A COMMERCIALLY AVAILABLE SIDEROPHORE-RECEPTOR AND PORIN-BASED VACCINE REDUCED THE PREVALENCE OF *ESCHERICHIA COLI* O157:H7 IN THE FECES OF BEEF CATTLE UNDER FIELD CONDITIONS IN 10 COMMERCIAL FEEDLOTS.

by

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Abstract

A total of 284,300 animals from 10 commercial feedyards in Nebraska and Colorado were used to evaluate the effectiveness of a siderophore-receptor/porin protein-based vaccine under commercial feedlot conditions. Individual feedlots were assigned to 1 of 2 treatments: 1) all incoming cattle injected with 2 ml of SRP *E. coli* O157:H7 vaccine subcutaneously at arrival and at time of re-implant (VAC) or 2) all incoming cattle were not vaccinated, and were used as negative controls (CON). Twenty freshly voided fecal samples were taken from 5 pen floors of market ready cattle at each feedyard once a month during May, June, July, and August of 2010. Pre-harvest blood samples were collected on 3 occasions throughout the summer (June, July, and August). For each sampling month, 1 lot of 5 animals representing each feedyard was sampled. Fecal and blood samples were shipped to Epitopix, LLC for subsequent microbiology and anti-SRP antibody testing. Samples were coded such that laboratory personnel were blinded to the location and treatment of samples. Cattle receiving VAC treatment had reduced prevalence of *E.coli* O157:H7 in their feces relative to the E. coli O157:H7 prevalence in the feces of CON cattle (12.83% vs. 20.25% for VAC and CON, respectively; $P = 0.07$). Anti-SRP antibody titer was higher in the serum from VAC cattle relative to the SRP titer levels in serum obtained from CON cattle (0.622 and 0.075 for VAC and CON, respectively; $P < 0.001$). These data suggest that vaccination of feedlot cattle with SRP upon arrival at the feedlot and again 70-100 days pre-harvest reduces shedding of *E. coli* O157:H7.

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Preface

Chapter two entitled “A commercially available siderophore-receptor and porin-based vaccine reduced the prevalence of *E. coli* O157:H7 in the feces of beef cattle under field conditions in 10 commercial feedlots.” was submitted for publication in the *Journal of Food Protection.* The text and figures within these chapters are formatted according to the guidelines that were specified by this journal.

Chapter 1 - Literature Review

Escherichia coli

In 1885, German Pediatrician Theodor Escherich described slender, rod shaped organisms growing in the feces of infants. Escherich named the organism *Bacterium coli commune,* but is known today as *Escherichia coli.* In 1945, Bray, an English scientist, found an antigenically homologous strain of *E. coli* to be responsible for an enteritis outbreak in babies. Four years later, Taylor investigated another outbreak in nursery babies and identified the epidemic strain as *E. coli* D433. Kauffmann (1947) began classifying *E. coli* based on serological characteristics, and later found that both Bray and Taylor's strains belong to newly established serogroup *E. coli* O111:B4 (Cooper 1956). The classification scheme was further developed, and based on heating and agglutination methods. Three different antigens were described; "O", "K", and "H." Since then, the "F" antigen has been added to the serological classification system (Gyles 1994).

E. coli belongs to the family *Enterobacteriaciae* and is a Gram negative facultative anaerobe. Some *E. coli* identification can be aided by whether the particular *E. coli* strain is motile or non-motile and/or ferments lactose. (Brenner 1984).

E. coli O157:H7

E. coli responsible for enteric disease has been divided into six different groups based on pathogenicity. This classification system recognizes six major groups: 1) enteropathogenic *E. coli* (EPEC), 2) enterohemmorhagic *E. coli* (EHEC), 3) enterotoxigenic *E. coli* (ETEC), 4) enteroaggregative *E. coli* (EAEC), 5) enteroinvasive *E. coli* (EIEC), and 6) diffusely adherent *E. coli* (DAEC) (Kaper 2004). Enterohemmorhagic *E. coli* has been the most studied group, and

includes *E. coli* O157:H7. Cattle are the major reservoir for *E. coli* O157:H7 in the United States. *E. coli* O157:H7 colonize in the intestines of cattle, and are naturally shed in the feces. *E. coli* O157:H7 shed in the feces of cattle is a source of carcass contamination at harvest (Griffin et al 1991). *E. coli* O157:H7 is responsible for human disease in the United States, including hemorrhagic colitis and hemolytic uremic syndrome. The key differentiation of EHEC from other groups of *E. coli* is the production of shiga toxins (Lior 1994). From a public health perspective, shiga toxin production is the most important virulence factor of *E. coli* O157:H7 infections. It is crucial to disease manifestation and pathogenesis in humans (Griffin et al 1991).

There are two shiga toxins associated with *E. coli* O157:H7. Shiga toxin 1 (Stx -1) is nearly identical to the shiga toxin produced by bacterium Shigella dystenteriae, differing by only one amino acid (Gyles et al 1994). *E. coli* O157:H7 produces a second shiga toxin (Stx – 2) which is 50% homologous to the first. It is possible for strains to produce Stx-1, Stx-2, or both. Despite their similarity, the two toxins are functionally very different. Studies have shown *E. coli* O157:H7 strains encoding for Stx-2 to be more pathogenic than those encoding Stx-1 (Jackson et al 1987, Fraser et al 2004). Children who have become infected by an *E. coli* strain possessing Stx-2 most commonly suffer from hemolytic uremic syndrome, while infection with a Stx-1 *E. coli* strain usually manifests as hemorrhagic colitis or is asymptomatic (Jenkins et al 2003).

Both Stx -1 and Stx -2 are AB5 toxins, which contributes to their affinity for epithelium of the gastro-intestinal and renal systems. The B subunit is responsible for attachment to Gb3/CD77 receptors on the host cells which are most prevalent on the intestinal epithelium and glomerular cells of the kidney in humans (Patton et al 2004). Once adhered, the A subunit portion of the toxin is endocytosed, and it inhibits protein synthesis within the host cell leading to apoptosis (Gyles 2007).

Human Disease

Humans can become infected with *E. coli* O157:H7 by several routes, the most common being from undercooked meat that was contaminated during slaughter. Other routes of infection include ingestion of contaminated milk, water, fruits, or vegetables. Finally, transmission from person to person has been reported in hospitalized children (Mead et al 1998).

Symptoms of an *E. coli* O157:H7 infection include abdominal cramps and bloody diarrhea (hemorrhagic colitis), with an average incubation time of 3-5 days prior to clinical signs. In certain populations, particularly in children under the age of 5, life–threatening complications can occur. These complications include renal failure, anemia, and thrombocytopenia leading to a potentially lethal condition termed hemolytic uremic syndrome (HUS). Approximately 10% of *E. coli* O157:H7 infections result in HUS, with a 3-5% fatality rate in these cases (CDC 2010).

Overall incidence of disease from *E. coli* O157:H7 infection was .99 per 100,000 people in 2009, with the highest incidence in young children and the elderly. This pales in comparison to incidence of other foodborne diseases. In January of this year, the CDC reported 1 in 6 people in the United States become ill due to a foodborne pathogen, leading to 3,000 deaths per year. Reported illnesses caused by *Campylobacter*, *Salmonella*, *Shigella*, or *Cryptosporidium* are all more common than those due to *E. coli* O157:H7 (CDC 2010). However, the potentially lethal complications of *E. coli* O157:H7 infection has lead to intensive investigation into the epidemiology of the organism and the development and implementation of control strategies in the beef production system. (Wileman 2010).

