PREVALENCE, CHARACTERIZATION AND INTERVENTION OF *ESCHERICHIA COLI* O157 IN FINISHING CATTLE

by

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B.S., Kansas State University, 2002
M.S., Texas A&M University, 2004

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
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Abstract

Escherichia coli O157 is a major foodborne pathogen. The bovine gut is the primary reservoir and the organism is shed in the feces, which serves as the major source of contamination. The objectives of our research on *E. coli* O157 were to 1) determine prevalence and characterize presence in the rectoanal mucosa of cattle, 2) rationalize preferential prevalence in the hindgut, 3) evaluate fecal prevalence and concentration in relation to carcass contamination 4) determine the efficacy of preharvest intervention strategies to reduce fecal prevalence and/or concentration in cattle. We determined that *E. coli* O157 isolated from the rectoanal mucosa were similar to fecal isolates. We evaluated methods of enumeration in cattle feces to identify fecal samples with > 10^3 and 10^4 CFU of *E. coli* O157/g. We observed that prevalence of *E. coli* O157 on carcasses was correlated with high-shedders. We identified gluconic acid as a substrate which stimulates growth of *E. coli* O157 in fermentations with ruminal microbial or fecal microbial inocula from cattle. This may explain the preference to persist or colonize the hindgut because gluconic acid is a component of colonic mucin. Additionally, we evaluated the effects of cattle diets (two grain types and two grain processing methods), which present different amounts of fermentable starch to the hindgut, on prevalence of *E. coli* O157. Dry-rolled grain-based diets reduced prevalence of *E. coli* O157, possibly because of increased flow of starch to the hindgut. Competitive exclusion cultures of *E. coli* in *in vitro* fermentations reduced *E. coli* O157 in ruminal microbial inoculum, but in fecal microbial inoculum cultures were only efficacious when gluconic acid also was added. Lastly, we evaluated a vaccine which targeted the siderophore receptor/porin proteins of *E. coli* O157. The vaccine reduced prevalence, the total number of days cattle tested positive, and the total number of days cattle were identified as high-shedders of *E. coli* O157. In summary, our research adds further knowledge to the literature about *E. coli* O157 in the hindgut, provides methods to identify high-shedding animals, demonstrates the importance of high-shedding animals, and offers information about potential preharvest interventions.

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Dedication

I dedicate this dissertation to my wife and family for their love, patience and support throughout my education regardless of the duration.

Preface

The purpose of this dissertation is to look at multiple aspects of *E. coli* O157 in finishing beef cattle. Chapter One focuses specifically on the presence of the organism in different locations of the hindgut at slaughter. This chapter has been submitted and accepted in "Foodborne Pathogens and Disease." The focus of Chapter Two is how to quantify the amount of *E. coli* O157 in bovine feces. This study enabled us to pursue further studies which evaluate factors that may increase or decrease the concentration of the pathogen. Chapter Two is published in Applied and Environmental Microbiology Vol. 73:5253-5260. The third chapter of this dissertation utilizes techniques from Chapter Two and identifies factors that impact the risk of carcass contamination at slaughter. Chapter Three is not yet submitted for publication. Chapter Four is an attempt to explain why *E. coli* O157 persists in the hindgut of cattle given various aspects of this region of the gastrointestinal tract. This chapter is not yet submitted for publication. Results from Chapter Four suggest that perhaps increasing fermentative activity in the hindgut may reduce survivability of *E. coli* O157, so Chapter Five evaluates the use of different grain-processing methods which alter the concentration of fermentable substrates delivered to the hindgut. This could be classified as a method of preharvest intervention of *E. coli* O157. Chapter Five is published in the Journal of Animal Science Vol. 85:1207-1212. In Chapters Six and Seven, which are not yet submitted for publication, two additional preharvest interventions were evaluated, competitive exclusion cultures of bacteria and an anti-*E. coli* O157 siderophore receptor/porin protein vaccine.

CHAPTER 1 - *Escherichia coli* O157 in the Rectoanal Mucosal Region of Cattle

The rectoanal junction (RAJ) mucosal region is the site of colonization of *E. coli* O157 in cattle. Our objective was to determine the relatedness of *E. coli* O157 in the mucosa of RAJ to isolates from colon contents and feces. Colon contents and rectums were collected from cattle at harvest. Rectums were opened and feces were sampled with a cotton swab. The mucosa of the rectum was cleansed free of visible feces with water and saline. The region, 2 to 5 cm proximal to the RAJ, was swabbed with a foam-tipped applicator and then incisions were made in this region and the submucosa was swabbed with an applicator. Isolation and identification of *E. coli* O157 was performed in accordance with well-documented methods. Prevalence of *E. coli* O157 in the colon contents, feces, rectal mucosa, and rectal submucosa was 21, 29, 54, and 34%, respectively. Pulsed-field gel electrophoresis was used to compare clonal similarity among isolates from different sampling regions. Sixty-seven cattle had *E. coli* O157 isolated from the rectal mucosa swab and feces of which 82% were clonally similar (dice similarity > 95%) within animal. *Escherichia coli* O157 isolates from feces and colon contents were similar in 76% of cattle, but *E. coli* O157 isolates from the rectoanal mucosal swab and colon contents were only similar in 61.4% of cattle. Our results suggest that *E. coli* O157 in the feces may be from two sources, colonized in the rectoanal mucosa or transient in the gastrointestinal tract.

Key Words: *Escherichia coli* O157, feces, rectoanal mucosa, cattle

INTRODUCTION

Escherichia coli O157:H7 is a food-borne pathogen that causes hemorrhagic colitis, hemolytic uremic syndrome and thrombocytopenic purpura in humans (Armstrong et al., 1996; Karmali, 2005; Park et al., 1999). The bovine gastrointestinal tract is the primary reservoir of *E. coli* O157:H7 (Bach et al., 2002; Rasmussen and Casey, 2001). The organism colonizes in the gastrointestinal tract, particularly in the hindgut, and is then shed in the feces. Prevalence of *E. coli* O157 in cattle is variable among herds and individual animals, and feedlot cattle prevalence ranges from 10 to 28% and may be as high as 80% in the summer months (Callaway et al., 2003). Though preventative measures such as HACCP plans are employed in feedlots and abattoirs, some contamination of beef carcasses does occur (Koohmaraie et al., 2005; Loneragan and Brashears, 2005).

