhibiting protein synthesis and causing lysis of the cells (Paton and Paton, 1998).

The STEC can produce shiga toxin I (*stx*$_1$), and/or, shiga toxin II (*stx*$_2$). Both of which have virtually the same biological activities (Strockbrine et al., 1986). The *stx*$_1$ is serologically identical to the *Shigella dysenteriae* toxin (O'Brien et al., 1987). The *stx*$_2$ can be serologically differentiated from *stx*$_1$ and *Shigella dysenteriae*, although all biological activities are similar (Strockbine et al., 1986). Studies have suggested that *E.coli* that produce *stx*$_2$ are more virulent than *E. coli* that produce *stx*$_1$ (Dorn et al., 1989; Wadolkowski et al., 1991; Arthur et al., 2002). The increase in virulence and association of *stx*$_2$ to HUS is thought to be from the increased transcription rate *in vivo*, as opposed to *stx*$_1$ and the combination of both (Paton and Paton, 1998).

The O26 STEC harbors the *stx*$_1$ gene most predominately, but some strains of O26 are able harbor the *stx*$_2$ or the combinations of both genes (Arthur et al., 2002). The *stx*$_1$ gene can be harbored by O103, O45, O111, O121 and O145. Some STEC can harbor both shiga toxins such as O45, O111, O111 and O145. Although able to harbor the

combination of both shiga toxins, O45 and O111 cannot exclusively harbor *stx*$_2$ alone (Kaper et al., 1998)

Certain strains of STEC are capable of producing A/E lesions on enterocytes that rearranges the host actin, disassembling the microvillus and forming actin pedestals (Francis et al., 1986). All genes, including shiga toxin genes required for the A/E adherence and lesions are located on a single pathogenicity island referred to as the locus for enterocyte effacement (LEE; Donnenberg et al., 1997). The LEE is a cluster of genes that encode for a type III secretion system which aids in expressing the *eae* genes (O'Brien et al., 1987). The type III secretion system secretes translocated effector proteins such as EspA, EspB and EspD which are responsible for the initiation of signal transduction events (Paton and Paton, 1998). The *eae* gene is responsible for encoding the outer membrane protein, *intimin* and its receptor *tir*, which the binding of both proteins is responsible for the intimate attachment to the enterocyte (O'Brien et al., 1987; Kenny et al., 1997). *Enterohemolysin* is expressed through the *eae* gene and lyses erythrocytes to acquire iron to support growth of STEC (Paton and Paton, 1998). These virulence factors are responsible for A/E of the intestinal surface and can aid in virulence, but are not necessarily the cause of severe human illnesses (Donnenburg, 1993). Although these factors are characteristics of O157:H7, research suggests these factors are more common in pathogenic non-O157 strains, such as STEC (Beutin et al., 1994).

In 2010, the incidences of O157:H7 decreased to reach the national target of ≤ 1 case per 100,000 (CDC, 2011). Implementing pathogen prevention strategies in the abattoir and further processing plants such as hide and carcass washes, antimicrobial treatments, HACCP policies, cleanliness and good sanitation practices have lead to decreased O157:H7 populations (Elder, 2000). While the O157:H7 related outbreaks in

humans and animals have decreased, there has been an observed increase in non-O157 incidences. In response to this increase in prevalence, starting June of 2012 the USDA-FSIS will classify the STEC serotypes O26, O45, O103, O111, O121 and O145 as adulterants in non-intact raw beef and prohibit sale of any contaminated products (USDA-FSIS, 2012).

Role of enterohemorrhagic *Escherichia coli* in ruminant animals

Enterohemorrhagic *E. coli* are capable of colonizing within the GI tract of many ruminants (Callaway, 2002). Cattle are considered a major reservoir of O157:H7 (Low et al., 2005; Hussein et al., 2006; Ferens and Hovde, 2011). Cattle are asymptomatic carriers and play a role in the epidemiology of human O157:H7 infections (Griffin and Tauxe, 1991; Sargeant et al., 2006). One fecal prevalence study's results have suggested that approximately 30% of cattle are carriers of O157:H7 (Elder et al., 2000; Callaway et al., 2002). However, this can vary due to individual animal and season (Elder et al., 2000).

Studies have suggested that the large intestinal environment of cattle is selective for O157:H7 growth (Enss et al., 1996; Fox et al., 2009). This selective environment is related to GI tract mucosal components: fucose, galactose, mannose, *N*-acetylgalactosamine, *N*-acetylglucosamine, galacturonic acid, glucuronic acid and gluconic acid (Peekhaus and Conway, 1998; Fox et al., 2009). Mucus and its glycoprotein constituent, mucin are secreted within the large intestine and are responsible for the defense mechanisms to prevent colonization of pathogenic bacteria. Although a study conducted by Enss et al. (1996) indicated that another STEC serogroup O55 (Ludwig et al., 1996), more specifically the O55:B5 strain was able to modify mucin,

thus creating a more favorable condition for growth of bacteria by aiding in A/E mechanisms. These results suggest that other STEC such as O157:H7 may alter mucin to increase specific carbohydrate constituents, hence permitting O157:H7 to survive in the large intestine of cattle (Fox et al., 2009). Research has suggested that these mucosal components promote colonization of *E. coli* (K-12 and F-18) in the mouse model, but not specifically O157:H7 (Peekhaus and Conway, 1998; Chang et al., 2004). Although a more recent study conducted by Fox et al. (2009) suggested that more specifically gluconic acid may serve as a growth prominent for O157:H7 in the large intestine of beef cattle. These data further enhance the hypothesis that the extracellular components of the mucosal cells, such as mucin are able to serve as growth factors for O157:H7 (Miranda et al., 2004).

Escherichia coli O157:H7 has also been isolated from the oral cavity (Keen and Elder, 2002), digesta (Laven et al., 2003), ruminal contents (Elder et al., 2000), and feces (Elder et al., 2002; Keen and Elder, 2002). Some studies have suggested that the colon (Grauke et al., 2002) and the lymphoid tissue located at the recto-anal junction (Naylor et al., 2003) may be the primary colonization sites within the ruminants. Researchers have suggested that the recto-anal junction is very closely involved with O157:H7 colonization (Greenquist et al., 2005; Davis et al., 2006; Lim et al., 2007) due to O157:H7 specificity for follicle-associated epithelium within the recto-anal junction (Naylor et al., 2003).

Various serogroups of STEC (O7, O91, O146, O113, etc.) have been isolated from beef cattle (Beutin et al., 1997; Leomil et al., 2003), manure (Lahti et al., 2002) and raw beef (Kahn et al., 2002). Hussein and Bollinger (2005) indicated that the prevalence of non-O157 STEC were increased compared to O157:H7 in packing plants, supermarkets and fast food restaurants.

Escherichia coli O157:H7 shedding in cattle

Shedding of O157:H7 in cattle appears to very sporadic (i.e. animal, season, time) and widespread (Meyer-Broseta et al., 2001). The amount and frequency of O157:H7 shedding varies greatly due to individual animal and season (LeJeune et al., 2004; Stanford et al., 2005). Although a number of various factors affect pathogen shedding, the only factor that has consistently been repeated is season (Edrington et al., 2006). In shedding prevalence studies, results suggest that typically, there is an increase in fecal shedding in spring, with potential peaking at 80% in feedlot cattle in the summer months; then falling drastically in the fall months with least amount of fecal shedding, 5-10% in the winter months (Elder et al., 2000; Barkocy-Gallagher et al., 2003; Edrington et al., 2006). Outbreaks of O157:H7 in humans seem to shadow this seasonal trend, primarily occurring in the summer months (Rangel et al., 2005). Cattle that are considered shedders of O157:H7 shed at a rate of $<10^2$ CFU/g of feces (Omisakin et al., 2003), whereas cattle that are considered super shedders can shed at least 10^4 CFU/g of feces (Chase-Topping et al., 2008).

Prevalence of *Escherichia coli* O157:H7 on cattle hides

Cattle can become infected and continually shed O157:H7 in feces up to 49 days (Wang et al., 1996). Therefore, O157:H7 can spread to other cattle through physical contact; such as animal-to-animal or animal-to-environment (Collis et al., 2004; Wetzel and LeJeune, 2006). These two routes are frequently the source of spread within the feedlot and transportation (Cuesta Alonso et al., 2007). Studies have indicated an increase in the prevalence of O157:H7 on cattle hides, specifically during transportation (Collis et al., 2004; Stanford et al., 2011).

Cattle within confined spaces with greater numbers of cattle tend to have more filth and fecal matter on hides (Arthur et al., 2009). Cattle hides at the time of harvest have been suggested to be one of the major sources of O157:H7 contamination on beef carcasses due to the increase fecal matter on the hides arriving at the abattoir (Nou et al., 2003; Bosilevac et al., 2005; Arthur et al., 2007). It has been reported that concentrations of *E. coli*, including O157:H7, can range from 10^2 to 10^7 CFU/g of feces at harvest (Jordan and McEwen, 1998). Cattle with potentially contaminated hides that arrive at the harvesting facilities increase the risk of microbial contamination of the meat (Bacon et al., 2000; Elder et al., 2000; Midgley and Desmarchelier, 2001). Even though carcasses are considered sterile after the hide has been removed, further contamination can occur through fabrication, viscera removal (Tutenel et al., 2003) and through contact with contaminated workers (Brackett, 1999).

Escherichia coli pre-harvest intervention strategies

Controlling and/or eliminating fecal shedding in cattle has been a major goal in pre-harvest food safety. Intervention strategies have been installed within harvesting facilities, but still do not completely guarantee elimination of O157:H7, thus why researchers have put an emphasis on pre-harvest intervention strategies. Currently there are no pre-harvest interventions strategies that have been successful because strategies have not been repeatable and consistent or cost effective to the producer. According to Callaway (2010), pre-harvest intervention strategies can be divided into three basic categories: management practices and transport, cattle water and feed management and live animal treatments.

Management practices and transport

Fundamental animal husbandry practices have long been essential to maximize production efficiency and ensure animal health and welfare. Although O157:H7 is commonly isolated from a host, O157:H7 can survive within the environment as well. Studies have reported that O157:H7 can survive anywhere from 3 to 130 days within the soil (Maule, 2000).

Biosecurity factors also play a role in the O157:H7 spread. Cattle are not the only animals that able to harbor O157:H7 and all EHEC, pest such as rodents, birds, insects and flies can also harbor O157:H7 and can aid in the spread and epidemiology (Rice et al., 2003; Wetzel and LeJune, 2006; Ahmad et al., 2007). Allowing other domesticated livestock animals on the farm (i.e. goats and sheep), pets (i.e. dogs and cats) and unintentional wild animals and fowl (i.e. deer, ducks, geese, and pheasants) have been associated with increased O157:H7 shedding within cattle (Synge et al., 2003; Gunn et al., 2007; French et al., 2010).

Subsequently, O157:H7 is commonly indicated within a group of cattle as opposed to an entire farm, therefore it is suggested that cattle be placed within contemporary groups throughout the duration on the farm. Overcrowding of cattle within a group or on a livestock trailer increases the possibility for fecal O157:H7 transmission. The trailers used during transportation of potential carriers of O157:H7 are effortless pathways for the spread of pathogens (Mather et al., 2007). The trailers used to transport cattle from the feedlot to packer are frequently reported to test positive for O157:H7 but washing trailers did not decrease prevalence (Cuesta Alonso et al., 2007; Reicks et al., 2007).

Cattle water and feed management

Water sources shared by cattle are a common place where O157:H7 can be isolated. *Escherichia coli* O157:H7 can survive for up to six months within water sources (Hancock et al., 1998; LeJeune et al., 2001). Chlorination of the water supply within cattle water troughs has been tested as a possible solution to controlling the spread of O157:H7. Consequently, outside environmental factors such as sunlight and organic matter contribute to the decrease of effectiveness of chlorination (LeJune et al., 2004). Chlorination has led to cattle refusal to drink treated water because of organic matter accumulation and the distinct chlorine smell and taste. Electrolyzing water is another intervention strategy implemented in cattle drinking water. This involves the electrolysis of deionized water with free-chlorine concentrations to decrease bacterial populations (Shimamune et al., 1996). Electrolyzing water has been suggested to reduce O157:H7 concentrations as an in-plant hide cleaning strategy (Bosilevac et al., 2005), but not as a water source strategy.