Pre – Harvest Intervention

Over the last 20 years, much of the time and resources has been spent on mitigating *E. coli* contamination within the packing plant. The program termed Hazard Analysis and Critical

Control Points (HACCP) was developed as a set of standard operating procedures designed to mitigate carcass contamination at critical control points identified in production systems. Although effective, it is proposed that reducing the *E. coli* load coming into the plant will increase the effectiveness of post-harvest procedures such as HACCP (Loneragan and Brashears 2005). In addition to protecting the meat supply, pre-harvest measures may help to reduce the environmental contamination of ground water and produce that has been linked to cattle (Rasmussen 2001).

Scientist found that the use of neomycin, an aminoglycoside, in the feed significantly reduced shedding of *E. coli* O157:H7 in the feces of feedlot cattle (Elder 2002). Despite this finding, Neomycin treatment has not been adopted as a pre-harvest intervention strategy because it is not approved for control of *E. coli* shedding in cattle in the United States. The use of antibiotics in the feed of animals is a socially sensitive topic due to fears of drug resistant organisms (Callaway 2009).

Another class of feed grade antibiotics are the ionophores, which are approved for growth promotion and coccidian control in the United States. Ionophores are not used in human medicine, so the issue of resistance is not as pertinent as with traditional antibiotics (Callaway et al. 2003). However, due to the microbiological properties of some of the foodborne pathogens, no detectable effect on *E.coli* O157:H7 shedding was seen in both in *vivo* and in *vitro* studies (Edrington et al. 2003; Edrington et al. 2006).

Another intervention tested for its ability to control *E. coli* O157:H7 shedding in feedlot cattle are probiotics. Probiotics are commensal bacteria that can be administered orally to alter rumen microflora. It is well documented in the literature that these “beneficial” bacteria can be used to out compete “harmful” bacteria. Other positive effects of probiotics are to support

immune function, as well as production of anti-bacterial compounds (Berry and Wells, 2010). By far the most studied pro-biotic in cattle has been *Lactobacillus* strains. Brashears et al (2003) evaluated the effect of *Lactobacillus acidophilus* NPC 747 on 180 feedlot steers. Steers receiving the probiotic treatment shed less *E. coli* O157:H7 in their feces compared to control cattle. Stephens et al (2007) conducted a similar study in which 500 yearling steers were placed in to cohorts and fed varying levels of *Lactobacillus* throughout the feeding period. The prevalence of *E. coli* O157:H7 in the feces of control animals was greater than that in cattle receiving *Lactobacillus* probiotic in the feed.

Administration of prebiotics for the reduction of *E. coli* O157 in the feces of cattle has been explored. A prebiotic is an organic compound or sugar that cannot be utilized by the animal, but can be utilized by the animal's resident microflora. In cattle, the theory is that these compounds bypass the rumen, and support intestinal bacteria directly. The concept is that the beneficial bacteria are able to outperform the pathogens by utilizing these specific sugars, giving them a competitive advantage (Schrezenmeir et al 2001).

Another compound that may be of some benefit in *E. coli* mitigation is sodium chlorate. *E. coli* O157:H7 is in the family Enterobacteriacea, which is made up of facultative aerobes, meaning they can utilize aerobic respiration as well as anaerobic fermentation. An enzyme that becomes activated during aerobic respiration is nitrate reductase, which actively converts chlorate to chlorite. Chlorite is lethal to *E. coli*, but isn't harmful to most other commensal bacteria (Anderson et al. 2000). In cattle given water supplemented with sodium chlorate there was a two log reduction in *E. coli* O157:H7 isolated from the feces after an oral inoculation challenge (Callaway et al. 2002).

Researchers have also proposed that diet may be a major contributing factor to the *E. coli* burden in the animal (Callaway et al 2009, Klopfenstein at al 2009, Reinstein et al 2009). In general, high energy carbohydrate rich feeds which are actively converted to volatile fatty acids in the rumen have been shown to increase the *E. coli* O157:H7 growth in the distal intestines. It has been documented that cattle on a forage based diet have consistently less *E. coli* shed in the feces than cattle on a grain-based diet (Tkalcik et al. 2000). Similarly, animal-level prevalence of *E. coli* O157 was higher in cattle fed distillers grains, compared to those with no distillers in the feed (Jacob et al. 2008). More specifically, the type of grain and the way in which the grain is processed may have an effect on *E. coli* O157 colonization. Studies have shown a greater *E. coli* burden in animals fed a barley-based diet compared to those on a corn based diet (Berg et al 2004). Also, cattle receiving a steam rolled corn diet were more likely to shed *E. coli* O157 in the feces compared to cattle fed a dry-rolled corn diet (Fox et al. 2007). Despite the fact that these studies implicated diet as a major contributing factor in the *E. coli* prevalence in cattle, they do not take into account the economic impact of changing form a high energy grain-based ration to a forage based diet. It is unlikely that the improvement in *E. coli* shedding would be worth the loss in cattle performance, feed efficiency, and yield grade.

Bacteriophages are small, highly specific viruses that incorporate their DNA into the host DNA, use host cell machinery to replicate, followed by destruction of the host cell (Sheng 2006). Exceptional efficacy of *E. coli* O157:H7 specific phages has been documented in *vitro* (Tanji et al 2005), unfortunately, there is very little documentation of the effectiveness of bacteriophages in the cattle industry. More in *vivo* trials in cattle will be necessary in order to truly evaluate the potential of phages as a pre-harvest mitigation tool (Rozema et al 2009).

Epidemiology

Over the last twenty years, much effort has been put forth into better understanding the epidemiology and ecology of the *E. coli* O157 organism. Hancock (1997) was one of the first scientists who examined *E. coli* prevalence at the feedlot level. He found *E. coli* to be widely distributed throughout the U.S., but at relatively low levels. One hundred feedlots in 13 states were sampled; in each feedlot had 30 fecal pat samples from 4 different pens collected. Overall, individual animal prevalence was only 1.8%, however feedlot level prevalence was estimated at 63% with no regional distribution of *E. coli* prevalence identified (Hancock 1997). In another study, individual animal prevalence in feces and on hides was 28% and 11%, respectively with a pen level prevalence of 72% and 38% for the feces and hides, respectively. (Elder et al. 2000). More recently, Dewell et al (2005) tested cattle from 12 feedlots in three different states and found 13 of 15 pens tested to have at least one positive sample, and within pen prevalence ranged from 3.3% to 77.8%.

Seasonality and environmental factors effecting *E. coli* O157 shedding has been a topic of interest as well. Hancock (1997) reported a higher prevalence among feedlot cattle in the summer months relative to those in the winter months. Reasons for this phenomenon are yet to be discovered, but Edrington et al (2006) suggested a correlation between day length ($r = .67$ $P = .0009$) and temperature($r = .43$ $P = .05$) and *E. coli* prevalence (Edrington et al. 2006). The effect of pen conditions on *E. coli* prevalence has also been subject to investigations with greater prevalence of *E. coli* O157:H7 being found in pens with muddy conditions, compared to pens with ideal conditions (Sargeant et al. 2004; Smith et al., 2001).

Another factor in the ecology of *E. coli* O157:H7 is the role of fomites. Van Donkersgoed et al. (2005) reporting *E. coli* O157 isolation from feedbunks (1.7%), water troughs (12%), and incoming water supplies (4.5%). These fomites may aid in the transmission of *E. coli* O157

between cattle and the environment. Further studies have looked into the possibility of carriers, such as *Musca domestica* (house flies). In 2006, eight calves were subjected to flies infected with *E. coli* O157:H7. All eight calves were fecal positive for up to 11 days following exposure with 62% of calves remaining positive until the end of the study (Ahmad et al. 2007).