The primary site of *E. coli* O157 colonization in cattle has been shown to be the mucosal epithelium at the terminal rectum (Low et al., 2005; Naylor et al., 2003). This region, 1 to 5 cm proximal to the rectoanal junction, is rich in lymphoid follicles and swabbing of this region using a foam-tipped applicator (Rectoanal Mucosal Swab or RAMS) has been shown to be a more sensitive sampling method for detecting *E. coli* O157 in cattle (Davis et al., 2006; Greenquist et al., 2005; Rice et al., 2003). The mucosal carriage of *E. coli* O157 at the rectoanal junction is associated with long-duration fecal shedding and high level of fecal excretion (Cobbold et al., 2007; Davis et al., 2006; Lim et al., 2007; Low et al., 2005). In persistently shedding animals, bacterial numbers in the feces were significantly higher than those samples taken from other sites of the gut (Naylor et al., 2003). Also, the mean surface counts of *E. coli* O157 were 1,000 fold higher than the core count of the freshly voided fecal pats suggesting that the feces gets coated as it passes through the rectoanal mucosal region (Naylor et al., 2003). Greenquist et al. (2005)

compared isolates from fecal culture and isolates from RAMS culture from the same animal for clonal similarity by pulsed-field gel electrophoresis (PFGE) and found that in 24 animals, 20 exhibited 100% clonal similarity and the remaining four animals exhibited > 95% clonal similarity. Because it is almost impossible to obtain RAMS without fecal contamination, it is difficult to conclude that the strains from each source are, in fact, the same. Further research is needed to determine if *E. coli* O157 isolates present in the contents of the gastrointestinal tract prior to reaching the rectal mucosa are similar to those in the feces and those colonized in the rectal mucosa. Therefore, the objective of this study was to compare the clonal similarity of *E. coli* O157 isolates from the colon contents, feces and rectoanal mucosa (within an animal) using PFGE.

MATERIALS AND METHODS

Experimental animals

Cattle used in this study were from four feedlots in the Midwest US. Animals were fed to meet the standards of natural-branded beef and diets consisted primarily of corn and silage. Samples were collected at slaughter in a commercial abattoir on five different dates during the summer months. A total of 374 slaughtered cattle were sampled in the study.

Sample collection

Rectums (n = 374) were cut at approximately 8 cm from the anus and the intact rectums with the anus were placed in large freezer bags. Colon contents were sampled (n = 289) by lancing the spiral colon and squeezing the intestine approximately 10 cm on both sides of the

incision and collecting the contents into Whirl-Pak bags (Nasco, Ft. Atkinson, WI.). After collection, samples were immediately placed on ice and transported to the laboratory. The dorsal aspect of each rectum was cut longitudinally to lay open the entire rectum. Rectal contents (n = 281) were sampled by swabbing with sterile cotton swabs. This method was used to standardize sampling among animals with varying amounts of feces present in the rectum. Following the rectal content sampling, rectums were cleansed free of visible feces with water and then rinsed with saline solution. The rectoanal mucosal area, proximal to the rectoanal junction up to approximately 3 or 4 cm, was then swabbed with a foam-tipped applicator (VWR International, Buffalo Grove, IL, catalog #10812-022, RAMS). After swabbing the rectoanal mucosa, three or four vertical incisions were made in the mucosa of the same region with a sterile scalpel to expose the submucosal region, and the submucosal tissue was swabbed with another foam-tipped applicator (VWR International).

Isolation of E. coli O157

Approximately 1 g of colonic contents or fecal swabs was placed into test tubes containing 9 ml of gram-negative (GN) broth (BD, Franklin Lakes, N. J.) containing cefixime, cefsulodin, and vancomycin (GNccv; Greenquist et al., 2005), vortexed for 1 min and incubated at 37°C for 6 h. Rectoanal mucosal swabs and submucosal swabs were placed into test tubes containing 3 ml GNccv (Greenquist et al., 2005), vortexed for 1 min and incubated at 37°C for 6 h. Following the 6 h enrichment, tubes were vortexed for 1 min and 1 ml from each tube was subjected to immunomagnetic separation (Dynal, Inc. New Hyde Park, NY), and plated onto sorbitol MacConkey agar (BD) containing cefixime (50 ng/ml) and tellurite (2.5 µg/ml). Plates were incubated overnight (16 to 18 h) and up to six sorbitol-negative colonies were transferred on to blood agar (Remel, Lenexa, KS) for 12 to 18 h at 37°C. Blood agar colonies were tested

for indole production, latex agglutination for the O157 antigen (Oxoid Limited, Basingstoke, Hampshire, England), and species were confirmed by API (Rapid 20E; Biomerieux, Inc., Hazelwood, Mo). *Escherichia coli* O157 positive isolates were preserved in Protect beads (Key Scientific Products, Round Rock, TX) and stored at -80°C for PFGE typing.

Pulsed-field gel electrophoresis

The pulsed-field gel electrophoresis (PFGE) procedure followed that described by PulseNet (CDC, http://www.cdc.gov/pulsenet/; Greenquist et al. 2005; Sargeant et al., 2006). Briefly, isolates from Protect beads were streaked onto blood agar plates and grown overnight at 37°C. Tris-EDTA buffer (100 mM Tris:100 mM EDTA, pH 8.0) was used to prepare the bacterial suspension to equal an absorbance of 1.3 to 1.4 at 610 nm. Two-hundred micro-liters of each suspension were mixed with 10 µl of Proteinase K (20 mg/ml; Fisher, Houston, TX) and 200 µl of 60° C 1% SeaKem Gold Agarose (BioWhittaker Molecular Applications, Rockland, MN) in TE buffer (10 mM Tris: 1 mM EDTA, pH 8.0) containing 1% sodium dodecyl sulfate. The mixture was then pipetted into disposable plug molds (Bio-Rad, Hercules, CA.). Following solidification, plugs were placed into 1.5 ml of lysis buffer (50 mM Tris: 50 mM EDTA, pH 8.0 + 1% Sarcosine) containing 40 µl of proteinase K and the lysing procedure (2 to 4 h) was performed at 54°C in a water bath shaking at 75 rpm. The lysis buffer was then removed and plugs were washed six times (two washes in 10 ml of distilled water and four washes in 10 ml TE buffer. Washes were performed in a shaking water bath at 50°C for 15 min per wash.. A 2-mm slice of each plug was digested (37°C, 2 to 5 h) using the restriction endonuclease, *Xba*I (Promega Corporation, Madison, WI). Plugs were loaded onto one percent SeaKem Gold Agarose gel and run with *E. coli* O157 strain G5244 (CDC) as the standard. The CHEF II system (Bio-Rad) was used for PFGE with the following run parameters: switch time of 2.2 and