Dietary manipulation has been a pre-harvest area researched in an attempt to reduce the O157:H7 burden before transportation and at the abattoir. Previous research by Wolin (1969) suggested that increased concentrations of volatile fatty acids (VFA) and a decreased pH could possibly inhibit *E.coli* growth. The toxicity of VFA on *E.coli* and other bacteria is due to the anion accumulation within the cell membrane when the pH is decreased (Russell, 1992). Whole cottonseed has been linked to reduce O157:H7 shedding because of the increase in short-chain VFA concentrations observed (Garber et al., 1995). Corn silage, addition of animal by-products (i.e. blood meal, meat meal, feather meal; Herriott et al., 1998) barley (Dargatz et al., 1997), distiller's grains (Jacob et al., 2008; Yang et al., 2010) and brewer's grain (Dewell et al., 2005) are feed

ingredients that have been linked to increased fecal shedding of O157:H7 in cattle. The fecal shedding is suggested to increase with use of these products because of the decreased effect on the short-chain VFA concentrations.

There has also been extensive research in the last decade on whether concentrate or forage diets are advantageous at reducing shedding of O157:H7. Concentrates are typically fed to feedlot cattle which are observed to have the greatest frequency of shedders (Tkalcic et al., 2000). Steam-flaked concentrates have indicated to increase O157:H7 shedding, while dry rolled concentrates have reduced shedding (Fox et al., 2007). Interestingly, greater concentrations of O157:H7 were observed within the feces of concentrate-fed animals compared to forage-fed animals (Tkalcic et al., 2000). Forage-fed animals were observed to shed O157:H7 for longer periods of time; 60 days for forage-fed as opposed to 16 days grain-fed (Van Baale et al., 2004). The O157:H7 proliferation due to potential variation in VFA concentrations suggests that cattle fed concentrates shed greater concentrations of O157:H7 (Tkalcic et al., 2000).

The amount and quality of forage also is important in dictating the amount and frequency of shedding. Poor quality forages (i.e. straw) exhibited a faster and greater death rate of O157:H7 compared to superior quality forages (i.e. grass silage; Franz et al., 2005). When cattle are suddenly changed from a full grain diet to a full forage diet, O157:H7 populations decreased (Diez-Gonzalez et al., 1998).

Feed additives have been researched to attempt to reduce the populations and patterns of O157:H7 shedding. Plant based products such as phenolics, essential oils and tannins have been evaluated for O157:H7 shedding reduction and/or elimination. Min et al. (2007) reported that secondary metabolites from natural phenolic compounds exhibit bactericidal activities in gram-negative bacteria. Research conducted with tannins has

reported to decrease populations of O157:H7 *in vitro*; although only concentrations of generic *E. coli* within the live animal were reduced (Min et al., 2007). The Federal Drug Administration has recognized essential oils as generally safe for human and animal consumptions (FDA, 2004). Essential oils contain microbial activity and are common components responsible for fragrance (Benchaar et al., 2008). Essential oils from plants are added to diets in attempt to reduce O157:H7 concentrations because of the toxicity exhibited on the cell wall of gram-negative bacteria such as O157:H7 (Dusan et al., 2006). Essential oils have reduced O157:H7 populations within cattle, while increasing fermentation (Benchaar et al., 2008).

Probiotics have previously been used by researchers to enhance production and health within the livestock (Tournut, 1989). Probiotics have also been proposed as a method to reduce pathogenic bacteria. By definition, probiotics are the use of live or dead cultures of microorganisms to alter the microbial environment of the GI tract. Probiotics within the GI tract, reduce pathogen concentrations by binding to the intestinal epithelium, subsequently preventing pathogens from binding (Collins et al., 1999). There are a few probiotic varieties that have been produced to aid in the reduction of shedding: direct-fed microbials (DFM), competitive exclusion (CE) and prebiotics.

Direct-fed microbials are fed to ruminants to improve ruminal fermentation. Direct-fed microbials are comprised of live or dead yeast, fungal or bacterial culture fermentation end-products (Callaway, 2009). Previous research suggests that *Streptococcus bovis*, *Lactobacillus gallinarum* (Ohya et al., 2001), *Lactobacillus acidilacti*, *Lactobacillus acidophilus* (Brashears and Galyean, 2002) and *Pediococus* (Rodriguez-Palacios et al., 2009), have decreased O157:H7 concentrations in cattle.

Although research in direct-fed microbials is ongoing, the limiting factor is cost to the producer.

The concept of CE includes introducing non-pathogenic bacteria of the same species to the GI tract with intentions of reducing pathogenic bacteria colonization and shedding within the animal (Nurmi et al., 1992). The mechanisms of CE to reduce bacterial populations are to first create a hostile environment for other bacteria to survive within, eliminate bacterial receptor sites, produce and secrete antimicrobial metabolites and select and competitively deplete essential nutrients for other bacteria (Rolfe, 1991). The use of CE has reduced O157:H7 shedding for O111:NM and O26:H11 (Zhao et al., 2003).

Prebiotics are organic compounds that are indigestible by animals. Prebiotics are able to pass through the GI tract until being utilized as nutrient sources by the microbial populations within a specific environment in the animal. Prebiotics provide a competitive environment by competing with the pathogenic bacteria for nutrients and binding sites in the intestine (Zopf and Roth, 1996). Research has proposed that a synbiotic relationship (coupling CE and prebiotics) could reduce O157:H7 concentrations; by combining mechanisms of a competitive environment, elimination of receptors, and production of antimicrobial and depletion of nutrients available to pathogens (Bomba et al., 2002). To date most probiotic treatments have not been implemented due to the great cost associated and lack of producer return.

Colicins are a toxic exoproteins produced by *E. coli* strains that contain very specific cell wall receptors that apply an inhibitory effect on O157:H7, thus destroying or hindering growth by destroying the cell wall (Konisky, 1982; Smarda and Smajs, 1998). Callaway et al. (2004) indicated that colicins were effective in reducing O157:H7 *in*

vitro, while Cutler et al. (2007) indicated that colicins were effective in preventing other *E. coli* besides O157:H7 *in vivo*. Also, colicins have been supplemented to cattle diets, and research has reported a 2 $\log_{10}$ CFU/g in reduction of O157:H7 populations (Nandiwada et al., 2004). Further research in this area is currently on-going but major barriers must be surmounted. Possible barriers include the ability of colicins to become resist to certain strains overtime (Alonso et al., 2000), immunity proteins produced by certain O157:H7 strains against colicins (Murinda et al., 1998), GI tract degradation and the cost associated (Lui et al., 2011).

Live animal *Escherichia coli* O157:H7 treatments

Aside from dietary manipulation, researchers have also investigated live animal pre-harvest treatments. Vaccinations, bacteriophages and sodium chlorate are the primary live animal treatments that are under investigation. The *E.coli* and *Salmonella* vaccinations utilized within swine and poultry have been effective in reducing pathogens (Johansen et al., 2000). Siderophore receptor and porin (SRP) protein vaccines are another vaccination being researched. *Escherichia coli* O157:H7 releases siderophore proteins within the intestinal environment to acquire iron. The SRP protein vaccination targets siderophores to obstruct the iron acquisition, thus leading to O157:H7 death. Recent research has indicated that the SRP injection has reduced O157:H7 concentrations by 98% in the feces (Thomson et al., 2009). The SRP protein vaccination is often not utilized because the cost is too great for producers to implement within production systems.

The bacterial extract vaccination is a three-dose regiment produced from O157:H7 extracts, particularly type III secreted proteins. The host protein type III

secretions release the *tir*, an adhesion protein that will integrate into the cell membrane. Once *tir* protein is established on the O157:H7 cell membrane it becomes a receptor for *intimin*, another adhesion protein necessary for the intimate colonization of O157:H7 (DeVinney et al., 1999). Research has reported that the *tir-intimin* binding is vital in O157:H7 A/E of the intestine (DeVinney et al., 1999; Cornick et al., 2002). The type III secretion vaccination introduces $intimin_{O157}$ antibodies within the host and have decrease A/E lesions and colonization of the intestine of pigs (Dean-Nystrom et al., 2002). Research has indicated that this vaccine reduced O157:H7 shedding within cattle (Potter et al., 2004) and proposed to decrease shedding (McNeilly et al., 2010). A study conducted by Smith et al. (2009) suggested a 92% less likely probability for cattle within feedlot environments to be colonized in the terminal rectum mucosa when administered as a two dose regimen of a type III secretion vaccine. Research suggests that type III secreted protein vaccines and SRP vaccines have reduced fecal prevalence of O157:H7 within cattle that are naturally exposed to O157:H7 (Snedeker et al., 2011). Although, the results of vaccinations are promising and are approved for use in the United States (SRP and bacterial extract vaccines) and Canada (bacterial extract vaccines), producer use will remain uncertain because of cost and economic return and lack of study consistency.

Bacteriophages (phages) are viruses that can invade bacteria within any organism. The idea behind implementing phages in pre-harvest food safety involves phages that have the ability to specifically target host bacteria. When present, phages take over the target bacteria and replicate, continually releasing daughter cells to repeat the process (Callaway et al., 2008b). Exponential growth will continue as long as the target bacteria are present. One limiting factor to phage use is that phages can easily develop resistance.

Bacteriophages have been implemented to decrease O157:H7 populations in the ruminant GI tract (Bach et al., 2003; Callaway et al., 2008b; Rozema et al., 2009) as well as for hide and surfaces washes. Although phage treatments have been successful in reducing O157:H7 population *in vivo* (Bach et al., 2009) implementation on the farm has not been successful due to the cost relative to the producer (Callaway et al., 2008b).

Sodium chlorate has enzymatic properties that allow for beneficial pre-harvest application. Through the nitrate reductase enzyme, chlorate has the ability to reduce nitrate to nitrite. Nitrite reductase also reduces chlorate to chlorite within the cell cytoplasm (Stewart, 1988). An increase in chlorite concentrations within the cytoplasm destroys O157:H7 (Stewart, 1988). The addition of sodium chlorate to ruminal fluid indicated a reduction in O157:H7 concentrations *in vitro* (Anderson et al., 2000). Sodium chlorate supplementations to water supplies have decreased O157:H7 throughout the GI tract in cattle as well as sheep (Anderson et al., 2002; Callaway et al., 2002; Callaway et al., 2003).

Citrus by-products

The citrus production generates by-products for various food and household products, such as pulp and peel that have nutritional value and exhibit antimicrobial properties. Citrus by-products have been added to diets of dairy and beef cattle because of palatability, nutritional value and the relative decreased cost (Arthington et al., 2002). Citrus by-products are usually supplemented at a rate of 5-15% of the ration (Arthington et al., 2002). Citrus by-products are considered an energy, calcium, dietary fiber, and low protein concentrate ingredient, and are fed to cattle in the wet, dry, powdered or pelleted form (Arthington et al. 2002). Citrus by-products are readily available within the citrus-

producing regions such as California, Texas and Florida (Callaway et al., 2008a) and can be stored and transported long distances (Volanis et al., 2006).

Research has suggested that citrus by-products display antimicrobial activity against foodborne pathogens within the host animal (Dusan et al., 2006; Viuda-Martos et al., 2008; Friedly et al., 2009). Phytochemicals, such as limonene and citrullene are the most common and have the greatest microbial activity within citrus by-products (Di Pasqua et al., 2006). These oils are toxic to bacteria by increasing cell membrane permeability (Kim et al., 2005; Fisher and Phillips, 2006; Gill and Holley, 2006; Di Pasqua et al., 2007). The lipophilic properties of essential oils are able to permeabilize the cell wall and cytoplasmic membranes within gram-negative bacteria (Bakkali et al., 2006). The change in the fluidity of the membranes due to the permeabilization, allows essential oils to coagulate the cytoplasm (Gustafson et al., 1998), depleting ATP (Di Pasqua et al., 2006) and leading to lysis of the cell (Kim et al., 2005; Fisher and Phillips, 2006; Gill and Holley, 2006; Oussalah et al., 2006; Di Pasqua et al., 2007).