From a public health perspective, eliminating carcass contamination at the time of harvest is most critical. Elder et al (2002) measured fecal prevalence and hide contamination at the feedyard and found fecal and hide prevalence to be significantly correlated with carcass contamination ($P = .001$). In another study conducted by Ransom et al (2003) cattle were divided into two groups based on *E. coli* O157:H7 prevalence in fecal pats. In pens with fecal prevalence greater than 20%, prevalence of *E. coli* O157 on hides, colons, pre-evisceration, post-evisceration, and in-cooler samples was 22.5%, 46.3%, 12.5%, 2.5%, and 0.6% respectively. However, pens in which fecal prevalence was less than 20%, prevalence of *E. coli* O157 on hides, colons, pre-evisceration, post-evisceration, and in-cooler was 5.7%, 7.1%, 7.1%, 0.0%, and 0.0% respectively. These studies illustrate two important points. One, in-plant procedures are very effective at eliminating the *E. coli* burden coming into the plant. Secondly, if the incoming pathogen load is significant, in-plant procedures are overwhelmed and carcass contamination occurs. It is reasonable to assume that reducing the *E. coli* burden pre-harvest will in turn reduce the likelihood of carcass contamination at harvest (Loneragan et al 2005).

Vaccination

Along with a Siderophore Receptor and Porin protein based vaccine, another product has been developed to utilize immunomodulation in the reduction of *E. coli* O157:H7 burden in production cattle. Type III secretory proteins (TTSPs) are used as antigens for the vaccine, these proteins play a key role in villi adherence in the intestines of cattle. Antibody production against

these proteins would prevent attachment and colonization of *E. coli* O157:H7 in the distal colon of cattle (Dziva et al 2007).

Initial field trials conducted by Potter et al (2004) showed a 58.7% reduction in fecal prevalence of *E. coli* O157:H7 in cattle vaccinated with the type III secretion proteins ($P = 0.04$). In a dose determination study, cattle vaccinated with one, two, and three doses of the type III secretion proteins were 68%, 66%, and 73% less likely to be shedding *E. coli* O157:H7 at the time of sampling when compared to unvaccinated cattle in the same feedyard ($P < 0.05$). This trial also revealed an effect of herd immunity as unvaccinated cattle who were in direct contact with vaccinated cattle were 59% less likely to be shedding *E. coli* O157:H7 than unvaccinated cattle who were housed separately (Peterson et al 2007). In 2008, Smith et al published a study conducted at 19 commercial feedlots in the United States. Using the ROPES method of sample collection, they found that pens with unvaccinated cattle were more likely to have a positive ROPES sample ($OR = 0.59$ $P = 0.004$) than pens that received vaccine. The ROPES method measures oral contamination of *E. coli* O157:H7, and has been described as being directly correlated to environmental contamination in the pen, and therefore can be used to evaluate shedding in the animal. In another study, Smith et al (2009) evaluated terminal rectal mucosal (TRM) samples in 21 pens of cattle (11 vaccinated with type III secretion proteins, 10 non-vaccinated), and found vaccinated cattle were 92% less likely to be colonized with *E. coli* O157:H7 than non-vaccinated cattle ($OR = 0.07$, $P = 0.0008$) (Smith et al 2009a). More recently, Smith et al (2009b) evaluated the effect of regional vaccination within the feedyard. Cattle were assigned to three treatment groups, all cattle in the pen received type III secretion protein vaccination (ALLVAC), half the cattle in the pen received vaccination (HALFVAC), or all cattle in the pen received placebo control (NOVAC). When comparing the ALLVAC vs. NOVAC

cattle, there was a significant reduction in *E. coli* O157:H7 fecal prevalence and hide contamination (63% and 55% $P < 0.05$). Interestingly, when comparing cattle within the HALFVAC pens, fecal contamination was significantly reduced, whereas hide contamination was not ($P = 0.33$). This suggests that environmental control and mingling of cattle are important factors in study design (Smith et al 2009b). Furthermore, vaccination of the entire feedlot population may be necessary to maximize the effectiveness of the vaccine to reduce contamination at harvest.

Snedeker et al (2010), did a meta-analysis of 8 published research trials conducted in the U.S. or Canada, evaluating the efficacy and heterogeneity of the type III secretory protein based vaccination. The odds of cattle being fecal positive for *E. coli* O157:H7 was significantly lower in cattle who received the vaccine vs. those who received placebo control ($OR = 0.38$, $CI = 0.29$-0.51). There was also statistically significant heterogeneity between trial results ($Q = 16.1$, $P = 0.024$). Overall, vaccination using type III secretion proteins may be used as an effective pre-harvest intervention, helping to reduce the *E. coli* O157:H7 contamination at harvest.

SRP Vaccine

Siderophores are low molecular weight proteins which many Gram negative bacteria utilize to compete with the host cells for free iron in low-iron environments (Neilands et al 1995). Siderophores are released by the cell and scavenge free iron and form a complex with ferric iron. The siderophore then carries the bound iron back to the bacterial cell wall where it binds with a siderophore receptor and is transported in the periplasmic space, converted to ferrous iron and is utilized by the cell (Prescott et al 2005).

In iron rich environments, these same gram negative organisms utilize another class of iron regulated outer membrane proteins called porins. Porins serve a similar function to

siderophore receptors, but are less sophisticated and can only be utilized when simple diffusion of iron into the cell is possible (Emery et al 2000).

Initially, scientists at Epitopix LLC created a novel vaccine utilizing siderophore receptors and porins from the outer membrane of *Salmonella* species. The technology has since been developed for *E. coli* O157, which also utilize siderophore receptors as a survival strategy. The proteins are used for antigenic stimulation and immunomodulation within the animal. The subsequent humoral immune response produces antibodies specific to the siderophore receptors and porins, which then bind to the siderophore receptors and porins of *E. coli* O157 bacteria and block iron uptake by the cell, effectively starving the organism of iron leading to cell lysis (Emery et al. 2000).

In the discovery stage of *E. coli* O157:H7 SRP vaccine, several trials were completed in turkeys with initial field trial results showing turkeys vaccinated with SRP had decreased carcass condemnation (31% decrease $P < 0.01$), decreased mortality (38% decrease $P < 0.01$) and increased weight gain (9% increase $P < 0.01$). (Emery et al 2000). These results led to further exploration and development of an *E. coli* O157:H7 vaccine to be used in cattle.

Kansas State University researchers have collaborated with scientists at Epitopix to conduct a series of research trials over the last 10 years. The initial study conducted by Thornton et al (2009) was a challenge model in which 30 calves were randomly assigned to one of two treatment groups (vaccinate or placebo). Calves were orally inoculated with nalidixic acid-resistant (Nalr) *E. coli* O157:H7 two weeks after the second vaccination. Fecal samples and rectoanal mucosal swabs were collected everyday for the first five days post-challenge, and then 3 times per week over the next 4 weeks. Fecal prevalence and enumeration of fecal concentration of (Nalr) *E. coli* O157:H7 was calculated and analyzed. Vaccination of cattle with the SRP

vaccine tended to ($P = 0.10$) decrease fecal concentration of *E. coli* O157:H7 and the number of calves fecal culture positive for *E. coli* O157:H7 was lower ($P = 0.05$) in the vaccinated group relative to the control group (Thornton et al 2009).