54.2 s; angle of 120°; voltage 200 v; temperature of 14°C; and a run time of 21 h. Gels were stained in 400 ml of distilled water containing 40 µl of 1% ethidium bromide solution for 30 min and then destained by washing three times in distilled water (20 min per wash). Images from each gel were captured with a Gel Doc 2000 system (Bio-Rad), and BioNumerics software (Applied Maths, Inc., Austin, Texas) were used to analyze and compare band patterns. Band-based Dice similarity coefficient and the unweighted pair group method for clustering were used with a position tolerance of 1.5% for optimization and position tolerance of 1.5% for band comparison. Isolates were grouped into subtypes and types based on banding pattern similarities. Pulsed field gel electrophoresis types are defined as isolates having fingerprint patterns of >95% Dice similarity.

Statistical analyses

Prevalence of *E. coli* O157 in each sampling region was calculated as the proportion of cattle testing positive in a given region divided by the total number of samples collected in that region. Prevalence of *E. coli* O157 among cattle was determined as the proportion of cattle culture positive for *E. coli* O157 in one or more regions divided by the total number of cattle sampled. Differences in prevalence of *E. coli* O157 among sampling locations were determined using logistic regression in PROC GENMOD of SAS (SAS Institute, Cary, NC) and accounted for the effect of multiple samples per animal. Two-way comparisons of prevalence in fecal swabs, colon contents, RAMS and submucosa swabs were assessed with LSMEANS of PROC GENMOD. The proportion of isolate pairs collected within animal but in different regions that shared a common PFGE type was calculated. Differences in these proportions between two-location combinations were assessed using LSMEANS of PROC GENMOD and the model accounted for multiple samples per animal.

RESULTS

Prevalence of E. coli O157

A total of 374 cattle were sampled from five separate visits to the commercial abattoir. The numbers of cattle harboring *E. coli* O157 in at least one of the regions of the hindgut sampled (colon, rectum, and rectoanal mucosal and submucosal regions) at each visit are shown in Table 1. *Escherichia coli* O157 isolates were obtained from 61 of 289 (21.1%) colon content samples, 81 of 281 (28.8%) fecal swab samples, 201 of 374 (53.7%) RAMS samples, and 127 of 374 (34.0%) submucosal swab samples. Prevalence was different ($P < 0.05$) among all sampling sites with the exception of fecal and submucosal swab samples (Fig. 1). *Escherichia coli* O157 was isolated from at least one region in 231 of 374 (62%), which was greater ($P < 0.05$) than prevalence in any single sampling region. In cattle harboring *E. coli* O157, isolates were detected in the RAMS sample (87.0%) more frequently ($P < 0.05$) than in any other sampling region (feces, 39.9%; colon, 30.0%; submucosa, 55.0%).

In cattle that had all four regions of the hindgut (colon, rectum, and rectoanal mucosal and submucosal areas) sampled ($n = 196$), 8.7% of cattle were positive in all four regions (Fig. 2). Sixty percent of cattle tested negative in colonic contents when *E. coli* O157 was present in feces and/or in the rectoanal mucosal region. In most cattle (89%), if *E. coli* O157 was detected in feces, colon, submucosa or any combination of the three, it was also detected by RAMS. *Escherichia coli* O157 was isolated from 200 cattle when feces, RAMS and submucosal swabs were collected and only eight (3.4%) had *E. coli* O157 isolated from the feces, but not from RAMS and submucosal swabs. Only a small percentage of cattle (1.5%) were *E. coli* O157 positive in colonic contents and negative in the rectum (feces, RAMS or submucosal area of the rectoanal junction; Fig. 2). A similar percentage of *E. coli* O157 positive cattle were positive in

the submucosal area and negative in all other locations tested in this study (Fig. 2). Interestingly, only a small percentage of cattle (1.5%) were only positive in feces and negative in all other sampling sites.

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis was performed on *E. coli* O157 isolates to compare clonal similarity of isolates obtained from different regions of the hindgut within cattle. Only isolates from cattle having two or more *E. coli* O157 positive locations were used (n = 145 animals, 377 isolates). Seventy-four, 55 and 16 cattle had O157 isolates in two, three or four locations of the gut, respectively. The proportion of isolate pairs within animals having clonal similarity (> 95% dice similarity) are shown in Table 2. The majority (59 to 82%) of *E. coli* O157 isolates obtained within the same animal shared a common PFGE type.

Escherichia coli O157 isolates were obtained from three regions of the hindgut in 55 cattle (Table 2). Fecal, RAMS and submucosal swab isolates were detected in 30 cattle. In 18 of these cattle all three isolates were the same PFGE type. Of the animals that had different PFGE types, four cattle had a fecal isolate of a different PFGE type, four had a submucosal swab isolate with a different type, two had a different RAMS isolate and two animals had three isolates all with different PFGE types. Fecal, colon and RAMS isolates were detected in seven animals. Four animals had isolates with a common PFGE type, two had colon isolates that were a different PFGE type and one had all three isolates with different PFGE types. Colon, RAMS and submucosal swab isolates were detected in 17 animals. Eight of these animals had isolates of a common PFGE type, six had a colon isolate with a different PFGE type and three had a RAMS isolate with a different PFGE type. One animal had *E. coli* O157 isolated from the colon, feces and submucosal swab and these isolates shared a common PFGE type.

Sixteen animals had *E. coli* O157 isolates in all four sampling regions. Examples of PFGE banding patterns from cattle with *E. coli* O157 isolated from all four regions sampled are shown in Fig. 3. In nine of these animals (56%) all four isolates had clonal similarity (Fig.3A). Three of the animals had clonal similarity in fecal, RAMS and submucosal swab sample isolates, but the colon content isolate was a different PFGE type (Fig. 3C). Two of the animals had isolates with clonal similarity in the colon content, fecal swab, and RAMS samples, but the submucosal swab isolate was different (Fig. 3D), and the remaining two animals the colon content and fecal swab isolates shared the PFGE type (Fig. 3B), which was different than the PFGE type shared by the RAMS and submucosal swab.