In vitro studies have suggested that the addition of >1% orange peel and pulp reduced O157:H7 and *Salmonella typhimurium* concentrations within rumen fluid (Callaway et al., 2008a). *In vivo* research conducted with sheep indicated that when feeding a 50/50 ration of orange peel and citrus pellets, the ration reduced inoculated O157:H7 and *S. typhimurium* populations in the rumen (Callaway et al., 2011a; Callaway et al., 2011b). Feeding orange peel and pulp to weanling pigs decreased ileal and cecal populations of diarrheagenic *E. coli*, while completely eliminating concentrations within the rectum (Collier et al., 2010). The *in vitro* and sheep *in vivo* data suggest that citrus by-products can reduce O157:H7 populations within cattle and sheep, more specifically within the rumen. Even though O157:H7 can reside in the rumen, the intestinal tract is

where colonization and shedding occurs (Laven et al., 2003). If citrus by-products can reduce O157:H7 populations within the rumen, it could subsequently reduce populations within the intestinal tract. This could make citrus by-products a dual purpose pre-harvest intervention strategy.

Future of pre-harvest

There have been numerous developments in pre-harvest intervention techniques. These strategies have been implemented on the farm and through the environment. Although cases of O157:H7 incidences have decreased, there has not been a strategy that has proven to completely eliminate O157:H7. The main limitation to pre-harvest food safety intervention techniques is the inability to successfully track pathogens. Biophotonics has been an area that researchers suggest could aid in the knowledge and pathogenesis of bacteria. Biophotonics introduces the idea of bacteria transformed with bioluminescent plasmids that can be detected under a specialized camera. Implementing the use of bioluminescence within the live animal model can serve as a research tool for comprehending the pathogenesis and physiological systems of healthy or diseased animals (Ryan et al., 2011). One of the main limitations to pre-harvest intervention strategies is the inability to recognize and diagnose an individual or a group of cattle that are carriers of O157:H7. Being able to physically track pathogens like O157:H7 will help researchers better understand what physiological events occur *in vivo*, allowing researchers to advance pre-harvest pathogen intervention strategies. Imaging of bioluminescent plasmids offers a rather straightforward, strong, lucrative and extremely sensitive resource for capturing biological *in vivo* processes (Dothager et al., 2009).

Biophotonics and bioluminescence

Biophotonics involves the introduction of a vector containing the *lux*CDABE operon, which encodes enzymes responsible for synthesis of the luciferase enzyme and the aldehyde substrate (Meighen, 1993) into the bacterial strain of interest. Biophotonics combines the scientific fields of biology, photonics and optical imaging. Bioluminescence is the voluntary or involuntary emission of visible light in living organisms that is arbitrated to an enzyme catalyst (Meighen, 1993). An oxidized reaction that involves a luciferase enzyme and a substrate is produced and thus emits light (Zhao et al., 2004). Unique to bioluminescent bacteria is the ability to self-synthesize all substrates within the bacteria themselves for the production of light (Close et al., 2010). In order to generate light, the luciferase protein first binds to the two naturally occurring products within the cell; reduced riboflavin phosphate ($FMNH_2$) and oxygen (O_2). After the protein binds to each individually, it then binds to the synthesized long chain endogenous aliphatic aldehyde, produced by lipid biogenesis. This permits the *lux* cassette to implement the endogenous materials within the cell to form intermediates, in turn oxidizing the intermediates to produce emission of a blue-green light (Meighen, 1991). The overall reaction that is observed can be reviewed as:

$$FMNH_2 + RCHO + O_2 \rightarrow FMH + H_2O + RCOOH + h\nu_{490nm}$$ (Meighen, 1991)

The light that is emitted can be detected using an intensified charge-coupled device camera (Contag et al., 1995) and this camera can detect as few at 50 colonies (Siragusa et al., 1999).

Bacteria transformed with photonic plasmids that constitutively express of *lux*CDABE operon do not exhibit alterations in growth kinetics (Beyer and Bohm, 1996), biochemical mechanisms, serology, or structure in comparison to non-transformed strains

(Chen and Griffiths, 1996). Bioluminescence from bacterial luciferase also correlates well with *in vitro* enzyme activity, as the amount of luciferase protein and *lux* mRNA generated directly correlates with cell viability (Close et al., 2010; Siragusa et al., 1999) bacterial plate counts (Siragusa et al., 1999) and intracellular numbers of bacteria in cell culture (Maurer et al., 2000).

Successful models

Recent studies have adapted this bioluminescent technology for investigating a variety of physiologically relevant systems, including single, living cells (Willard et al., 1999), whole plants (Anderson et al., 1995), *Drosophila* (Brandes et al., 1996), and rodents (Contag et al., 1995, 1997). The ability to track bioluminescent pathogens *in vivo* through the GI tract has previously been successful in pigs, mice and fish (Willard et al., 2002; Burns-Guydish et al., 2005; Karsi et al., 2006; Moulton et al., 2009a). Successful *in vivo* tracking of *Salmonella typhimurium* transformed with the pAK1-*lux* bioluminescent plasmid was achieved within the swine (Willard et al., 2002; Moulton et al., 2009a) and *S. typhimurium* and *E.coli* transformed with various *lux*CDABE operons within the murine (Burns-Guydish et al., 2005; Foucault et al., 2010) GI tract models. *Ex vivo* tracking of *E. coli* transformed with Xen-14 plasmid has been successful in the bovine (Curbelo et al., 2010) and *E. coli*+pAK1-*lux* within the ovine (Moulton et al., 2009a), gerbils (Disson et al., 2008) and equine reproductive tracts (Ryan et al., 2010).

Advantages of bioluminescence

Real-time imaging using these luciferase reporter genes can aid in pre-harvest pathogen intervention *in vivo* by reducing the number of animals used in studies and providing answers to the pathogenesis of bacteria (Ryan et al., 2005). Luciferase reporter

genes applied to bioluminescent imaging are used to help monitor real-time physiological events related to colonization and pathogenesis of bacterial pathogens (Klerk et al., 2007; Luker and Luker, 2008; Ryan, 2011). Employing bioluminescence in live animal models will facilitate tracking of pathogens (Contag et al., 1997) and indicate the physical location of pathogens (Siragusa et al., 1999), which will increase researchers' understanding of pathogenic processes (Contag et al., 1995). Live animal models, which include *in vivo* cellular and molecular imaging, are being implemented through new technologies (Contag, 2002). Although technology like biophotonics does not seem to be appropriate for cattle producers or consumer use, it has the ability to help pre-harvest researchers in the pathogenic bacteria analysis and mode of action and transmission (Moulton et al., 2009a).

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CHAPTER III

SURVIVAL OF *ESCHERICHIA COLI* O157:H7 (ATCC 43888) TRANSFORMED WITH EITHER THE PAK1-*LUX* OR PXEN-13 PLASMIDS IN BOVINE MIXED RUMINAL AND FECAL MICROORGANISM FLUID

Abstract

The use of luminescent plasmids in bacteria may serve as a viable model for the real-time validation of various pre-harvest interventions on the colonization or shedding patterns of *Escherichia coli* O157:H7 within cattle. The objective of this study was to determine if the growth characteristics of *E. coli* O157:H7 (ATCC 43888) in mixed ruminal and fecal microorganism fluid cultures was altered when transformed with one of the two luminescent plasmids: pAK1-*lux* or pXEN-13. Transformants harboring the luminescent plasmids were compared to the non-transformed parental strain (WT) in mixed ruminal and fecal microorganisms fluid media for 6 h in triplicate (n = 3). The transformants and WT grew similarly in the presence of ampicillin (50 μg/mL). Within mixed ruminal microorganism fluid fermentations all transformants grew similarly through the 6 h study. The three transformants also grew similarly within mixed fecal microorganism fluid. The RLU (reflective light units; photon/pixel second) photonic emissions of each plasmid within ruminal fluid differed at 0 h ($P < 0.002$) and 2 h ($P < 0.02$) and within fecal fluid 0 h ($P < 0.009$) and 2 h ($P < 0.04$). The RLUs remained the same within rumen fluid at 4 h ($P < 0.22$) and 6 h ($P < 0.80$) and within fecal fluid at 4 h ($P < 0.06$) and 6 h ($P < 0.29$). Growth of *E. coli* O157:H7 transformed with the

bioluminescent plasmids was not altered in comparison to the WT, suggesting that both plasmids may serve as useful models for *in vivo* studies.

Introduction

One of the largest food safety threats facing the cattle industry today is the foodborne pathogenic bacterium *Escherichia coli* O157:H7 and other shiga toxin-producing *E. coli* (STEC; Gyles, 2007; Karmali et al., 2010). This bacterium is a natural member of the gastrointestinal microbial ecosystem of cattle; it does not cause illness in cattle, but can cause severe illness and even death in human consumers (Gyles, 2007; Karmali et al., 2010). Many intervention strategies aimed at reducing this critical pathogen have been implemented at the abattoir at a cost of more than $2 billion (Kay, 2003; Koohmaraie et al., 2005; Arthur et al., 2009). *Escherichia coli* O157:H7 can also be transmitted directly by contact to farm workers and visitors, and it can be carried by water run-off to nearby water supplies or irrigated crops (Chapman et al., 2000). In 2010, STEC illnesses were estimated to cost $1 billion dollars due to medical costs associated with foodborne illnesses and deaths in the United States (Scharff, 2010). Because of the link between this pathogen in live cattle and human illness, there has been an increased focus on the development of pre-harvest intervention strategies that reduce pathogenic bacteria in live food animals (Sargeant et al., 2007).

A limiting factor to pre-harvest intervention development has been the inability to physically track STEC or *E. coli* O157:H7 in a real-time fashion. Since contamination on meat carcasses could potentially originate from fecal material, punctured gastrointestinal contents and from feces on hides, a means of tracking of this pathogen through the use of biophotonics would provide a valuable model (Lohr, 1996). Detection of bioluminescent

cells is extremely sensitive when using an intensified charged-coupled device camera, with as few as 50 colony forming units being distinguishable (Siragusa et al., 1999). Bacteria transformed with luminescent plasmids that constitutively express the *lux*CDABE operon have been reported to have no alterations in growth kinetics compared to parental strains (Beyer and Bohm, 1996), and no significant alterations biochemically, serologically, or structurally (Chen and Griffiths, 1996).

The ability to track bioluminescent pathogens *in vivo* through the gastrointestinal tract has previously been successful in pigs and mice (Willard et al., 2002; Burns-Guydish et al., 2005; Moulton et al., 2009a). Employing bioluminescence in live animal models will facilitate tracking of pathogens (Contag et al., 1997) and can indicate the physical location of these pathogens (Siragusa et al., 1999) which will increase researchers' understanding of pathogenic processes (Contag et al., 1995). However, limited information is available for the capability of this technology for monitoring *E. coli* colonization and shedding *in vivo*. In particular, it is not known whether bioluminescent plasmids can be retained within the stressful, competitive environment of the gastrointestinal tract. Therefore, the objectives of this study were to analyze the parental strain *E. coli* O157:H7 ATCC 43888 wild type (WT) transformed separately with two different bioluminescent plasmids, pAK1-*lux* and pXEN-13, in order to determine whether the growth of *E. coli* O157:H7 was altered in bovine mixed ruminal microorganism fluid or fecal microorganism fluid media in relation to the non-transformed parental strain. The present study is a preliminary step in the validation of the use of bioluminescent *E. coli* O157:H7 as a novel real-time model for foodborne pathogen contamination.

Materials and Methods

Bacterial Cultivation Conditions

Escherichia coli ATCC 43888 is an O157:H7 serotype that does not possess the shiga toxin I or shiga toxin II genes. This bacterium was routinely cultured on Tryptic Soy Agar (TSA; Difco Co., Corpus Christi, TX) at 37°C. The transformation of *E. coli* O157:H7 with the plasmid pXEN-13 (Caliper Life Sciences, Hopkinton, MA), cells were washed three times with 10% glycerol and electroporated with 50 µg of the pXEN-13 plasmid (Sambrook and Russell, 2001). The *E. coli* O157:H7 ATCC 43888 was transformed with pAK1-*lux* as previously described by Moulton et al., 2008. Both transformed *E. coli* O157:H7 were selected for growth on 50 µg/mL of ampicillin (AMP) at 37°C; bioluminescence was confirmed by analyzing transformed strains with a Berthold/NightOwl camera equipped with the WinLight 32 software, version 2.51.11901 (Berthold Technologies; Oak Ridge, TN). The 43888 strain was passed through a series of increasing concentrations of novobiocin until the cells were made resistant to 10 µg/ml novobiocin.