In addition to an oral inoculation challenge, vaccine efficacy has been measured in cattle naturally shedding *E. coli* O157:H7 (Fox et al., 2009). A group of 600 calves were screened for fecal prevalence of *E. coli* on two separate occasions, and cattle found to be shedding *E. coli* O157 were enrolled in the study. Cattle were randomly divided into 3 treatment assignments: placebo control, 1,000 µg SRP vaccine, 1,500 µg SRP antigen. For each treatment assignment two doses were given 21 days apart. Rectoanal mucosal swabs as well as fecal samples were collected 2 to 3 times per week for 8 weeks following vaccination. Concurrently, serum samples were collected weekly and antibody response to SRP vaccination was determined. The 1,500 µg dose significantly reduced *E. coli* O157:H7 fecal shedding (17.7% vs. 33.7%; $P < 0.01$) and the number of days cattle tested positive for *E. coli* O157:H7 in either a RAMS or fecal samples when compared to controls ($P < 0.05$). Similar numerical differences for all of the outcome variables regarding *E. coli* O157:H7 prevalence were seen in the 1,000 µg dose group, however the differences were not statistically significant. Both treatment groups (1,000 µg and 1,500 µg) had significantly higher anti-SRP antibody titers than controls in all weeks except for week 0 ($P < 0.01$) (Fox el al. 2009).

Subsequently, two field studies were conducted in commercial feedlots in Nebraska (trial 1 n= 1,252) and Kansas (trial 2 n=1,284). In each trial, cattle in large pens (> 120 head per pen) were randomly assigned to one of two paired treatment pens of 60-70 cattle (20 pens total for each trial). In trial 1, cattle in half of the pens received 2 ml SRP *E. coli* O157:H7 on day 0 and 27 (two doses), while cattle in the opposing paired pen received a placebo control. In the second

trial, cattle in half of the pens received 3 doses of the SRP vaccine (day 0, 21, and 42), cattle in the opposing pens received a placebo control. In both trials, cattle were weighed periodically throughout the trial, and feed intake was monitored. In trial 1, samples from freshly voided feces were collected on day 21, 35, and 70; and on day 85 fecal, recto-anal mucosal, and hide samples were also collected. Across all days and sampling methods, *E. coli* prevalence was lower among vaccinates compared to controls ($P = 0.04$). In trial 2, fecal samples were collected at 42 and 98 days on feed. On day 98, an 85% reduction in *E. coli* O157:H7 prevalence was observed in cattle vaccinated with SRP ($P < 0.01$). Furthermore, vaccination was associated with a 98.2% reduction in *E. coli* concentration in the feces of vaccinated animals relative to controls ($P < 0.01$). In both trials, there was no treatment effect on average daily gain, daily feed intake, or gain efficiency (Thomson et al 2009).

Researchers have investigated the usefulness of the *E. coli* O157: H7 SRP vaccine in other areas of the beef production system. In 2010, Wileman et al. inquired as to whether cows vaccinated with the *E. coli* SRP pre-partum would successfully transfer antibodies to their calves via the milk. Twenty cross-bred commercial cows were selected from the Kansas State cow-calf herd and were randomly divided into one of two treatments. Ten cows received SRP vaccination 30 and 60 days prior to the start of calving season, while 10 cows received a placebo control. Blood samples were taken from calves at pre-suckle, 6, 12, and 24 hours; as well as 7, 14, and 21 days post-partum. Anti- SRP antibody and total protein fractions were measured for each calf. There was a significant treatment by sample time interaction for SRP antibody level in the calves ($P = 0.01$). This can be explained by the fact that there was no difference in titer level between treatment groups pre-suckle, then a significant increase in the SRP antibody levels post –suckle in calves born to cows vaccinated with SRP (Wileman et al. 2010).

Another avenue for the *E. coli* O157:H7 vaccination explored in recent research has been the cross-protection of SRP *E. coli* O157:H7 vaccination to the K99+ strain of *E. coli*, a major cause of scours in neonatal calves. Eleven Holstein male neonatal calves were assigned to one of two treatment groups; colostrum administration from a heifer vaccinated with *E. coli* O157:H7 SRP, or colostrum from a non-vaccinated heifer. Calves were challenged with a K99+ strain of *E. coli* one hour after colostrum administration. Calves were then monitored twice per day for one week; fecal, respiratory, hydration, and appetite scores were recorded. Calves given colostrum from cows treated with the SRP vaccine had significantly better fecal consistency scores than those given colostrum from non-vaccinated heifers ($P < 0.05$). Concurrently, fecal samples were collected from all calves at arrival and once per day until completion of the trial. The concentration of K99+ *E. coli* cultured during the sampling period was significantly higher in the control calves than those treated with SRP colostrum ($P < 0.05$) (Wileman et al. 2010) .

Cattle production systems in the United States are very dynamic, there is a plethora of options regarding integration of the SRP vaccine into the industry. Scientists at Kansas State University compared the efficacy of the vaccine when given at different stages of the supply chain. A group of 492 cows were stratified by age, and then randomly assigned to either SRP vaccination (administered 30 and 60 days prior to calving) or placebo control. Calves were then blocked by dam treatment assignment and randomly assigned to placebo control or SRP *E. coli* vaccination at branding, pre-conditioning and at feedlot arrival; thus a 2x2 factorial was created. The four treatment groups were as follows: unvaccinated cow, unvaccinated calf (CON); unvaccinated cow, SRP vaccinated calf (CALFVAC); SRP vaccinated cow, unvaccinated calf (COWVAC), or SRP vaccinated cow, SRP vaccinated calf (BOTH). Among the four treatment groups, fecal *E. coli* O157 prevalence at the time of slaughter was not significantly different

between cattle from the different vaccine treatments (Wileman et al. 2010). Results of this study, in combination with the results of the study conducted by Fox et al. (2009) suggest that vaccine administration during the feedyard level may have greater efficacy than when given at other stages.

Snedeker et al (2011) performed a meta-analysis of published literature evaluating the efficacy of the SRP *E. coli* O157:H7 vaccine. The analysis included three trials (Thornton et al. 2009, Fox et al. 2009, Thompson et al. 2009) and found overall that SRP vaccinated cattle were less likely to shed *E. coli* O157:H7 in the feces compared to placebo control (*OR*=0.42, *CI*=0.25-0.73). Clearly, there is a significant amount of literature suggesting the SRP vaccine may be used as an effective tool for pre-harvest mitigation of *E. coli* O157:H7. However, all studies to this point have used individual animals or pens of animals as the experimental unit. These study designs may yield inaccurate estimates of efficacy due to effects of herd immunity or the effect of contamination of the environment by unvaccinated animals (Peterson et al. 2007, Smith et al. 2009b). Keeping in mind the ecology of *E. coli* O157:H7, this thesis project compared pens within feedyards in which all animals are vaccinated to pens within feedyards in which no animals were vaccinated. As mentioned before, the SRP vaccine stimulates strong titer responses in vaccinated cattle, however, a question yet to be answered is if this response is measurable at the time of slaughter. The objectives of this thesis project were:

1. Examine the ability of the SRP *E. coli* vaccine to control *E. coli* O157 in feedlot cattle where all cattle within a feedlot are vaccinated or not vaccinated.
2. Determine the ability to measure vaccine administration compliance through observing serological responses to *E. coli* SRP vaccine.

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Chapter 2 - A commercially available siderophore-receptor and porin-based vaccine reduced the prevalence of *E. coli* O157:H7 in the feces of beef cattle under field conditions in 10 commercial feedlots.