DISCUSSION

In this study, *E. coli* O157 was isolated from slaughtered cattle in different regions of the hindgut on different sampling dates. The overall prevalence in cattle (62%) was considerably higher than prevalence reported by several studies (Bach et al., 2002; Elder et al., 2000; Gansheroff and O'Brein, 2000; Sargeant et al., 2003). Cattle were sampled during June and July of 2004 and it appeared that prevalence was greater in July than in June, but because source of cattle was different for each sampling day, this was not compared. In a study evaluating *E. coli* O157 in feedlot pens during summer months, a 1°C increase in a 7-day mean air temperature increased the likelihood of a pen testing positive for *E. coli* O157 (odds ratio = 1.04; $P < 0.01$; Smith et al., 2005).

The bovine terminal rectum is the primary site for colonization of *E. coli* O157 (Low et al., 2005; Naylor et al., 2003). Localization at this site is in contrast to other *E. coli* serotypes, which are present throughout the hindgut (Cobbold et al., 2007). The mechanism for this tissue

tropism is not known. Apparently, the presence of lymphoid tissue in this region was not the reason for this tropism (Lim et al., 2007). The frequency of isolation and the numbers of *E. coli* O157 are higher at the site (1 to 5 cm) proximal to the rectoanal junction than the other areas of the rectum (Low et al., 2005; Naylor et al., 2003). The finding is also supported by necropsy analysis of the gastrointestinal tract of sheep and cattle experimentally inoculated with *E. coli* O157 (Alali et al., 2004; Grauke et al., 2002; Van Baale et al., 2004). Rectal swab administration of *E. coli* O157 has been shown to result in consistent, long-term colonization in cattle (Sheng et al., 2004). The evidence is mounting that mucosal carriage at the terminal rectum is also associated with high concentration of fecal shedding (Low et al., 2005). The RAMS technique is designed to sample the mucosal surface of the rectoanal region (Rice et al., 2003). Studies comparing RAMS to traditional fecal culture for detection of *E. coli* O157 in live cattle, found that in nearly all cases, the RAMS was as sensitive, or more sensitive than fecal culture of either 1 or 10 g (Davis et al., 2006; Greenquist et al., 2005; Rice et al., 2003). The increased sensitivity of detection of *E. coli* O157 is generally attributed to direct sampling of the colonized site and fewer competing organisms in the sample (Greenquist et al., 2005; Rice at al., 2003). In our study, we isolated *E. coli* O157 more often in RAMS samples than in the feces, colon contents or rectal submucosal area. Differences in the number of competing organisms between feces and RAMS samples were maximized because of rinsing of the rectums free of visible feces prior to swabbing. Because we used cotton swabs to sample contents of all rectums collected at slaughter, the amounts of fecal sample used for isolation was obviously different between cattle and were less than 1 g that we routinely have used (Alali et al., 2004, Greenquist et al., 2005; Van Baale et al., 2004), or the 10 g used by others (Grauke et al., 2002). The method of sampling may have contributed to lower prevalence in feces compared to RAMS.

However, it is interesting that the difference in prevalence between feces and RAMS observed in this study agrees with other studies that have used higher amounts of feces (Davis et al., 2006; Greenquist et al., 2005; Rice et al., 2003). The cotton swab method to sample rectal contents has been used previously (Sanderson et al., 1995); however, our primary intention to use the cotton swab was not to estimate prevalence of *E. coli* O157 in cattle, rather it was to assure sampling of all rectums regardless of the amount of contents so that isolates from multiple regions could be compared genetically.

Naylor et al. (2003) evaluated fecal samples from cattle that were experimentally inoculated with *E. coli* O157 and reported that the concentration of *E. coli* O157 was greater on fecal surface samples than in fecal core samples and suggested that the feces became coated with *E. coli* O157 colonized in the rectal mucosa as it passed through this region before excretion. In support of this theory, Greenquist et al. (2005) found that *E. coli* O157 isolates in the feces and RAMS from the same animal (n = 24) were of the same genetic type (> 95% clonal similarity determined by PFGE) in all cattle evaluated.

Pulsed-field gel electrophoresis has been used to compare genetic types of *E. coli* O157 in human infections (Rios et al., 1999; Swaminathan et al., 2001; Welinder-Olsson et al., 2002) as well as to compare genetic types among colonized cattle (Avery et al., 2004; Barkocy-Gallagher et al., 2001; Davis et at., 2003; Lahti et al., 2003; LeJeune et al., 2004; Rice et al, 1999; Sanderson et al., 2006; Sargeant et al., 2006). Of the 374 cattle sampled, 145 had *E. coli* O157 isolates in two or more gut locations. Pulsed-field gel electrophoresis of *Xba*1 digested DNA was used to compare these isolates within animals. Overall, fecal and RAMS isolates shared a similar PFGE type (> 95% of Dice similarity) in 82% of animals. For cattle in which *E. coli* O157 was not isolated from the colon, RAMS and fecal isolates shared a common PFGE

type in 81% (26 of 32) of animals. These percentages are less than those reported by Greenquist et al. (2005) who found fecal and RAMS isolates were genetically similar (> 95% of Dice similarity) in all (n = 24) live animals with *E. coli* O157 isolated from feces and RAMS. A possible explanation for this difference is that in the current study, the rectal mucosa was cleansed prior to swabbing to remove feces from this area. Regardless, the majority of the isolates were of the same genetic type, which supports the theory by Naylor et al. (2003) that *E. coli* O157 colonized in the rectal mucosa inoculate the feces during passage. Isolates from the RAMS and submucosal swabs were similar in 80% of the cases. The remaining 20% provides evidence that multiple genetic types of *E. coli* O157 co-exist in the rectal mucosa. Faith et al. (1996) obtained multiple *E. coli* O157 isolates from the feces of individual animals and PFGE analysis revealed that 24% of these animals had isolates with different restriction endonuclease digestion profiles.

Colon content isolates shared a common PFGE type with rectoanal mucosal isolates, mucosal surface or submucosa, in 59 and 61% of cattle, respectively. The fact that approximately 40% of cattle had fecal isolates of a different genetic type suggests that some animals may be colonized with a strain of *E. coli* O157 in the rectal mucosa, but a different strain is either colonized in a different gut location within the animal or the organism is transiently passing through the animal. In support of this, 7 out of 16 cattle that had *E. coli* O157 isolates from all four gut locations had a different genetic type of *E. coli* O157 in at least one sampling region.