Pure Culture Growth

Overnight cultures of the parental strain 43888, 43888 containing pAK1-*lux*, and 43888 containing pXEN-13 were cultured in triplicate at 37°C with constant agitation (140 rpm) in TSB supplemented with 50 µg/mL AMP or 10 µg/mL novobiocin. Overnight cultures (2 mL) were pelleted for 2 min at 10,000 x *g*, washed once with 2 mL of 1X phosphate buffer solution (PBS), and resuspended in 2 mL of TSB in order to remove the antibiotics. Cultures were then diluted 1:100 in TSB supplemented with 50 µg/mL AMP or 10 µg/mL novobiocin, and 200 µL aliquots were transferred, in duplicate,

to a 96-well microtiter plate. The OD_{600} for each culture grown in both conditions at 37°C was measured hourly for 24 h to verify that the plasmids did not slow growth rate, using a BioTek (Winooski, VT, USA) Synergy HT multi-mode microplate reader and analyzed with Gen5 data analysis software. Maximum specific growth rate was measured during exponential phase, while OD_{600} was less than 0.6 OD_{600} units and calculated according to Callaway and Russell (1999).

Mixed Ruminal and Fecal Microorganism Contents Collection

Rumen contents (1000 mL) were collected from the rumen ventral sac of a 362 kg cannulated steer at the Henry Leveck Animal Research Center at Mississippi State University. Fecal contents (6000 mL) were collected rectally from six Holstein cows at the Joe Bearden Dairy Research Center at Mississippi State University. Particles were separated from the ruminal and fecal fluid by passing contents through nylon paint strainers as previously described by Leyendecker et al., 2004. After separation, rumen and fecal fluids were incubated for 30 min at 37°C, which resulted in the formation of three visible layers. The middle layer of both ruminal and fecal fluid were removed and utilized for the foundation of media. The base medium utilized for both rumen and fecal fluid contained (per liter): 6.0 g KH_2PO_4, 6.0 g KH_2PO_4, 12.0 g $(NH_4)_2SO_4$, 12.0 g NaCl, 2.5 g $MgSO_4$•7 H_2O, 1.6 g $CaCl_2$•2H_2O, 0.04 g cysteine HCl; base medium was sterilized by autoclaving. Then 33.0 mL of an 8% solution of Na_2CO_3 and 333 mL of ruminal fluid or fecal fluid were added and homogenized by mixing. The pH was adjusted to 6.5 with 1 M of NaOH solution and bubbled with CO_2. Both fully prepared media (Cotta and Russell, 1982) were incubated in an orbital shaker at 140 rpm at 37°C for 12 h.

Mixed Ruminal and Fecal Microorganism Contents Fermentations

Fresh overnight cultures, in triplicate prepared as described above, of the parental strain 43888, 43888 containing pAK1-*lux*, and 43888 containing pXEN-13 grown in TSB supplemented with the appropriate antibiotic were then diluted 1:100 in 2 mL of TSB and allowed to incubate with agitation (140 rpm) at 37°C until mid-log phase (OD_{600} ~0.50; Broadway, 2011) was achieved. Cultures were then split and aliquotted; one tube for ruminal fluid and one for fecal fluid, pelleted by centrifugation at 10,000 x *g* for 2 min, and resuspended in 5 mL of either media containing ruminal or fecal fluid medium. After inoculation, cultures were incubated with constant agitation (140 rpm) at 37°C for 6 h. At each 120 min interval, four 100 mL aliquots of each culture were transferred to a 96-well plate (Costar® Black Sterile Polystyrene; Corning Incorporated, Corning, NY) and subsequently imaged. Photonic emissions were recorded following a 2 min acquisition phase and were measured based on reflective light units [RLU; photons/pixel per second (ph/pix s)]. Additionally, aliquots of each culture were acquired at 0 h and continuously at 120 min intervals for 6 h, diluted in 1X PBS, and plated onto the appropriate selective media: parental strain 43888 on TSA+ 10 mg/mL novobiocin, 43888 containing pAK1-*lux* or pXEN-13 on TSA+50 mg/mL AMP. Plates were then incubated at 37°C for 12 h prior to analysis, viable plate counts were obtained. Plates were also imaged using a Berthold/NightOwl camera to validate luminescence.

Statistical Analysis

Data were analyzed as a completely randomized design with repeated measures using PROC MIXED in SAS (SAS Inst. Inc., Version 9.2; Cary, NC). Experimental unit was defined as tube, and significance was declared at $P < 0.05$. Pair-wise differences

among least squares means at various sample times were evaluated with the PDIFF statement.

Results

Growth of *E. coli* 43888 containing pAK1-*lux* or pXEN-13

To determine whether pAK1-*lux* and pXEN-13 plasmids exhibited similar growth rates, growth was monitored over a 24 h period using a BioTek Synergy HT plate reader (Winooski, VT, USA) [Figure 3.1]. The pAK1-*lux* (1.05 h^{-1}) and the pXEN-13 (1.02 h^{-1}) containing-transformants both exhibited similar maximum specific growth rates as the parental strain 43888 (0.99 h^{-1}; Figure 3.1A). No differences were evident between the growth rates of pXEN-13 and the parental strain. Growth of both the pAK1-*lux* and pXEN-13 containing-transformants were also examined in the presence of the selective pressure of ampicillin (Figure 3.2B). The growth patterns of both transformants were similar, pAK1-*lux* (1.41 h^{-1}) and pXEN-13 (1.44 h^{-1}) and with no differences observed.

Growth in Ruminal Fluid Fermentations

To determine whether differences were evident in the ability of the two plasmids to be retained within the rumen; both strains containing either pAK1-*lux* or pXEN-13 were grown in the presence of freshly prepared mixed rumen microorganism fluid medium. The parental strain was also grown under these conditions to determine whether the presence of the plasmids would alter the ability of *E. coli* O157:H7 to survive. The *E. coli* O157:H7 and the two transformants grew similarly within mixed ruminal microorganism fluid (Figure 3.2). Growth was monitored by viable plate counts and populations were recorded in $\log_{10}$ (CFU/mL) populations. There was no difference ($P >$ 0.18) observed within populations between the WT and the pXEN-13 plasmid throughout

the study. The only difference observed within the study was at 4 h, when pAK1-*lux* had greater populations than pXEN-13 ($P < 0.004$), but was not different from the WT ($P > 0.10$).

Growth in Fecal Fluid Fermentations

To determine whether the presence of either plasmid would affect the ability of *E. coli* O157:H7 to survive within mixed fecal microorganism fluid, strains containing either pAK1-*lux* or pXEN-13 were grown in the presence of freshly prepared fecal fluid medium and compared to growth of the non-transformed parental strain. The WT and the two transformants grew similarly within mixed fecal microorganism fluid fermentations (Figure 3.3). There was no difference ($P > 0.14$) observed within populations between the WT and pXEN-13 for the duration of the study. The only difference observed was at 6 h when pXEN-13 suggested greater populations than pAK1-*lux* ($P < 0.01$) but was not different than the WT ($P < 0.18$).

Comparison of Photonic Emissions by pAK1-*lux* and pXEN-13

To determine whether photonic light emission would be sustained by both pAK1-*lux* and pXEN-13, RLU photonic emissions were monitored over a 6 h period in both mixed rumen and fecal microorganism fluid media. Photonic emissions gradually decreased from 0 h for both plasmids within ruminal fluid, indicating that there was a time effect ($P < 0.001$). The RLU values are measured in photons/pixel second (ph/pix s). The RLU values for pAK1-*lux* ranged from 16225.97 ph/pix s to 880.21 ph/pix s (data not reported in tabular form). The pXEN-13 plasmid had the greatest RLU values at 0 h at 292.28 ph/pix s and the lowest at 6 h 86.25 ph/pix s. The pAK1-*lux* plasmid had greater RLU values and was statistically different at 0 h ($P < 0.002$) and 2 h ($P < 0.02$)

and as photonic emissions decreased over time both plasmids emitted the similar values at 4 h ($P < 0.22$) and 6 h ($P < 0.80$), suggesting that there was only a tendency ($P < 0.07$) for a transformant effect. There was a transformant by time effect ($P < 0.001$) because both time and transformant affect photonic emissions.

Within mixed fecal microorganisms fluid media both plasmid RLU values decreased from 0 h, indicating that time impacted photonic emissions ($P < 0.001$). The pAK1-*lux* plasmid ranged from 5513.65 ph/pix s to 1404.42 ph/pix s (data not reported in tabular form). The pXEN-13 plasmid had decreased RLU values with a range from 112.92 ph/pix s to 34.61 ph/pix s. The two plasmids were different from 0 h ($P < 0.009$) and 2 h ($P < 0.04$), with a tendency to be different at 4 h ($P < 0.06$). At 6 h the plasmids were not different ($P < 0.29$), further suggesting that a tendency for transformant ($P < 0.06$) to impact photonic emissions, while indicating a transformant by time interaction ($P < 0.001$).

Discussion

To examine the application of bioluminescent technology in a large animal study, we analyzed the growth and luminescence of *E. coli* 43888 containing either the pAK1-*lux* or the pXEN-13 plasmid. Bioluminescence studies have suggested correlations with *in vitro* enzyme activity; the amount of luciferase protein and *lux* mRNA generated directly correlates with cell viability (Close et al., 2010). The two plasmids utilized in this study were selected on the basis of harboring the *lux*CDABE operon. The pAK1-*lux* plasmid was developed as a wide range host plasmid for gram-negative bacteria (Karsi et al., 2006). Research with this plasmid has suggested stability and luminescence, without antibiotic pressure for at least 8 d; while decreasing the percentage of photonic emissions

from 0 d (Moulton et al., 2008). The pXEN-13 plasmid carries the original *Photorhabdus luminescens* operon for production of luminescence in gram-negative bacteria (Harms et al., 2009). The pXEN-13 plasmid has been introduced into *E. coli* strains previously and emitted photonic emissions within the mouse model (Harms et al., 2009). The WT containing either pAK1-*lux* or pXEN-13 exhibited similar growth rates under antibiotics pressure.

Both *lux*CDABE containing plasmids grew similarly and no differences were noted in the growth of the plasmid-equipped bacteria in the presence of AMP, indicating that both plasmids grow similarly to the WT parental strain in the absence of AMP, and similarly under AMP pressure. This suggests that even within a live animal model without antibiotic pressure, both transformants will function similarly to the WT.

Many studies have demonstrated that *E. coli* O157:H7 can be cultured from the rumen, but does not grow rapidly or extensively in the rumen (Grauke et al., 2002). To further investigate the growth and stability of WT and the two bioluminscently transformed *E. coli* O157:H7, pAK1-*lux* and pXEN-13 were inoculated and grown in mixed microbial ruminal fluid microorganism fermentations. There was no difference observed in the two transformants compared with the controls when grown in mixed microbial ruminal fluid fermentations, thus suggesting that both transformants can survive the harsh rumen conditions and grow in a fashion similar to the WT.

Escherichia coli O157:H7 can contaminate beef carcasses via fecal spread from hide or spilled digesta and it has been suggested that 30% of cattle harbor *E. coli* O157:H7 or other EHEC in feces, depending on season (Elder et al., 2000; Callaway et al., 2003). Transformants and WT grew similarly in the presence of a mixed fecal microorganism fluid environment throughout the duration of the study. This suggests

that transformants functioned similarly within the fecal fluid environment and are capable of surviving the competitive fecal environment.

Transforming bacteria with the bioluminescent plasmids has been suggested to be a successful means of tracking bacteria in real-time (Moulton et al., 2008; Moulton et al., 2009b; Curbelo et al., 2010). The present data suggest that the pAK1-*lux* and pXEN-13 transformants are stable and can persist in rumen and fecal-like environments. Furthermore, while grown in these harsh, competitive environments, these transformants still display luminescence and could be a useful method for pathogen tracking in real-world environments (Moulton et al., 2008). The use of luciferase reporter genes in real-time imaging can aid in pre-harvest pathogen intervention *in vivo* by reducing the number of animals needed for use in studies, as well as by providing the physical location of pathogen contamination routes (Ryan et al., 2005).