Introduction

Despite the low incidence of foodborne disease traced to beef products (Bean 1997), *E. coli* O157:H7 has had a significant economic impact on the beef industry (Kay 2009). *E. coli* O157 colonizes in the distal colon and is shed in the feces of cattle (Griffin et al 1991), which leads to contamination of the environment, including the pens, feedbunks, and water. The bacterium is transmitted by the fecal-oral route. Fecal contamination of the environment leads to infection of other cattle. *E. coli* O157 is a source of carcass contamination at harvest and leads to foodborne illnesses in humans that consume contaminated beef (Mead et al 1998). The most common manifestation of an *E. coli* O157:H7 infection in humans is hemorrhagic colitis. Clinical signs include fever, dehydration, and bloody diarrhea. In some cases, a life threatening complication known as Hemolytic Uremic Syndrome (HUS) can occur (Griffin 1991). If severe, HUS can lead to kidney failure and death, with greatest risk in immunocompromised individuals, young children or the elderly. Both disease states are due primarily to shiga toxin produced by the O157 strain of *E. coli* (WHO 2005).

Most previous research efforts have focused on controlling *E. coli* within the abattoir (Loneragan 2005). Over the last 10 years, the National Cattleman's Beef Association has estimated *E. coli* O157 to cost the industry 2.67 billion dollars (Kay 2009). Control of *E. coli* O157 in cattle prior to arrival has been identified as an essential hurdle in the process of

producing a safer beef product. Many pre-harvest interventions have been researched in commercial settings to control E. coli O157 in cattle: traditional antibiotics (Elder 2002), feed-grade antibiotics (Callaway 2003), pro-biotics (Brashears 2003), sodium chlorate (Anderson 2000), phages (Sheng 2006).

One technology of particular interest is the utilization of vaccines to control this foodborne pathogen. Recently a novel vaccine technology became the only product licensed in the United States to control *E. coli* O157 in cattle. This vaccine targets the siderophore receptor and porin proteins (SRP) of *E. coli* O157, which are essential for iron uptake by the bacteria. Upon attachment of SRP antibodies to *E. coli* O157, the cell is essentially starved for iron and unable to thrive (Emery 2000). The *E. coli* O157 SRP vaccine has been shown to reduce fecal shedding of *E. coli* in cattle in laboratory conditions (Emery et al 2000; Thornton et al 2009) as well as field conditions (Fox et al. 2009; Thomson et al., 2009). However, no studies have compared at the efficacy of the SRP *E. coli* O157 technology when all cattle in the facility are either vaccinated or not with the product to control environmental exposure of cattle throughout the feedyard.

Therefore, the objectives of this study were to: 1) Examine the ability of the SRP *E. coli* vaccine to control *E. coli* O157 in feedlot cattle where all cattle within a feedlot are vaccinated or not vaccinated. 2) Determine the ability to measure vaccine administration compliance through observing serological responses to *E. coli* SRP vaccine.

Methods and Materials

Cattle

Cattle housed in 10 commercial feedlots (284,300 head capacity) located in Nebraska and Colorado were used in the field trial. All feedlots utilized in the study procured similar types of cattle during similar times of the year. These feedlots were recommended for use in this study because they were all members of a cattle marketing group that sold most cattle to a common commercial packing plant in Colorado. Feedlots were randomly assigned to 1 of 2 treatments: 1) all incoming cattle within a feedlot injected subcutaneously with 2 ml of SRP *E. coli* O157:H7 vaccine at arrival and again approximately 100 days prior to harvest (VAC) or 2) all incoming cattle within a feedlot did not receive the vaccine (CON). Feedlots were assigned letters (A-J), and samples were labeled with the appropriate feedyard letter code, sample number, and sampling date to blind laboratory personnel to treatment assignment.

Vaccination Protocol

Upon arrival, cattle were unloaded into an arrival pen and allowed to rest for 24 hours prior to processing. The next morning, cattle were processed. Cattle in VAC feedyards received a 2 ml dose of SRP *E. coli* O157 vaccine, in addition to normal processing procedures (vaccination, deworming, and administration of a steroid implant) while cattle in CON feedyards received the same processing protocol except for the SRP *E. coli* O157 vaccine. As part of the 2 dose SRP vaccine regimen, cattle in VAC feedyards received a second, 2 ml dose of the SRP vaccine at time of re-implant, which varied between 1 and 3 months after initial vaccination (approximately 80-100 days before harvest). Cattle in CON feedyards were reimplanted at similar intervals but did not receive an SRP vaccination.

Fecal Sampling Procedure

Freshly voided pen floor fecal samples were collected from cattle at feedyards at 4 sampling intervals over the course of the summer (May, June, July, and August) of 2010. Within each feedyard, 5 pens of market-ready cattle were selected for sampling at the time in which fecal samples were collected. To be eligible, the cattle within a pen had to be scheduled for shipment to the abattoir the week following sampling or sooner. Twenty freshly voided fecal pats were sampled in each pen using a clean spoon, cup and lid. A 10 g fecal sample was collected, and the sample cup was labeled and sealed in a plastic bag. The sealed bags containing the sample cups were stored on ice in coolers. Separate coolers were designated for use in each feedyard for the entire study, marked with the letter corresponding to the feedyard code. After sampling, coolers were shipped overnight to the Epitopix, LLC laboratory in Willmar, MN for microbiological analysis.

Fecal Microbiology

Laboratory personal began the process of *E. coli* O157:H7 recovery the morning upon which coolers were received at the Epitopix, LLC laboratory. Two grams of fecal material were placed in a filter bag (Nasco; Ft. Atkinson, WI.), along with 20 mL of GNccv liquid media. One mL of mixture from each bag was placed in a well of a 96 well plate and the location of the sample within the plate recorded. Plates were incubated overnight at 37ºC. Next, 20 µL of anti-*E. coli* O157 paramagnetic beads (Invitrogen, Carlsbad, CA) were added to each well of the 96 well plate. These beads were coated with anti-*E. coli* O157 antibody. Plates were then sealed and placed on a shaker tray (300 rpm) for 15 min at room temperature. During sample/bead incubation, 4 wash plates were set up for each sample plate, each containing1 mL of buffer per well. Also, a plate containing 100µL of buffer was set up for placing the beads after the washing

process. After shaking, magnetic beads were retrieved from *E. coli* enrichment using a Pick-Pen device (PickPen; Bio-Nobile, Turku, Finland). Beads were collected by placing the device in wells for 30 sec, using a swirling motion to ensure all beads were utilized. Beads were then washed by release and recapture within each of the 4 wash plates. Finally, beads were dropped in the plates containing 100 µL of buffer. Next, 50 µL of each sample was plated onto CT-CHROMagar (Chromagar, Paris, France). All plates were then incubated overnight at 37ºC. The next morning, *E. coli* O157:H7 positive plates were identified by the characteristic magenta colored colony. Suspect colonies were confirmed using a Remel latex agglutination test. Positive colonies were re-cultured on CT-CHROMagar and again incubated overnight. If the result was a pure culture and O157 was confirmed, colonies were inoculated in seed-stock vials and frozen at -80 ºC.

Blood Sampling Procedure

Blood samples were obtained for serological examination of SRP antibody response in vaccinated and non-vaccinated cattle. The blood samples in this study were taken from a subset of cattle at the time of exsanguination during the slaughter procedure. Determination of cattle to be sampled corresponded with cattle eligible for fecal sampling at the feedyard prior to shipment to the abattoir. Blood samples were collected in serum tubes on 3 occasions throughout the summer (June, July, and August). For each sampling month, 5 animals representing each feedyard were sampled (25 blood samples per treatment within each month; 150 blood samples total). Blood samples were centrifuged, serum was collected and frozen in uniquely identified tubes. Serum samples were shipped frozen on dry ice to Epitopix, LLC in Willmar, MN for anti-SRP antibody determination (ELISA).