Rectoanal mucosal swabs have been shown as a superior method for detection of *E. coli* O157 with the assumption that direct swabbing of the rectal mucosa will detect the organism that is colonized in this area. However, fecal contamination of the RAMS samples may complicate

the ability to evaluate genetic differences of these isolates. This study provides evidence that in majority of the cases, fecal and rectoanal mucosal isolates belong to the same PFGE type and variation in PFGE types within an animal exists and the variation is greater with increased distance of sampling regions (colon vs rectum) within the hindgut. We conclude that *E. coli* O157 in the feces may be from two sources in the gut. One source, probably more prevalent but not exclusive, is from colonization in the rectoanal mucosa and the other is a transient source in the gastrointestinal tract, possibly the proximal hindgut or rumen. Because RAMS appears to be a superior method of sampling compared to fecal sampling, RAJ may serve as a sensitive sentinel location to detect cattle that are culture positive for *E. coli* O157.

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Table 1.1. NUMBER OF CATTLE SAMPLED AND PREVALENCE OF *ESCHERICHIA COLI* O157 IN AT LEAST ONE REGION OF THE HINDGUT SAMPLED

Samples collected	No. of cattle sampled	No. of cattle harboring *E. coli* O157 (%)
Colon, Rectoanal mucosal surface and Submucosal areas	289	200 (69.2)
June 8	76	26 (34.2)
June 14	17	2 (11.8)
July 13[1]	96	80 (83.3)
July 20[1]	100	92 (92.0)
Feces, Rectoanal mucosal surface and Submucosal areas	281	200 (71.2)
June 30	85	28 (32.9)
July 13[1]	96	82 (85.4)
July 20[1]	100	90 (90.0)
Colon, feces, Rectoanal mucosal surface and Submucosal areas	196	175 (89.3)
July 13[1]	96	82 (85.4)
July 20[1]	100	93 (93.0)
Total	374	231 (61.8)

[1]All four regions were sampled in cattle on the indicated dates, the number of animals found positive differ depending on the areas evaluated in that portion of the table.

Table 1.2 PROPORTIONS OF PAIRS OF ISOLATES OF *ESCHERICHIA COLI* O157 WITH CLONAL SIMILARITY, ISOLATED IN TWO OR MORE LOCATIONS OF HINDGUT FROM CATTLE

Region sampled	Proportion[1] with > 95% clonal similarity (%)	Number of cattle
Rectoanal mucosal and submucosal areas	80.2[bc]	111
Feces and rectoanal mucosal area	82.1[c]	67
Feces and rectoanal submucosal area	70.0[ab]	50
Colon and rectoanal mucosal area	61.4[a]	44
Colon and rectoanal submucosal area	59.5[a]	37
Colon and feces	76.0[abc]	25

[1]Proportions not sharing a common superscript are different ($P < 0.05$).

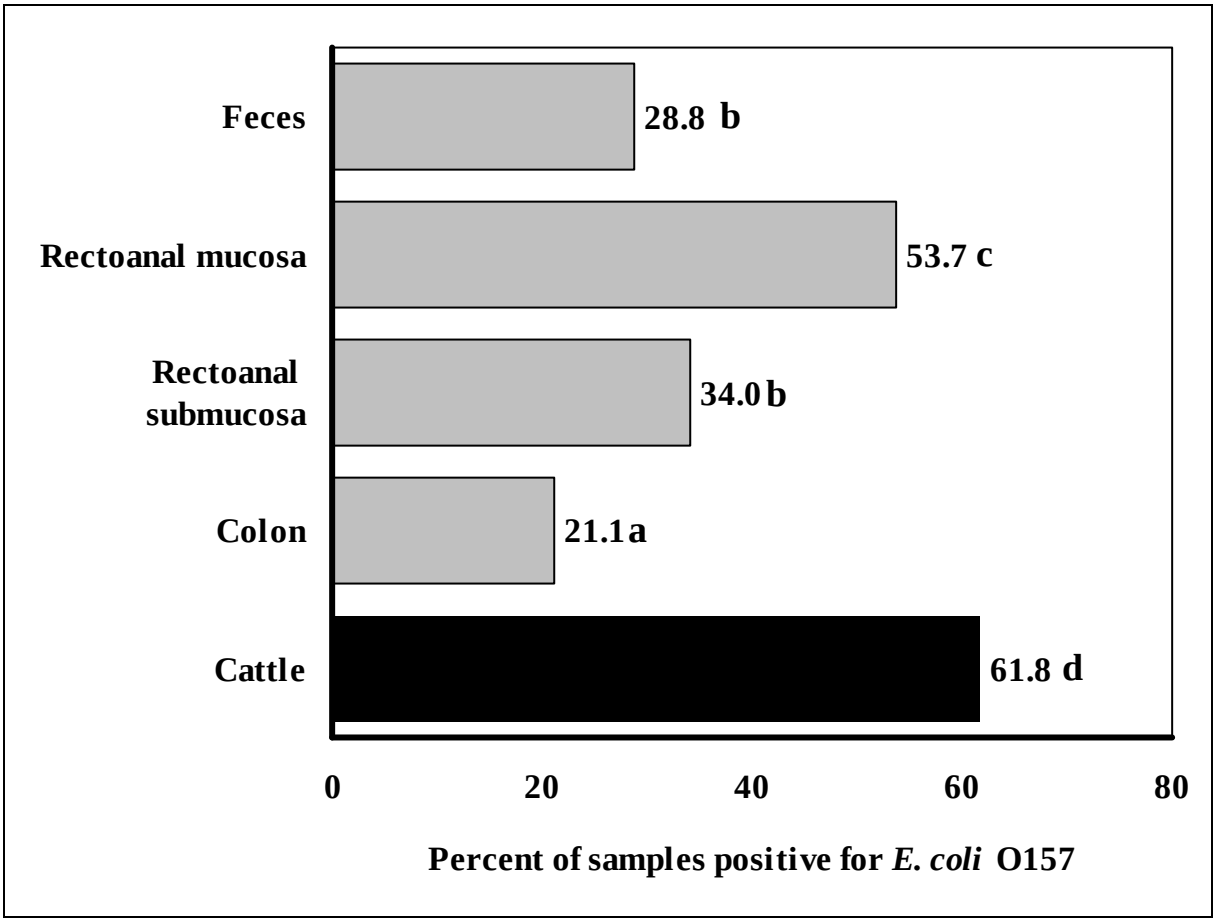

Figure 1.1 Detection of *Escherichia coli* O157 from each hindgut region. Bars with different letters represent differences in prevalence ($P < 0.05$).