Conclusions

Transformation of *E. coli* O157:H7 with the pAK1-*lux* gene cassette or the pXEN-13 gene cassette did not alter the growth of the transformants compared to the WT (ATCC 43888) within mixed ruminal or mixed fecal microorganism incubations. Our results suggest that both plasmids could be implemented for a model for pre-harvest pathogen intervention in ruminant animals.

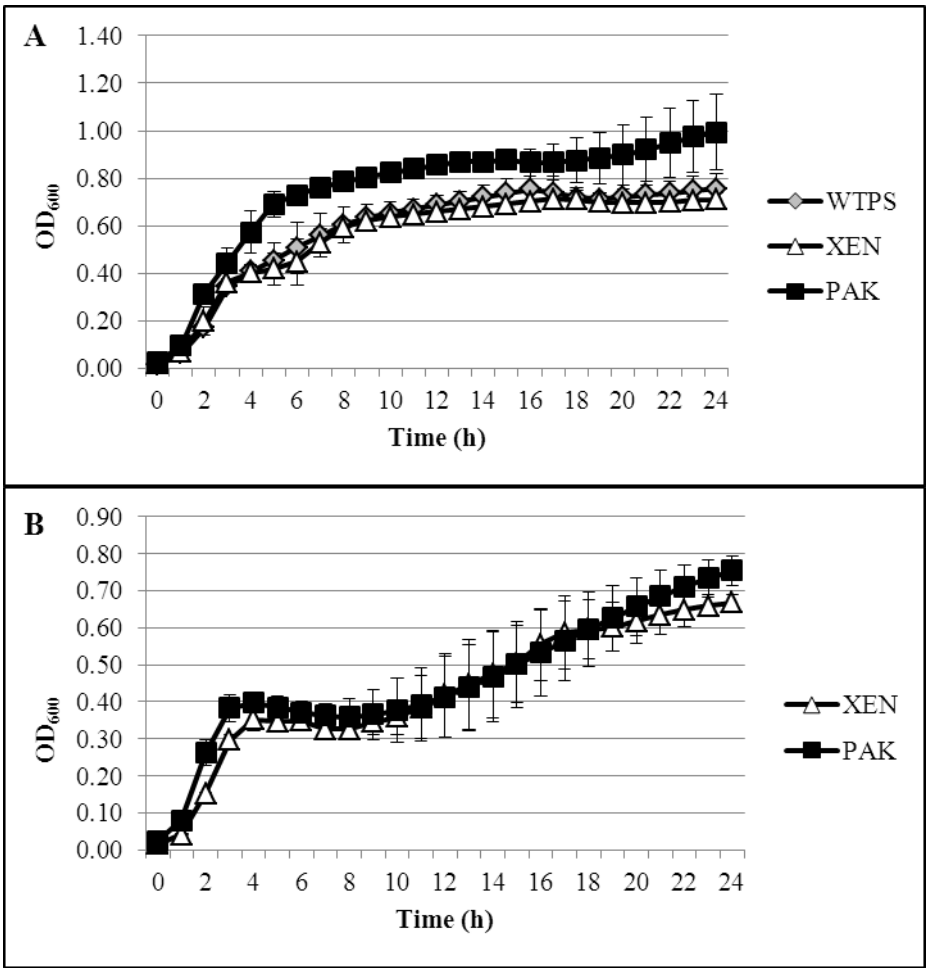
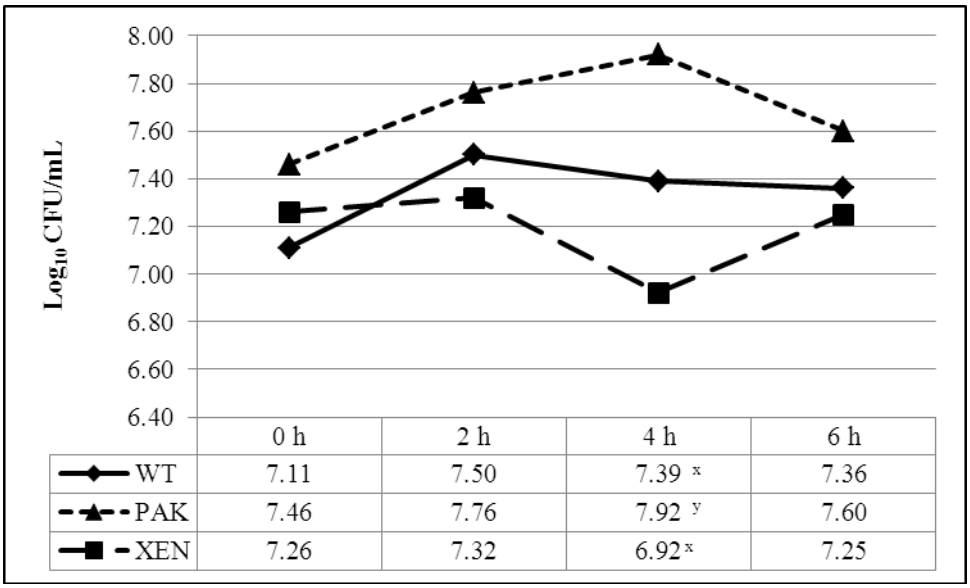


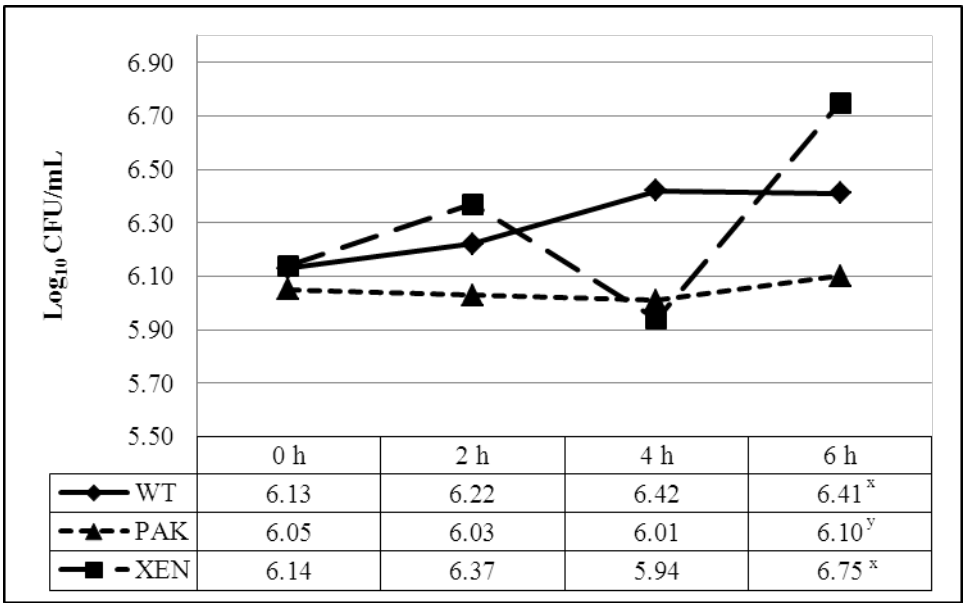
Figure 3.1 The OD_{600} of cultures of the wild type (WT) *Escherichia coli* O157:H7 ATCC 43888 (♦) and the two bioluminescently transformed variants, 43888 containing the plasmid pXEN-13 (▲) or pAK1-*lux* (■). Cultures were grown in the absence of (**A**) and presence (**B**) of ampicilloin (50 mg/mL) to provide selective pressure



	0 h	2 h	4 h	6 h
WT	7.11	7.50	7.39 [x]	7.36
PAK	7.46	7.76	7.92 [y]	7.60
XEN	7.26	7.32	6.92 [x]	7.25

Figure 3.2 The least square means of *Escherichia coli* O157:H7 (ATCC 43888) populations (log_{10} CFU/mL) in bovine mixed ruminal microorganism incubations. Growth was monitored by viable plate counts with the parental strain (♦; WT) or 43888 containing the plasmid pAK1-*lux* (▲; PAK) or pXEN-13 (■; XEN) in bovine mixed ruminal fluid for 6 h. Values represent the log_{10} CFU/mL values from three independent replicates

[x, y] Lsmeans lacking a common subscript within column differ ($P < 0.05$)



	0 h	2 h	4 h	6 h
WT	6.13	6.22	6.42	6.41 [x]
PAK	6.05	6.03	6.01	6.10 [y]
XEN	6.14	6.37	5.94	6.75 [x]

Figure 3.3 The least square means of *Escherichia coli* O157:H7 (ATCC 43888) populations (log_{10} CFU/mL) in bovine mixed fecal microorganism incubations. Growth was monitored by viable plate counts with the parental strain (♦; WT) or 43888 containing the plasmid pAK1-*lux* (▲; PAK) or pXEN-13 (■; XEN) in bovine mixed fecal fluid for 6 h. Values represent the log_{10} CFU/mL values from three independent replicates

[x, y] Lsmeans lacking a common subscript within column differ ($P < 0.05$)

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CHAPTER IV

EFFECTS OF CITRUS BY-PRODUCTS ON GROWTH OF O157:H7 AND NON-O157 *ESCHERICHIA COLI* SEROTYPES WITHIN BOVINE MIXED RUMINAL MICROORGANISM FLUID

Abstract

Citrus by-products (CBP) are utilized in citrus regions of the United States as a low cost, highly palatability and nutritional component of cattle diets. Researchers have suggested that inclusion of CBP both *in vivo* and *in vitro* can inhibit the growth of both *Escherichia coli* O157:H7 and *Salmonella* (Nannapaneni et al., 2008; Collier et al., 2010). The objective of this study was to examine the effects *in vitro* that varying concentrations of CBP in the powdered or pelleted variety impose on enterohemorrhagic *Escherichia coli* (EHEC) serotypes; O26:H11, O103:H8, O111:H8, O145:H28, O157:H7 and O157:H7 *Δstx$_1$stx$_2$* in mixed ruminal microorganism media. Mixed ruminal microorganism fluid media were supplemented with either powdered or pelleted CBP at concentrations of 0%, 5%, 10%, 20% and EHEC growth ($\log_{10}$ CFU/mL) were evaluated at 0, 2, 4, 6 h. The O26:H11, O111:H8, O145:H28 and O157:H7 serotypes did not exhibit a change in populations throughout both CBP varieties. The O103:H8 serotype displayed an approximate 1 $\log_{10}$ reduction in O103:H8 populations at 5% powdered CBP over 6 h. There was an approximate 1.5 $\log_{10}$ reduction in O157:H7 *Δstx$_1$stx$_2$* populations at 10% CBP and an approximate 5 $\log_{10}$ reduction observed in O157:H7 *Δstx$_1$stx$_2$* populations at 20% powdered CBP over the 6 h study. There was an

approximate 1 $\log_{10}$ reduction in O103:H8 populations when supplemented with 20% pelleted CBP. These results suggest that the serotypes of EHEC grow differently within mixed ruminal microorganism fluid media when supplemented with CBP in the powdered or pelleted variety. Further research is needed to indicate the affect that CBP have on non-O157 EHEC serotypes.

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) are capable of naturally colonizing within the gastrointestinal tract of cattle, without causing illness (Wells et al., 1991). Human consumption of products contaminated with EHEC can cause illness and more severely hemorrhagic colitis and hemorrhagic uremic syndrome (Griffin and Tauxe, 1991; Stanford et al., 2005). The most notorious EHEC within the meat industry has been *E.coli* O157:H7. Due to increased surveillance and pre- and post- harvest intervention, the occurrence of O157:H7 infections have been reduced to ≤ 1 case per 100,000 people (CDC, 2011a). However, there now appears to be an increase in the occurrence of foodborne outbreaks due to non-O157 EHEC (CDC, 2011a). According to the Center for Disease Control (CDC) an estimated 265,000 cases of EHEC were reported a year; of these, approximately 67% are attributed to non-O157 EHEC (CDC, 2011b). With increased concerns related to the prevalence of non-O157 outbreaks the USDA-FSIS will be labeling non-O157 EHEC serotypes O26, O45, O103, O111, O121 and O145 as adulterants in fresh non-intact beef products (USDA-FSIS, 2012).