Serological Analysis

Serum processing and anti-*E. coli* O157:H7 antibody titer determination was completed at Epitopix, LLC in Willmar, MN using a capture ELISA system. Two hundred fifty ng of *E. coli* O157 antigen with carbonate buffer (pH 9.6) was added to each of the 96 wells on the Nunc Maxisorp plate (Nalge Nunc International; Rochester, NY). Plates were then covered and incubated for 24 hours at 4ºC. The next day, plates were dumped and blocked using 1% PVA/PBS, covered in plastic film and incubated for 1 hr at 37ºC. Paired dilutions of serum samples were prepared at 1:500 using 1% PVA/PBS, plates covered and incubated at 37ºC for 1 hr. Next, the plates underwent a series of 3 washes with 0.05% PBS-Tween 20. After washing, the conjugate was added in 1:1600 dilution; using HRP sheep anti-bovine IgG (The Binding Site San Diego, CA). Plates were again covered and incubated for 1 hr at 37ºC to allow for conjugation to occur. Before reading the plates, there was another series of 3 wash steps, using 2,2' azino-di-3-ethyl-benzthiazoline-6-sulfonate (ABTS; KPL, Inc. Gaithersburg, MD). A 405-490nm ELISA reader was used to measure absorbance of the wells. For each sample, the average absorbance of the duplicates was calculated. Then, the average absorbance of the negative controls was subtracted from all reagent values, giving the relative absorbance of each sample. Finally, the sample absorbance was divided by the average positive control absorbance. This value was known as a sample to positive (S:P) titer, which was used as the dependent variable for immunological response in this study.

Statistical Analysis

Pen-level prevalence of *E. coli* O157:H7 was measured as a binomial outcome and included the number of positive and the number of samples taken per pen per sample collection. Data were entered into an electronic spreadsheet and analyzed using a commercial software

package (The SAS System 9.2, The SAS Institute, Cary, NC). The logit link function was utilized to develop generalized linear mixed models. The analysis was a repeated measure within feedyard to account for within level clustering. Various fixed and random effects were incorporated into each model.

For fecal samples, 2 statistical models were found to be relevant in this trial. The first model included fixed effects of vaccine status and sampling time, and their interaction. In the second model we assumed prevalence varied over time; therefore, we forced these terms into the model as random variables. Means were considered different using a protected F-test with an $\alpha = 0.10$.

Results

There was a significant vaccine status by sampling time interaction for prevalence of *E. coli* O157:H7 in the feces of cattle ($P = 0.0004$). Fecal samples from cattle vaccinated with SRP *E. coli* O157 vaccine had lower prevalence of *E. coli* O157:H7 in their feces compared to cattle from feedlots that were not vaccinated with the SRP vaccine technology during the months of May, June and August (Figure 2-3). However during July, there was no advantage in lower fecal *E. coli* O157:H7 prevalence in fecal samples from cattle vaccinated with SRP *E. coli* O157 vaccine compared to cattle not vaccinated with SRP technology. There was clearly a greater vaccine effect in May, June, and August when compared to July (Figure 2-3).

Overall, the prevalence of *E. coli* O157:H7 was lower in the feces of vaccinated cattle (12.83%) when compared to feces of control cattle (20.25%; $P = 0.07$) (Figure 2-1). The prevalence *E. coli* in feces of feedlot cattle was lower in May compared to June, July and August ($P<0.0001$) (Figure 2-2).

Anti-SRP antibody titer level was higher in vaccinates (0.622) vs. controls (0.075) ($P < 0.001$) (Figure 2-4). Titers were also compared within the vaccinate cohort by days on feed since last vaccination. Cattle were grouped into 1 of 3 time periods based on the length of time (days on feed) since receiving the last vaccination prior to slaughter. The number of days prior to slaughter had no effect on the titer responses to SRP *E. coli* O157 vaccine in feedlot cattle ($P = 0.31$) (Figure 2-5).