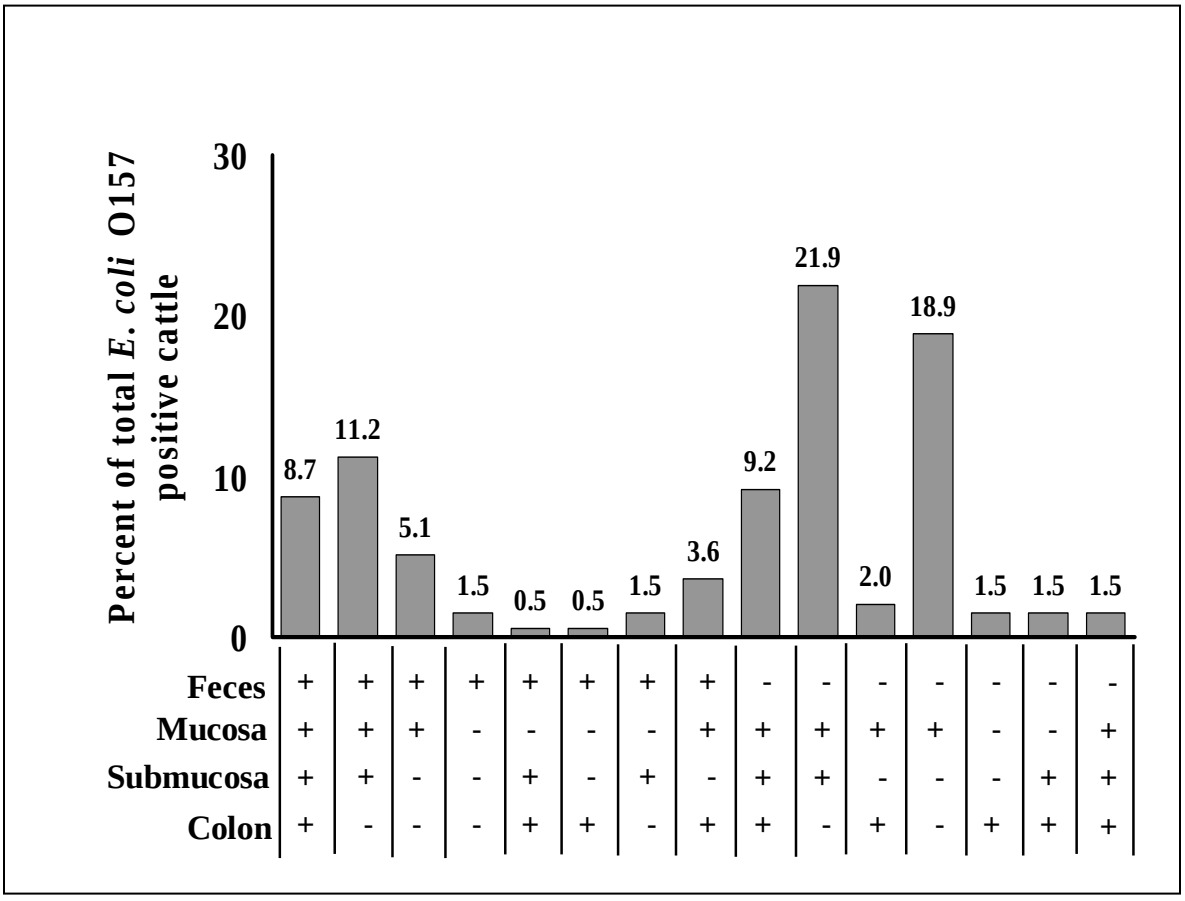

Figure 1.2 Grouping of *Escherichia coli* O157 positive cattle (+ = positive, − = negative for the respective region) from different regions of the hindgut. Bars represent the percent of cattle in each category.