The production of citrus for various food and non-food products generates by-products such as the pulp and peel from citrus fruit. These citrus by-products (CBP) have been utilized by dairy and beef cattle producers in regions of the United States as an

inexpensive nutritionally dense feed source (Callaway et al., 2008). The incorporation of CBP into diets for cattle also may aid in the reduction of foodborne pathogens due to antimicrobial aspects of the by-products. Citrus products and by-products contain essential oils that possess antimicrobial activities that are able to damage the cell wall due to lipophilic properties. The lipophilic properties of essential oils are able to permeabilize the cell wall within gram-negative bacteria (Bakkali et al., 2006). The change in the fluidity of the membranes due to the permeabilization, allows essential oils to coagulate in the cytoplasm (Gustafson et al., 1998), depleting ATP (Di Pasqua et al., 2006) and resulting in lysis of the cell (Kim et al., 2005; Fisher and Phillips, 2006; Gill and Holley, 2006; Oussalah et al., 2006; Di Pasqua et al., 2007). Research pertaining to essential oils within CBP has indicated severe damage to *E. coli* serotypes (Kim et al., 1995; Dusan et al., 2006). The rumen and intestinal gram-negative microbial populations of cattle can be altered due to this antimicrobial activity within cattle (Nam et al., 2006). Since CBP contain antimicrobial properties and are readily available at low costs within citrus-producing areas and has nutritional value, it is being investigated as a potential pre-harvest pathogen intervention strategy to reduce EHEC concentrations within the gastrointestinal tract of cattle. Therefore, the objective of this study was to examine the effects of powdered and pelleted citrus by-products have on growth of the EHEC serotypes: O26:H11, O103:H8, O111:H8, O145:H28, O157:H7 and O157:H7 $\Delta stx_1 stx_2$ in mixed ruminal microorganism media.

Materials and Methods

Ruminal Fluid Collection and Medium Preparation

Ruminal contents (1000 mL) were collected from the rumen ventral sac of a 544 kg cannulated steer at the Henry Leveck Animal Research Center at Mississippi State University. Rumen particles were separated from the ruminal fluid by passing contents through nylon paint strainers as previously described by Leyendecker et al., 2004. After separation, rumen fluid was incubated for 30 min at 37°C, to allow the fluid to separate into three distinct layers. The middle layer of the rumen fluid was extracted and utilized for the mixed ruminal microorganism fluid. The medium utilized for the mixed ruminal microorganism fluid contained (per liter): 6.0 g KH_2PO_4, 6.0 g KH_2PO_4, 12.0 g $(NH_4)_2SO_4$, 12.0 g NaCl, 2.5 g $MgSO_4$•7 H_2O, 1.6 g $CaCl_2$•$2H_2O$, 0.04 g cysteine HCl; base medium was sterilized by autoclaving (Cotta and Russell, 1982). To the base medium, 33.0 mL of an 8% solution of Na_2CO_3 and 333 mL of ruminal fluid were added and homogenized by mixing. The pH was adjusted to 6.5 with 1 M of NaOH solution, and bubbled with CO_2. The fully prepared media was incubated in an orbital shaker at 140 rpm at 37°C for 12 h.

Bacterial Serotypes and Cultivation Conditions

Six serotypes of *E. coli* included in this study were purchased from the American Type Culture Collection (ATCC): O157:H7 (ATCC 43895), O103:k:H8 (ATCC 23982), O145:H28 (BAA-2129), O26:H11 (BAA-1653), O111:H8 (BAA-179) and O157:H7 *Δstx$_1$stx$_2$* (ATCC 43888). All *E. coli* serotypes were routinely cultured in Luria-Bertani medium (LB; Difco Co.; Corpus Christi, TX) at 37°C. All serotypes were transformed with the bioluminescent pXEN-13 plasmid (Caliper Life Sciences; Hopkinton, MA), as a

marker for means of antibiotic selection for identification following incubation. For transformation with the pXEN-13 plasmid all serotypes were made competent by washing mid-log cultures four times with ice cold 10% glycerol. Competent cells were then transformed with pXEN-13 by electroporation and cultured in LB supplemented with 100 mg/mL of ampicillin (AMP) using standard techniques (Sambrook and Russell, 2001).

Citrus By-Product Trial

Isolates from fresh streaks of each serotype transformed with pXEN-13 on LB agar supplemented with 100 μg/mL AMP were used to inoculate a 5 mL starter culture in LB broth+AMP at 16 h at 37°C. Cultures were then diluted 1:100 in LB broth+AMP and allowed to grow to mid-log phase, after which cultures were pelleted, residual medium was removed, and cells were resuspended in an equal volume of mixed ruminal microorganism fluid media supplemented with 0, 5, 10 or 20% CBP. Cultures were incubated at 37°C at 140 rpm. Aliquots were removed at various times, diluted in 1X phosphate buffered saline (PBS), plated onto LB agar supplemented with 100 μg/mL AMP, and incubated overnight at 37°C. The pH values were measured from each serotype at each time interval at the various CBP concentrations recorded.

Statistical Analysis

Data were analyzed as a completely randomized design with repeated measures using PROC MIXED in SAS (SAS Inst. Inc., Version 9.2; Cary, NC). Experimental unit was defined as tube, and significance was declared at $P < 0.05$. Pair-wise differences among least squares means at various sample times were evaluated with the PDIFF statement.

Results

Citrus By-Products pH Values

The pH values of each CBP variety at 0%, 5%, 10% and 20% at each time interval were recorded. The pH was adjusted to 6.5 at -2 h prior to the addition of CBP in either variety. From -2 h to 6 h, pH increased within all serotypes from 6.5 to 7.5 for 0% CBP. At 0 h the pH was 5.5 and decreased to 4.0, in the 5%, 10% and 20% CBP throughout the study for both varieties. The pH values of the powdered CBP decreased according to the increasing concentrations over the time intervals. The pelleted CBP variety indicated a similar trend, with pH values decreasing with the increase in pelleted CBP concentrations across the 6 h study.

Powdered Citrus By-Product

The growth of the various EHEC populations ($\log_{10}$ CFU/mL) within mixed ruminal microorganism fluid media indicated differences within serotypes (Table 4.1). The O26:H11 and O145:H28 serotypes grew similar ($P < 0.11$) within the powdered CBP. The only difference observed in O26:H11 serotype's decreased ($P < 0.006$) populations at 4 h supplemented with 20% powdered CBP throughout the study. The O103:H8 serotype exhibited approximately a 1 $\log_{10}$ reduction in populations over the 6 h study, when mixed ruminal microorganism media was supplemented with 5% powdered CBP. The O157:H7 *Δstx$_1$stx$_2$* and O157:H7 serotypes had decreased ($P < 0.04$ and $P < 0.05$, respectively) populations in comparison to the other serotypes at 0 h. Although both O157 serotypes tended to grew similarly ($P < 0.06$) throughout 4 h, there was a difference observed at 6 h when O157:H7 *Δstx$_1$stx$_2$* had greater decreases ($P < 0.03$) in populations in comparison to O157:H7. When supplemented with 10% powdered CBP

the O157:H7 *Δstx$_1$stx$_2$* serotype displayed approximately a 1.5 $\log_{10}$ reduction in populations, and at 20% powdered CBP there was approximately a 5 $\log_{10}$ reduction in populations over the 6 h course of the study.

Pelleted Citrus By-Product

The growth of the EHEC populations within mixed ruminal microorganism fluid media indicated differences between the various serotypes (Table 4.2). The pelleted CBP tended to have no change ($P < 0.07$) in populations of O145:H28 from 0 h to 6 h. While O157:H7 had decreased ($P < 0.02$) at 0 h, there were no differences in populations observed between the O103:H8 and O157:H7 ($P < 0.11$) the remainder of the study. Populations of O103:H8 were decreased ($P < 0.02$) at 0 h, while populations tended to be similar ($P < 0.06$) to O111:H8 throughout the study. When mixed ruminal microorganism media was supplemented with 20% pelleted CBP there was approximately a 1 $\log_{10}$ reduction observed over the 6 h study. The O26:H11 serotype populations decreased ($P < 0.05$) throughout the study, exhibiting the least populations at 4 h. *Escherichia coli* O157:H7 *Δstx$_1$stx$_2$* exhibited the most decreased ($P < 0.05$) EHEC populations at 0 h and throughout the study.

Discussion

A study conducted by Free et al. (2012) utilized the same serotypes within this study O26:H11, O103:H8, O111:H8, O145:H28, O157:H7 and O157:H7 *Δstx$_1$stx$_2$*. The results of this study suggested that all serotypes were capable of growing with mixed ruminal microorganism fluid media; with decreased concentrations of serotypes O103:H8 and O145:H28 after 24 h compared to O157:H7 (Free et al., 2012). These data suggests the possibility that not all non-O157 serotypes function similarly within cattle.

The O157:H7 *Δstx$_1$stx$_2$* data indicated decreased populations within the mixed rumen microorganism fluid media supplemented with powdered CBP, while O103:H8 indicated decreased populations within both varieties of CBP. These results are in accordance with previous studies that have suggested a decrease in O157:H7 populations using other varieties of CBP. Callaway et al. (2008) supplemented mixed ruminal microorganism fluid media with 0%, 0.5%, 1% and 2% dried orange pulp and *E. coli* O157:H7 populations decreased according to increasing concentrations. While another Callaway et al. (2011) study supplemented sheep rations with 0%, 5%, 10% orange peel and reduced *E. coli* O157:H7 populations *in vivo*.

The CBP was added to the mixed ruminal microorganism fluid media 2 h before the serotypes were added to the mixture (0 h). Although a decrease in O103:H8 and O157:H7 *Δstx$_1$stx$_2$* populations were observed, other EHEC populations were not affected. Given that this study was only conducted for 6 h, the effects of CBP within the mixed ruminal microorganism fluid media and EHEC serotypes may not had been fully observed within the short time frame. Others studies indicate that CBP decrease *E. coli* O157:H7 populations from 24 h to 72 h (Callaway et al., 2008; Callaway et al., 2011). This study was only conducted for 6 h; an increased duration of the study could have been more beneficial to observe the effects of CBP on the various serotypes.

The essential oils within the CBP can permeabilize the bacterial cells walls and cytoplasm, leading to bacterial lysis, thus shifting the rumen environment leading to an increase in short-chain fatty acids while decreasing the pH. The acidic environment creates less favorable conditions for microbial populations to survive and replicate within, thus decreasing the possibility of *E. coli* O157:H7 populations. Although our research has reported a decrease in pH values with increasing CBP concentrations and an

observed decrease in O103:H8 and O157:H7 *Δstx$_1$stx$_2$* populations, this same trend was not observed within other EHEC serotypes. Further research is needed to determine how the various EHEC serotypes affect *E. coli* populations within mixed ruminal microorganism fluid media when supplemented with CBP.

Conclusion

Enterohemorrhagic Escherichia coli are foodborne pathogens that can survive within the gastrointestinal tract of cattle without imposing illness. Essential oils are the main component of citrus by-products and have been suggested to contain antimicrobial properties. Although decreased populations were observed within the O103:H8 and O157:H7 Δstx1stx2 serotypes other EHEC serotypes were not affected. Further research is needed to conclude how CBP affect non-O157 EHEC serotypes within mixed ruminal microorganism fluid media.