Figure 2-1

Effect of SRP technology on *E. coli* O157:H7 prevalence in feeder cattle

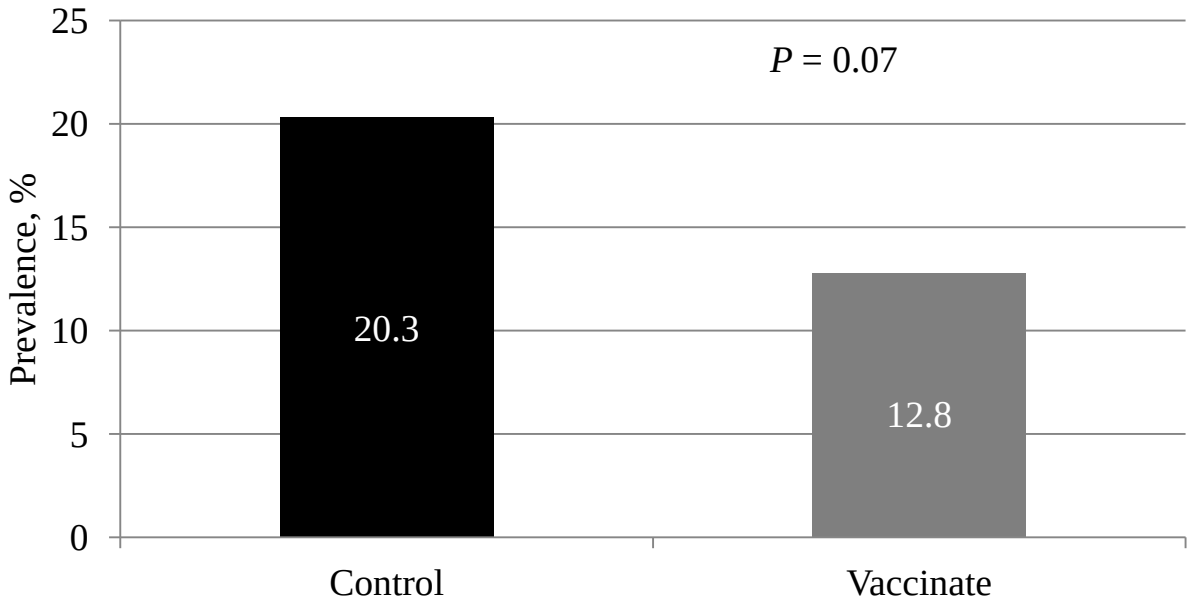


Figure 2-2

Prevalence of E. coli O157:H7 in feeder cattle during summer months

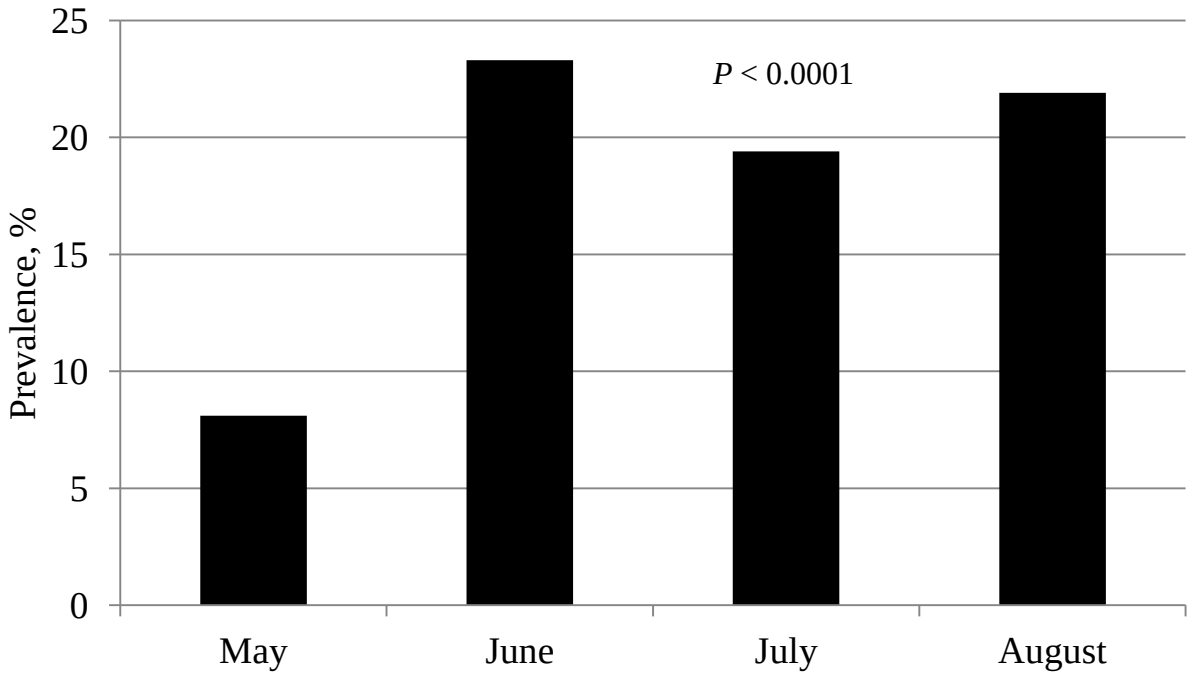


Figure 2-3

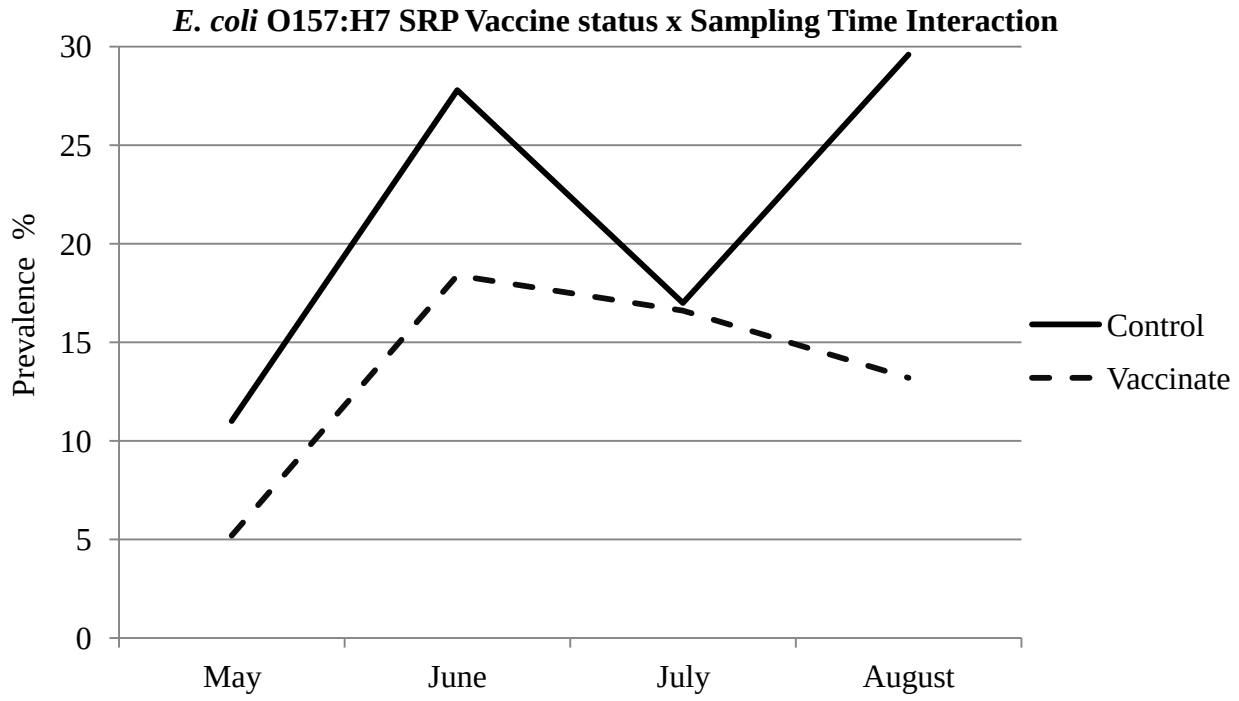

E. coli O157:H7 SRP Vaccine status x Sampling Time Interaction
Prevalence %
30
25
20
15
10
5
0
May
June
July
August
Control
Vaccinate

Figure 2-4

Effect of SRP technology on serum anti- *E. coli* O157:H7 S:P ratio in feeder cattle

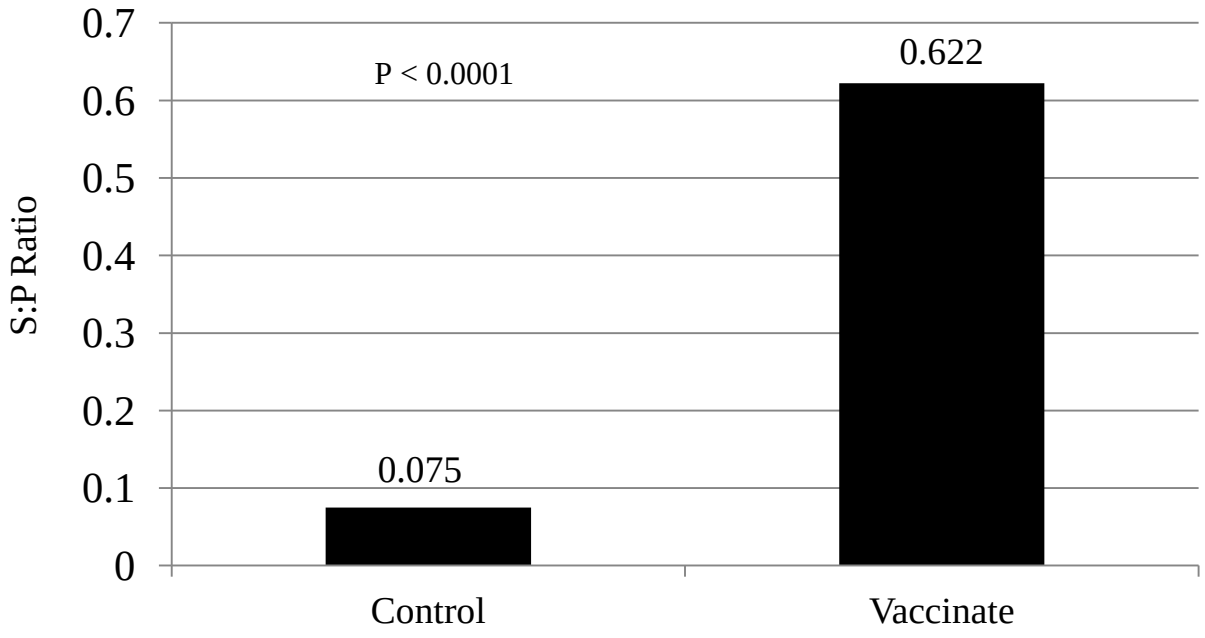
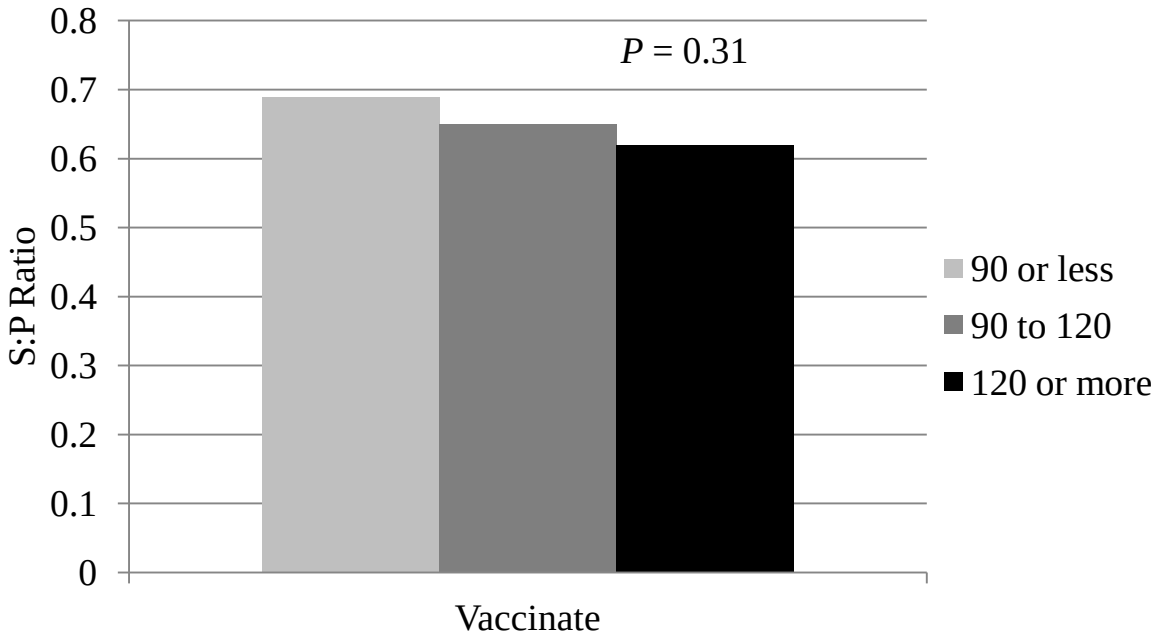