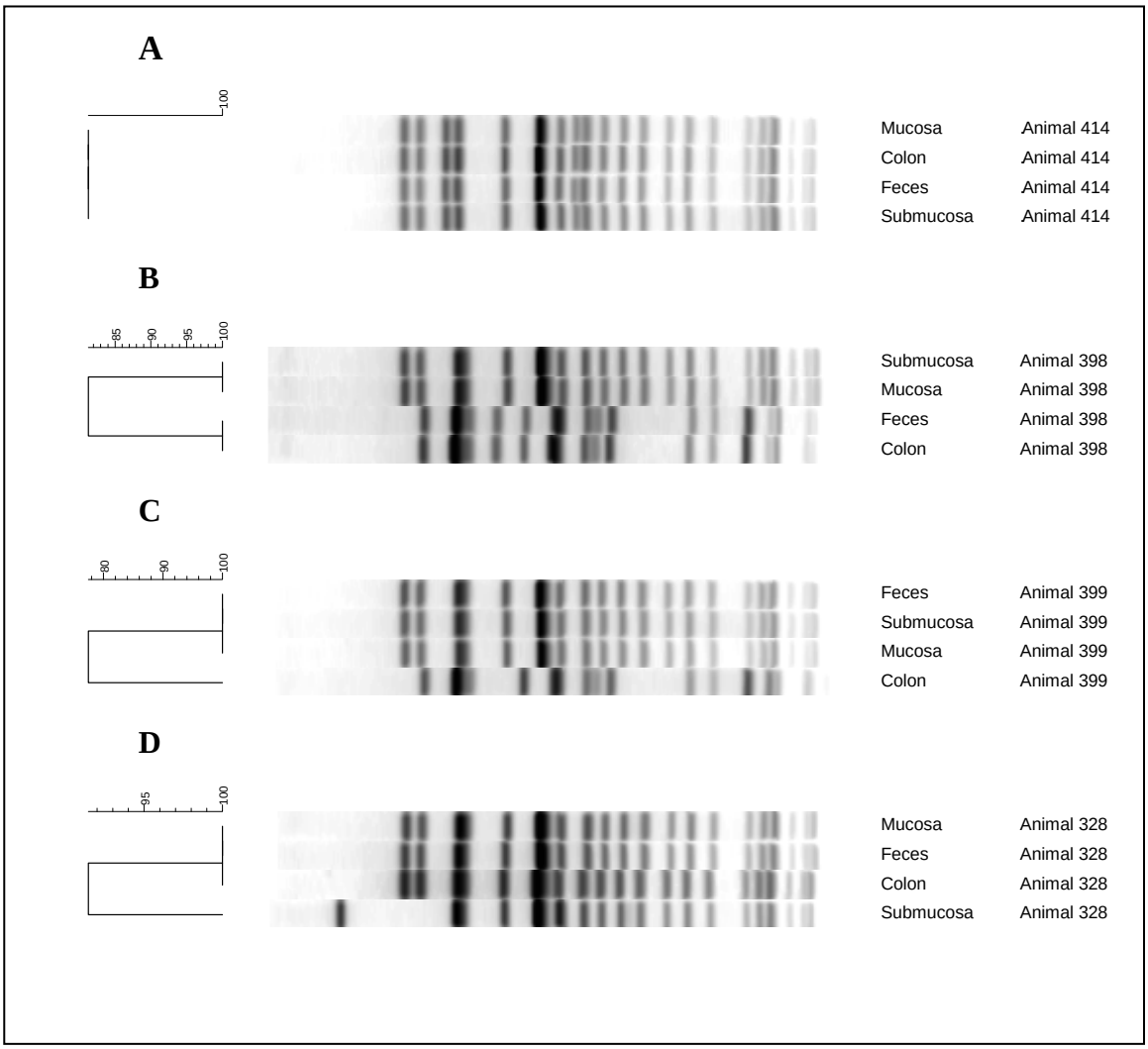

Figure 1.3 Examples of pulsed-field gel electrophoresis banding patterns of *Escherichia coli* O157 isolates from slaughtered cattle in which the organisms were isolated from colon contents, fecal swabs, rectal mucosa and submucosa. These represent examples of animals in which all isolates were clonally similar (> 95% of Dice similarity; A); rectal mucosal and submucosal isolates were genetically different (< 95% of Dice similarity) than colon contents and fecal isolates (B); the colon content isolate was genetically different than the fecal isolate (C); or the rectal submucosal isolate was different than colon content, fecal and rectal mucosal isolates (D).

CHAPTER 2 - Evaluation of culture methods to identify cattle feces with high concentrations of *Escherichia coli* O157

Our objective was to evaluate methods for identifying cattle with high concentrations of *E. coli* O157 in their feces. In two experiments, feces were collected from cattle orally inoculated with nalidixic acid (*Nal*) resistant *E. coli* O157, and direct plating of diluted feces on sorbitol MacConkey agar with cefixime and potassium tellurite (CT-SMAC) containing *Nal* was considered the gold-standard method (GS). In experiment one, methods evaluated were direct pre-enrichment streak, immunomagnetic separation with most probable number (MPN), and direct post-enrichment streak with MPN; all using CT-SMAC. Mean concentration of *Nal* resistant *E. coli* O157 in samples (n=59) by the GS was 3.6 $\log_{10}$ CFU/g. Pre-enrichment streak detected > 3.0 $\log_{10}$ CFU/g samples with a 74.4% sensitivity and 68.8% specificity. Direct post enrichment streak-MPN and immunomagnetic separation-MPN concentrations were significantly correlated with GS concentrations (r = 0.53 and r = 0.39, respectively). In experiment two (n=480), direct pre- and post-enrichment streaks performed in triplicate and spiral plating on CT-SMAC were evaluated. For pre-enrichment streaks, sensitivity was 79.7% and specificity was 96.7% for detecting > 3.0 $\log_{10}$ CFU/g when the criterion was positive cultures on at least two plates. For spiral plating at that concentration, sensitivity and specificity were 83.9% and 56.3%, respectively. Post-enrichment streaks performed relatively poorly. Triplicate pre-enrichment streaks of 1:10 diluted feces on CT-SMAC may be useful for identifying cattle shedding high concentrations of *E. coli* O157. Estimates of sensitivity and specificity enable appropriate application of methods and interpretation of results, and may enhance applied research, surveillance and risk assessments.

Key Words: *E. coli* O157, Enumeration, High shedders, Cattle feces, Test validation

INTRODUCTION

Escherichia coli O157 is a foodborne pathogen that continues to cause severe outbreaks of human infection despite many years of research. Cattle are a known carrier of *E. coli* O157 and previous evidence suggests that prevalence of the organism on carcasses at harvest is correlated with prevalence in feces or on hides (1). More recent literature suggests that not only prevalence, but also concentration of *E. coli* O157 in the feces is indicative of a risk of carcass contamination (3). There is evidence that within a given population of cattle, most animals shed *E. coli* O157 intermittently and at relatively low concentrations, but some animals may be shedding at high concentrations (i.e. greater than 3.0 $\log_{10}$ CFU/g of feces) (14). Additionally, animals shedding *E. coli* O157 at higher concentrations are thought to transmit infection to a greater number of animals compared to animals shedding the organism at low levels (8, 9), thus effectively increasing prevalence within the population. These facts suggest the importance of identifying high shedding animals prior to harvest as well as identifying these animals when evaluating intervention strategies. Furthermore, quantifying the prevalence of these high-shedding animals in a given population may improve the accuracy of microbial risk assessments (4).

Many methods for determining the concentration of *E. coli* O157 have been described. Direct plating of serially diluted fecal samples is a popular method of enumerating *E. coli* O157 (6, 7, 11). The most probable number (MPN) technique also is used to enumerate *E. coli* O157 and immunomagnetic separation (IMS) often is combined with this method (2, 16, 17). Spiral plating of diluted fecal samples (13,15) and real-time polymerase chain reaction-based quantification (5) are additional methods that have been evaluated for enumerating *E. coli* O157 in feces.

These methods may lead to quantitative or semi-quantitative estimates of *E. coli* O157 in feces, but equipment, material, and labor costs for some methods may become substantial when large numbers of samples are considered. In addition, many previous evaluations of these methods have failed to report sensitivity and specificity estimates, which can severely limit the accurate application of diagnostic tests and interpretation of subsequent results for research and risk assessments (4). Our objectives were to evaluate different methods for detecting cattle shedding high concentrations of *E. coli* O157 in their feces and compare these methods to a commonly used and accepted method (i.e. gold-standard method).