Table 4.1 Least squares means for growth of enterohemorrhagic *Escherichia coli* (EHEC) O26:H11, O103:H8, O111:H8, O145:H28, O157:H7 and O157:H7 *Dstx$_1$stx$_2$* within bovine mixed rumen microorganism fluid medium, supplemented with 0%, 5%, 10% and 20% powdered citrus by-product (CBP) over time (h)

CBP (%)	Time (h)	O26:H11	O103:H8	O111:H8	O145:H28	O157:H7	O157:H7 *Δstx$_1$stx$_2$*
0%							
	0 h	7.55	7.11	6.93	7.55	6.03^{v}	6.46
	2 h	7.68	7.05	6.70	7.58	6.91^{w}	7.00
	4 h	7.60	6.81	6.92	8.06	6.91^{w}	7.19
	6 h	7.73	6.65	7.07	7.83	7.24^{w}	6.98
5%							
	0 h	7.40	7.34^{y}	7.31^{v}	7.57	6.73	6.63
	2 h	7.47	6.86^{yz}	7.06^{v}	7.64	6.65	6.41
	4 h	7.43	6.95^{yz}	5.85^{w}	7.80	6.77	7.09
	6 h	7.59	6.55^{z}	7.22^{v}	7.80	7.02	6.74
10%							
	0 h	7.43	7.28	6.64	7.73	6.83	6.82^{vw}
	2 h	7.43	6.90	7.33	7.56	7.22	6.18^{w}
	4 h	7.36	6.99	7.18	7.64	6.96	7.13^{v}
	6 h	7.66	6.69	7.25	7.63	6.98	4.19^{x}
20%							
	0 h	7.12^{v}	7.13	7.02	7.60	7.03	7.03^{v}
	2 h	7.54^{v}	7.33	6.78	7.52	6.94	6.53^{v}
	4 h	5.96^{w}	7.07	6.65	7.44	7.30	7.34^{v}
	6 h	7.19^{v}	6.66	6.70	7.66	7.00	1.99^{w}

$^{v, w, x}$ Lsmeans within a column, within a treatment, without a common subscript are different between treatment groups if ($P \leq 0.05$)

$^{y, z}$ Lsmeans within a column, within a treatment, without a common subscript within column tend to differ ($P < 0.09$)

Table 4.2 Least squares means for growth of enterohemorrhagic *Escherichia coli* (EHEC) O26:H11, O103:H8, O111:H8, O145:H28, O157:H7 and O157:H7 *Dstx$_1$stx$_2$* within bovine mixed rumen microorganism fluid medium, supplemented with 0%, 5%, 10% and 20% pelleted citrus by-product (CBP) over time (h)

CBP (%)	Time (h)	O26:H11	O103:H8	O111:H8	O145:H28	O157:H7	O157:H7 *Δstx$_1$stx$_2$*
0%							
	0 h	9.27^{x}	7.01^{x}	8.47^{x}	9.61	8.48	6.46
	2 h	8.08^{y}	7.01^{x}	9.01^{xy}	9.48	8.24	6.28
	4 h	9.45^{x}	8.64^{y}	8.43^{x}	9.67	8.79	6.26
	6 h	9.64^{x}	8.77^{y}	9.48^{y}	9.50	9.01	6.51
5%							
	0 h	9.27^{xy}	8.21	8.40^{xy}	9.51	9.23^{x}	6.07
	2 h	8.71^{x}	8.26	8.93^{x}	9.60	7.94^{y}	5.92
	4 h	7.29^{z}	7.93	7.98^{y}	9.59	7.96^{y}	6.23
	6 h	9.71^{y}	8.28	8.77^{x}	9.69	8.93^{x}	5.97
10%							
	0 h	9.07^{xz}	8.76	8.76^{xy}	8.98	8.73^{xy}	6.83
	2 h	8.43^{xy}	8.50	9.03^{xy}	9.01	8.73^{xy}	6.94
	4 h	7.90^{y}	8.37	8.66^{x}	9.55	8.40^{xy}	6.64
	6 h	9.59^{z}	8.07	9.73^{y}	9.71	9.20^{y}	6.68
20%							
	0 h	9.47^{x}	8.52^{x}	9.03	9.29^{x}	8.00^{x}	6.78
	2 h	8.98^{x}	8.51^{x}	8.92	9.79^{xy}	8.95^{y}	6.77
	4 h	7.69^{y}	8.57^{x}	9.09	8.95^{y}	8.71^{xy}	6.76
	6 h	9.54^{x}	7.43^{y}	9.46	9.48^{xy}	9.05^{y}	7.13

$^{x, y, z}$ Lsmeans within a column, within a treatment, without a common subscript are different between treatment groups if ($P \leq 0.05$)

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CHAPTER V

EVAULATION OF THE COLONIZATION OF *ESCHERICHIA COLI* O157:H7 WITHIN THE SWINE GASTROINESTINALTRACT

Abstract

The objective of this study was to monitor the colonization and survival of *Escherichia coli* O157:H7 (ATCC 43888) in the swine gastrointestinal (GI) tract through the use of bioluminescent technology and viable plate counts. Twelve weanling pigs (18-21 d) were orally inoculated with 7.0 x 10^9 CFU/mL of *E. coli* O157:H7 containing the bioluminescent plasmid pAK1-*lux* at the following intervals: -10 d, -7 d, -2 d and -1 d pre-mortem (0 d). Colonization of *E. coli*+pAK1-*lux* was then analyzed in the duodenum, jejunum, ileum, cecum, colon and rectum by viable plate counts and also through luminescent imaging. The bacterial populations ($\log_{10}$ CFU/mL) increased ($P < 0.001$) as the incubation period decreased, with the 1 d incubation period exhibiting the greatest bacterial populations within all intestinal sections analyzed, with the exception of the jejunum. Following the removal of intestinal contents, each section was individually rinsed to remove any *E. coli* O157:H7+pAK1-*lux* that may have adhered to the intestinal endothelial lining and subsequently analyzed. Data suggest that upon initial exposure to *E. coli* O157:H7, there is a propensity for the bacteria to colonize throughout the GI tract within 1 d. Although the 1 d exposure displayed the greatest populations within the GI rinse, 1 d also displayed similar populations ($P < 0.21$) within all the sections of the 1 d treatment. This study demonstrates that *E. coli*+pAK1-*lux* were successfully monitored

through the GI tract of swine, and a colonization pattern was observed. Further research is needed to validate if *E. coli* O157:H7 transformed with the pAK1-*lux* bioluminescent plasmid could serve as a valid model for long-term *in vivo* studies.

Introduction

In 2011 *E. coli* O157:H7 was responsible for an estimated 1 million pounds of ground beef recalled in the United States due to possible contamination (USDA-FSIS, 2011). Controlling and eliminating fecal shedding of *Escherichia coli* O157:H7 in food animals has been a major issue in pre-harvest food safety and has prompted investigations into intervention strategies to reduce colonization and shedding in livestock (Genovese et al., 2000; Callaway et al., 2003). Many pre-harvest intervention strategies have proven to reduce *E. coli* O157:H7 populations but are not economically feasible to the producer or are not implemented due to the lack of producer utilization and acceptance. Therefore, to date there has been no confirmed method to fully control or eliminate *E. coli* O157:H7 shedding (Huffman, 2002; Callaway et al., 2009).

Historically, swine have not been commonly linked to enterohemorrhagic *E. coli* (EHEC) because unlike cattle, swine are susceptible to EHEC infections (Anderson et al., 2001). *Escherichia coli* O157:H7 is capable of attaching to swine's large intestine epithelial cells in a mechanism similar to that observed in humans (Francis et al., 1986; Tzipori et al., 1986), thus effacing the microvilli and inducing illness (Donnenberg et al., 1993). However, data is surfacing that suggest swine may be asymptomatic carriers of the *E. coli* O157:H7 pathogen (Borie et al., 1997; Anderson et al., 2001; Callaway et al., 2004). For instance, in 1997, 68% of pigs that were sent to harvest in Santiago, Chile, were harboring EHEC (Borie et al., 1997; Anderson et al., 2001). In 1999, *E. coli*

O157:H7 was isolated from swine that were sent to harvest in the Netherlands (Heuvelink et al., 1999). Also in 2007, a nationwide spinach-associated *E. coli* O157:H7 outbreak was linked to feral swine in California (Jay et al., 2007).

Real-time tracking of pathogens can aid in the monitoring and understanding of physiological events, pathogenesis and colonization patterns of bacteria both *in vivo* and in vitro (Contag et al., 1995; Contag et al., 1997; Siragusa et al., 1999; Ryan et al., 2010). Successful *in vivo* tracking of *Salmonella typhimurium* transformed with the pAK1-*lux* bioluminescent plasmid was achieved within the swine model (Willard et al., 2002; Moulton et al., 2009b). Moulton et al. (2009b) inoculated swine with *S. typhimurium*+pAK1-*lux* and monitored tracking within the small intestinal tract within a 12 h period. Additionally, *S. typhimurium* and *E. coli* transformed with various *lux*CDABE operons have been successfully implemented within murine gastrointestinal (GI) tract models (Burns-Guydish et al., 2005; Foucault et al., 2010). *Ex vivo* tracking of *E. coli* transformed with the Xen-14 bioluminescent plasmid has been successful in the bovine (Curbelo et al., 2010) and *E. coli* containing pAK1-*lux* within the ovine (Moulton et al., 2009a) reproductive tracts, suggesting that an *in vivo* application of this technology in a swine model is possible. Therefore, the objective of this study was to determine whether *E. coli* O157:H7 transformed with the pAK1-*lux* bioluminescent plasmid could be successfully monitored within the GI tract of weanling pigs.

Materials and Methods

Bacterial Cultivation Conditions

Escherichia coli O157:H7 ATCC 43888 ($\Delta stx_1 stx_2$) was transformed with the pAK1-*lux* plasmid by methods previously described by Moulton et al. (2009a). The *E.*

coli O157:H7+pAK1-*lux* is routinely cultured on tryptic soy agar (TSA; Difco Co., Corpus Christi, TX) supplemented with 50 µg/mL ampicillin (AMP) at 37°C. Luminescence was verified after transformation using a Berthold/NightOwl camera equipped with the WinLight 32 software, version 2.51.11901 (Berthold Technologies; Oak Ridge, TN). A fresh, 24 h culture of *E. coli* O157:H7+pAK1-*lux* cultivated in tryptic soy broth (TSB; Difco Co., Corpus Christi, TX) supplemented with 50 µg/mL AMP at 39°C was used for each inoculation challenge.

Swine Trial

All experimental procedures were performed in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching and approved by the Institutional Animal Care and Use Committee of the USDA-ARS. Twelve weanling Yorkshire-Landrace crossbred pigs 18-21 d of age were obtained for this study. Three pigs were orally inoculated via a 10 mL dose oral gavage to achieve an approximate inoculation per pig of 7.0×10^9 CFU/mL at -10 d, -7 d, -2 d, and finally at -1 d prior to euthanization (0 d). Pigs were humanely euthanized and the duodenum, jejunum, ileum, cecum, colon and rectum were aseptically removed from each individual pig; the anterior and posterior ends of each respective section of the GI tract were tied using sterile zip ties.

Gastrointestinal Tract Sections

Each section from each individual weanling pig was stored at 4°C and analyzed within 24 h of collection. Contents from each section were collected, homogenized, and either transferred to a 96-well black sterile plate (Costar®, Corning Inc.; Corning, NY) for imaging or serially diluted in 1X phosphate buffer saline (PBS) and plated onto TSA

supplemented with 50 µg/mL AMP in duplicate for validation of *E. coli*+pAK1-*lux*. A 10 min acquisition period was used for imaging contents from each section using a Berthold/NightOwl camera. Photonic emissions were recorded in RLU, which are measured in photons/pixel second (ph/pix s). Plates were incubated for 12 h at 37°C prior to analysis and were also subsequently imaged to validate luminescence.

Following the collection of contents, the jejunum, ileum, and duodenum were divided into subsections in order to image the entire portion of the tract. The jejunum and ileum were both divided into three sections: posterior, middle and anterior. The duodenum was divided into two sections. Each section or subsection was incubated briefly to reach an internal temperature of 37°C and then subsequently imaged. The RLU values were recorded as previously described above for the intestinal contents analysis.

Gastrointestinal Tract Rinse

Following imaging, each section was stomached (Seward Stomacher® 400 Circular; West Sussex, United Kingdom) at 240 rpm for 1 min in 5 mL of 1X PBS. An aliquot of each dissected section was serially diluted in 1X PBS and plated on TSA supplemented with 50 µg/mL AMP and incubated for 12 h at 37°C. An additional 2 µL aliquot was transferred to a 96-well plate and imaged to measure photonic emissions as described above. After incubation, plates were analyzed and subsequently imaged to validate luminescence.

Statistical Analysis

Data were analyzed as a completely randomized design with repeated measures using PROC MIXED in SAS (SAS Inst. Inc., Version 9.2; Cary, NC). Experimental unit was defined as section, and significance was declared at $P < 0.05$. Pair-wise differences

among least squares means at various sample times were evaluated with the PDIFF statement.