Figure 2-5

S:P ratio of vaccinates relative to days on feed since last vaccination



Discussion

E. coli O157:H7 has consistently presented the greatest economic issue for meat packers and retailers compared to all other foodborne pathogens associated with beef production (Kay, 2009). Cattle naturally shed *E. coli* O157:H7 in their feces and it is a source of carcass contamination at harvest. If the contaminated trim enters the food supply and is subsequently prepared incorrectly, it can lead to the human condition known as hemorrhagic colitis (Ransom et al., 2003). In children or elderly people, an *E. coli* O157:H7 infection may present as the more serious form known as hemolytic uremic syndrome, which is potentially lethal (Taylor et al 1986). Although the majority of previous research has been dedicated to reduction in contamination post-harvest, recent focus has shifted to pre-harvest mitigation of *E. coli* O157:H7 (Fox et al., 2008).

The results of this study showed an interaction between treatment effect and sampling time. There was a greater effect of SRP vaccination in May, June, and August, when compared to July. This interaction was included as a random variable in the second model. There are several possibilities as to why the interaction took place. The two major factors that come to mind are pen conditions and stress of the animals.

It is possible that a lack of treatment response in July can be accounted for by a peak in temperatures, leading to heat stress. The effect of stress on an animal's ability to fight disease is due to changes in immune function. This relationship has been researched thoroughly, and it is proposed that stress alters the hypothalamic-pituitary-adrenal axis, which in turn hinders the immune response to antigenic stimuli (Kelley 1980). Several studies in dairy cattle have found decreased levels of circulating IgG in vaccinated cattle exposed to elevated environmental temperatures, when compared to antibody titers of the non heat stressed group. (Kelley et al. 1982, Do Amaral et al. 2011). However, a stress-induced reduction in efficacy may be due to

factors other than antibody production. A study in chickens measured the effect of heat stress on *Salmonella* shedding in hens vaccinated with a *Salmonella enteritidis* bacterin. Post-heat stress, vaccinated chickens maintained levels of circulating antibody, but shed significantly more *S. enteritidis* (Nakamura et al. 1994). While there is documentation of the impact heat stress has on vaccine efficacy, further investigation is warranted to uncover why this occurs. For example, studies looking at the effect of stress on antibodies reaching the target tissues.

Another possibility for a lack of treatment response in July is the effect of pen conditions on *E. coli* ecology. Peak prevalence of *E. coli* O157:H7 of occurs in the summer months (Hancock et al. 1997). Muddy pen conditions contribute to increased growth *E.* coli in the environment. Two studies have described a positive correlation between fecal *E. coli* prevalence and pens with muddy conditions (Sargeant et al. 2004; Smith et al., 2001). It is possible that pen conditions varied between treatment groups in this study, due to variation in rainfall across the 10 feedyards. This could be a potential contributor to the lack of a treatment effect seen in July. Clinical research aimed to uncover the direct relationship between SRP vaccine efficacy and pen conditions may be indicated.

Across the 4 month sampling period, *E. coli* O157:H7 prevalence was reduced from 20.3% in the control group to 12.8% (P =0.07) in the vaccinate group (Figure 2-1). This finding is in line with the field trial completed in 2006, in which *E. coli* prevalence was reduced by a similar magnitude (Thomson et al., 2009). The combination of these 2 trials suggest pre-harvest mitigation of *E. coli* O157:H7 may be possible using the 2 dose regimen of the SRP vaccine.

Another important finding from this research is a strong seasonal effect on *E. coli* O157:H7 prevalence in feeder cattle. First recognized by Hancock et al. (1997), the association was further investigated by Edrington et al., (2006), who found a correlation of *E. coli*

prevalence with both day length and mean ambient temperature. Although not one of the primary objectives, this study found similar results. Across both treatment groups, prevalence of *E. coli* O157:H7 in the feces of cattle was higher in June, July, and August compared to May (Figure 2-2). This coincides with a peak in foodborne disease outbreaks due to *E. coli* O157 during the summer (Siegler et al., 2004).

There was a strong treatment effect on anti-SRP antibody titer (Figure 2-4). Fox et al., (2009) reported that cattle naturally shedding *E. coli* O157:H7 demonstrated increased antibody titers following vaccination with SRP. Wileman (2010) demonstrated passive transfer of SRP antibody in calves born to cows vaccinated with the SRP; calves from vaccinated dams had higher circulating anti-*E. coli* O157:H7 antibody, confirming colostral absorption.

Because vaccinated cattle demonstrated elevated titers when sampled at the packing plant during the slaughter procedure, the vaccine is effective at inducing a prolonged immune response throughout the feeding period. There was no difference in titer response with increasing days on feed following the final vaccination (Figure 2-5). Regardless of how many days prior to harvest cattle were vaccinated, there was a measurable anti-SRP antibody response at the time of slaughter. Results of this study suggest that a simple ELISA blood test at harvest could be used as a tool for vaccination compliance.

In conclusion, the results of this research suggest using a siderophore receptor and porin-based vaccine can be an effective pre-harvest tool for controlling *E. coli* O157:H7 in feedlot cattle prior to slaughter. The reduction of *E. coli* O157:H7 contamination of beef products starts with interventions that prevent carriage of the pathogen into the abattoir. The economic viability of the beef industry through consumer confidence in food safety is important for all beef producers. Further investigation into the carryover effects of pre-harvest interventions, such as

cattle vaccination with SRP *E. coli* O157 technology, on post-harvest beef contamination needs to be completed in the future.

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