Results

Gastrointestinal Tract Contents

To analyze the colonization of *E. coli* O157:H7 throughout the GI tract of swine, the duodenum, jejunum, ileum, cecum, colon and rectum were collected and contents were removed and analyzed for the presence of *E. coli* O157:H7+pAK1-*lux*. Analysis was based on viable plate count populations in $\log_{10}$ (CFU/mL) within each section, as well as on incubation periods within each section (Table 5.1). The 1 d incubation period had the greatest viable plate count populations observed within the ileum, cecum, colon and rectum. Incubation period ($P < 0.001$) decreased viable plate counts as incubation period increased. There were differences observed within the duodenum ($P < 0.02$), cecum ($P < 0.02$) and rectum ($P < 0.03$) when comparing the 1 d and 2 d incubation periods. There were similar populations observed within 7 d and 10 d populations with the only difference observed within the duodenum ($P < 0.001$), with 10 d displaying decreased populations. The *E. coli*+pAK1-*lux* were initially observed within the jejunum, ileum, cecum and rectum within 1 d incubation period. There were greater populations observed within the jejunum and ileum in the 2 d, 7 d and 10 d incubation periods. There were no differences observed within the colon and rectum populations of the 2 d ($P < 0.90$), 7 d ($P < 0.63$) and 10 d ($P < 0.36$).

Photonic emissions from the *E. coli*+pAK1-*lux* were measured in RLU (ph/pix s) using the Berthold NightOwl camera. The RLU values of the GI content ranged from 38.67 ph/pix s to 53.41 ph/pix s (data not reported in tabular form). The incubation

period ($P < 0.74$) and section ($P < 0.52$) did not affect the capabilities of the pAK1-*lux* plasmid to emit photons, thus exhibiting luminescence. There was no difference in RLU values observed within each individual section; duodenum ($P < 0.27$), jejunum ($P < 0.35$), ileum ($P < 0.19$), cecum ($P < 0.23$), colon ($P < 0.32$) and rectum ($P < 0.12$), respectively.

Gastrointestinal Tract Rinse

After the GI content was removed, each section was stomached in 1X PBS to remove any *E. coli* O157:H7 cells that might have adhered or colonized along the intestinal endothelial lining. The 1 d incubation period had the greatest populations within all rinse of the GI tract and had similar populations when compared to the 2 d incubation period except within the duodenum ($P < 0.003$; Table 5.2). The 7 d and 10 d incubation period data indicated that no difference within populations of the section with the exception of the duodenum ($P < 0.009$), which had greater populations observed at 7 d.

The *E. coli*+pAK1-*lux* initially were observed within the cecum, jejunum and ileum within 1 d and 2 d incubation periods. Greater populations of adhered cells were observed within the cecum, colon and rectum at 1 d incubation period in comparison to the other incubation periods, thus suggesting the possibility of *E. coli* O157:H7 being shed.

The RLU values from the GI sectional rinse were similar to the GI content values. The GI rinse values ranged from 37.09 ph/pix s to 43.09 ph/pix s (not reported in tabular form). The incubation period ($P < 0.61$) or the section ($P < 0.21$) did not affect the photonic emissions or luminescent capabilities of the pAK1-*lux* plasmid. There was no

difference in RLU values observed within each individual section; duodenum ($P < 0.63$), jejunum ($P < 0.27$), ileum ($P < 0.17$), cecum ($P < 0.46$), colon ($P < 0.16$) and rectum ($P < 0.63$).

Discussion

Since most *E. coli* O157:H7 infections are acquired through the consumption of contaminated food products, there have been numerous pre- and post- harvest intervention strategies implemented to reduce prevalence of this dangerous bacterium. Here, we utilized bioluminescent technology to determine pathogen survival and movement within the GI tract of a simple stomached animal. By employing bioluminescence as a monitoring device, researchers can obtain a better understanding of pathogen location and will have potential capabilities of real-time tracking and monitoring of pathogens.

When comparing the GI content treatments over the course of a 1 d infection, significant changes in the colonization pattern of *E. coli* O157:H7 were observed within each section. Overall, there were greater *E. coli* O157:H7 populations observed within the GI rinse than compared to the GI content. This data indicates that the *E. coli* O157:H7 was more apt to adhere to the intestinal endothelial lining rather than remain loose or sloughed within the GI tract.

The greatest populations of *E. coli* O157:H7 within the GI content were observed in the small intestine (duodenum, jejunum, ileum). These data indicate that *E. coli* O157:H7 is able to colonize successfully within the small intestine within 1 d post-exposure. The 1 d incubation period exhibited greater populations that had adhered to the intestinal lining, while the 10 d incubation period exhibited decreased populations within

the cells that did not adhere. This correlates with the increase observed initially within the rectum populations, suggesting that that non-adhered populations are being shed. These data propose that *E. coli* O157:H7 is able to resist bacterial properties within the GI tract such as bile, and still colonize and cause infection. This suggests that upon initial exposure to the pathogen, there is a propensity for the bacteria to colonize throughout the GI tract within 1 d. The *E. coli* O157:H7 populations with an increased incubation period such as 7 d and 10 d post-exposure tend to exhibit decreased populations being colonized and sloughed through the GI tract.

The GI content and GI sectional rinse exhibited similar photonic emission values and results, indicating that each section exhibited relatively the same amount of photonic emissions. All sections exhibited luminescence with little difference observed across incubation groups. Although, the photonic emissions that were obtained were relatively low, these data are in accordance with results previously obtained from our lab, which indicate that photonic emissions from the pAK1-*lux* plasmid decrease over time (Duoss, unpublished). It is possible that the stability of the pAK1-*lux* plasmid is compromised in the absence of AMP *in vivo*, resulting in a decrease of photonic emissions. This is supported by results reported by Moulton et al. (2009a) that indicated in the presence of AMP, there were 100% photon emitting bacteria; and in the absence of AMP there was a significant decrease after 1 d. Although no section exhibited luminescence upon imaging, photonic emissions and viable plate counts ensure that the plasmid, pAK1-*lux* was retained and *E. coli* O157:H7 were present. The use of a more sensitive coupled-camera device, such as the Stanford Photonic Imaging System (XR/MEGA-10Z; Stanford Photonics Inc., Palo Alto, CA), might be more viable for this study, than the NightOwl/Berthold camera used. Therefore, it is still possible that biophotonic

technology could be beneficial to understanding bacterial pathogenesis and colonization patterns *in vivo*. Further research is needed, though, to increase the sensitivity of the procedure used.

Conclusion

Transformation of *E. coli* O157:H7 with the pAK1-*lux* gene cassette did exhibit luminescence within the GI tract implemented within the swine model. Considering that pathogen tracking was successful in the monogastric, simple stomached model, this gives inspiration that pathogen tracking could be utilized within the ruminant, more complex stomached model and be successful within a restricted time frame. Further research is needed to validate if *E. coli* O157:H7 transformed with the pAK1-*lux* bioluminescent plasmid could serve as a valid model for long-term *in vivo* study.

Table 5.1 The least square means of *Escherichia coli* O157:H7+pAK1-*lux* (ATCC 43888) populations (log_{10} CFU/mL) within contents collected from the gastrointestinal tract of swine

	Incubation Period (d)									
	***Log*$_{10}$ CFU/mL**				***P-values (between incubation periods)***					
Sections	**1 d**	**2 d**	**7 d**	**10 d**	**1 d vs 2 d**	**1 d vs 7 d**	**1 d vs 10 d**	**2 d vs 7 d**	**2 d vs 10 d**	**7 d vs 10 d**
Duodenum	6.52^{a}	4.26^{a}	4.40^{ab}	0.12^{a}	0.02	0.01	<0.001	0.93	<0.001	<0.001
Jejunum	7.73^{ab}	8.41^{b}	4.75^{be}	5.11^{b}	0.41	0.009	0.003	<0.001	0.003	0.66
Ileum	8.80^{b}	8.77^{b}	7.21^{d}	6.48^{b}	0.97	0.06	0.005	0.07	0.006	0.28
Cecum	8.24^{b}	6.17^{c}	3.88^{ae}	4.75^{bc}	0.02	<0.001	0.002	0.009	0.10	0.32
Colon	6.75^{a}	6.30^{c}	2.97^{ac}	3.59^{c}	0.60	<0.001	0.005	0.003	0.002	0.46
Rectum	8.29^{b}	6.40^{c}	2.30^{c}	3.23^{c}	0.03	<0.001	<0.001	<0.001	0.005	0.27
P-value	0.05	0.03	0.04	0.04						

$^{a, b, c, d, e}$ Lsmeans without a common subscript within column differ ($P < 0.05$)

Table 5.2 The least square means of *Escherichia coli* O157:H7+pAK1-*lux* (ATCC 43888) populations (log_{10} CFU/mL) within rinse obtained from the gastrointestinal tract of swine

	Incubation Period (d)									
	***Log*$_{10}$ CFU/mL**				***P-values (between incubation periods)***					
Sections	**1 d**	**2 d**	**7 d**	**10 d**	**1 d vs 2 d**	**1 d vs 7 d**	**1 d vs 10 d**	**2 d vs 7 d**	**2 d vs 10 d**	**7 d vs 10 d**
Duodenum	7.72	2.91^{a}	7.34^{a}	4.15^{x}	0.001	0.57	0.005	0.002	0.33	0.009
Jejunum	8.05	7.04^{b}	6.39^{ab}	5.82^{y}	0.27	0.08	0.02	0.50	0.21	0.53
Ileum	7.59	7.30^{b}	6.09^{ab}	5.38^{xy}	0.74	0.11	0.02	0.18	0.04	0.43
Cecum	7.65	7.11^{b}	4.55^{bc}	3.52^{x}	0.73	0.06	0.02	0.11	0.03	0.51
Colon	7.23	5.89^{b}	3.46^{c}	5.02^{xy}	0.39	0.03	0.16	0.13	0.57	0.32
Rectum	6.34	6.23^{b}	2.75^{c}	3.28^{xy}	0.94	0.03	0.17	0.03	0.20	0.35
P-value	0.21	0.03	0.04	0.07						

$^{a, b, c}$ Lsmeans without a common subscript within column differ ($P < 0.05$)

$^{x, y}$ Lsmeans without a common subscript within column tend to differ ($P \leq 0.10$)

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CHAPTER VI

CONCLUSION

Due to various pre- and post- harvest intervention strategies implemented throughout harvesting and processing plants, the United States food supply is one of the safest in the world. Although safe, foodborne pathogenic bacteria, such as enterohemorrhagic *Escherichia coli*, (EHEC) are still a threat to human health and well-being. Pre-harvest intervention strategies are extensively being researched to discover methods to reduce and/or eliminate EHEC and *E. coli* O157:H7 populations in cattle and decrease the potential for spread and contamination.

Researchers have suggested that the incorporation of citrus by-products within cattle diets may be a successful EHEC and *E. coli* O157:H7 pre-harvest intervention strategy. The essential oils within the citrus by-products possess antimicrobial activities that assert lipophilic properties on pathogens, by increasing permeabilization of the bacteria cell wall and cytoplasm, thus destroying the bacteria. Although previous research suggests that all EHEC function similarly, results presented in this thesis suggest that when citrus by-products in the powder or pelleted variety were supplemented to mixed ruminal microorganism fluid media, EHEC populations were affected differently. While the EHEC serotypes O26:H11, O111:H8, O145:H28 and O157:H7 populations were not affected, the O103:H8 and O157:H7 $\Delta stx_1 stx_2$ (ATCC 43888) had decreased populations. Further research is needed to indicate the effects of citrus by-products on EHEC serotypes and the differences observed within the various EHEC serotypes.

One of the main limitations to pre-harvest food safety intervention strategies is the inability to successfully monitor and track pathogens in real-time. The transformation of *E. coli* O157:H7 with bioluminescent plasmids could aid in the tracking of pathogens. *Escherichia coli* O157:H7 transformed with the bioluminescent plasmids pAK1-*lux* and pXEN-13 grew similarly within mixed ruminal microorganisms fluid media, suggesting that bioluminescent plasmids do not alter the abilities of *E. coli* O157:H7 to thrive in rumen-like conditions of cattle. The implementation of bioluminescent plasmids can also serve as a research tool for comprehending the pathogenesis, pathogen location and physiological systems *in vivo*. *Escherichia coli* O157:H7 transformed with the pAK1-*lux* bioluminescent plasmid was successfully monitored through the gastrointestinal tract of weanling pigs with incubation periods of 10, 7, 2, and 1 day. Successful pathogen tracking within the monogastric, simple stomached model, gives inspiration that pathogen tracking could be utilized within more complex stomached model, such as cattle and be successful within a restricted time frame. Further research is needed to validate if *E. coli* O157:H7 transformed with the bioluminescent plasmids can serve as a valid model for long-term *in vivo* studies.