MATERIALS AND METHODS

Overall Approach and Methods

Objectives were accomplished with two separate experiments both utilizing fecal samples from cattle orally inoculated with nalidixic acid (Nal) resistant *E. coli* O157. The first experiment evaluated direct streaking of samples prior to enrichment, immunomagnetic separation (IMS) with most probable number (MPN) enumeration, post enrichment direct streaking with MPN enumeration, and standard IMS. Results from the first experiment lead to the re-evaluation of the pre-enrichment direct streak technique with modifications and standard IMS. A spiral plating technique also was included in experiment two. Gold-standard methods used to assess the precision and accuracy of other methods in both experiments were based on direct plating of diluted feces on selective medium containing nalidixic acid. Culture media and methods to confirm *E. coli* O157 were similar in both experiments.

Gram-negative broth (Becton Dickinson Co., Franklin Lakes, NJ) containing cefixime (50 ng/ml), cefsulodin (10 µg/ml) and vancomycin (8 µg/ml; GNccv) was used for enrichment. Sorbitol MacConkey agar (Becton Dickinson Co., Franklin Lakes, NJ) supplemented with cefixime (50 ng/ml) and potassium tellurite (2.5 µg/ml; CT-SMAC) was used for plating samples. For determination of gold standard concentrations of nalidixic acid resistant *E. coli* O157, CT-SMAC was supplemented with nalidixic acid (Sigma-Aldrich, St. Louis, MO), at either 20 (CT-SMACNal^{20}; experiment one) or 50 (CT-SMACNal^{50}; experiment two) µg/ml. For confirmation of *E. coli* O157 on CT-SMAC, isolates were grown on blood agar (Remel, Lenexa, KS) and tested for indole production and latex agglutination of the O157 antigen (Oxoid Limited, Basingstoke, NH).

Experiment One

Animals and bacterial inoculation

Cattle (n = 10) were orally inoculated via a stomach tube with a mixture of three bovine fecal strains (FRIK920, FRIK1123, and FRIK2000) of *E. coli* O157 made resistant to 20 µg/ml of nalidixic acid (Sigma). Cattle were commercial beef calves of mixed breeds, of either sex, weighing between 200 and 300 kg body weight. Fecal samples were collected via rectal grab sampling from these animals two times per week for three weeks giving a total of 60 fecal samples for evaluation of the procedures. Samples were transported in sterile plastic bags to the laboratory and further procedures were performed within 1 h of collection. Each sample was considered as an independent experimental unit for assessment of the enumeration procedures.

Gold standard

For each sample, approximately two g of feces was placed into 18 ml of GNccv broth in large, pre-weighed test tubes. Tubes were then weighed again to determine the amount of feces and vortexed for one minute. Two-hundred µl aliquots from the fecal slurry tubes (pre-enrichment) were placed into 1.8 ml GNccv in a 96-well assay block (Corning Inc., Corning, NY) in triplicate. Each of these sub-samples underwent four serial 10-fold dilutions of 200 µl into 1.8 ml GNccv to yield dilutions from 10^{-2} to 10^{-6} in the assay block. The gold standard concentration of *E. coli* O157 in each fecal sample was determined by spread plating 100 µl of the original fecal slurry in triplicate and each dilution in the assay block onto CT-SMACNal[20]. Following overnight incubation (37°C) of plates, sorbitol-negative colonies on CT-SMACNal[20] plates were counted to determine the concentration of *E. coli* O157 (CFU/g) in each sample. Two sorbitol-negative colonies from each plate were tested for confirmation of *E. coli* O157 as described above.

Direct streak of pre-enriched sample

Prior to enrichment, loopfuls (broad tip [10 µl] of a sterile bacterial loop; Catalog #13-075-4a, Fisher Scientific, Palantine, IL) of sample slurry in GNccv broth were streaked onto CT-SMAC plates. Following overnight incubation of plates at 37°C, up to two sorbitol-negative colonies were tested for confirmation of *E. coli* O157.

Standard IMS

Tubes containing feces in GNccv broth were incubated for 6 h at 37°C. Following enrichment, tubes were vortexed for one minute and one ml was pipetted into a 1.5 ml microcentrifuge tube (Fisher Scientific) containing 20 µg of Dynabeads (anti-*E. coli* O157; Dynal Biotech ASA, Oslo, Norway). Following IMS, 50 µl were spread plated onto CT-SMAC

and incubated (37°C) overnight. Up to six sorbitol-negative colonies were tested to confirm *E. coli* O157.

IMS - MPN

Following sample dilution in assay blocks and transfer of inocula onto plates for gold standard enumeration, blocks were incubated for 6 h at 37°C. After enrichment, one ml from 10^{-2}, 10^{-4}, and 10^{-6} dilutions was subjected to IMS as described above and plated on CT-SMAC. Assay blocks were refrigerated (4°C) during the overnight incubation of plates. After overnight incubation, if sorbitol-negative colonies were evident on CT-SMAC from the 10^{-2} or 10^{-4} dilutions, then 1 ml of the next higher dilution (10^{-3} or 10^{-5}) was subjected to IMS on the following day (2). Isolation and identification of *E. coli* O157 was performed as described above. Based upon the number of wells that were positive for *E. coli* O157 in each dilution, MPN values were determined with MPN Build 23 (Mike Curiale, http://i2workout.com/mcuriale/mpn/index.html) and concentration was expressed as MPN/g.

Post enrichment direct streaking - MPN

Following enrichment (6 h at 37°C), loopfuls of GNccv-fecal slurry, and of each dilution and replication in the assay block were then streaked onto CT-SMAC plates. Confirmation of *E. coli* O157 was performed as described above. Again, using the number of wells that tested positive per dilution, MPN was determined as described above.

Experiment Two

Animals and bacterial inoculation

Commercial mixed-bred beef calves (n = 30) weighing approximately 180 kg (5 to 6 mo of age), were orally inoculated via a stomach tube with a mixture of five bovine fecal strains

((FRIK920, FRIK1123, FRIK2000, 01-2-10561, and 01-2-08970) of *E. coli* O157 made resistant in the laboratory to 50 µg/ml of nalidixic acid. Fecal samples were obtained from the animals three times a week for approximately five weeks for a total of 480 samples, and as in experiment one, samples were transported in plastic bags and further procedures were performed within one hr of collection. Each sample was again identified as an independent experimental unit for assessment of the enumeration procedures.

Gold standard

In contrast to the gold standard determination in experiment one, serial dilution of feces in GNccv was not performed in triplicate, yet spread plating of each dilution onto CT-SMACNal[50] was done in triplicate. Procedures for determining the concentration of *E. coli* O157 in each sample were similar to experiment one. In order to detect *E. coli* O157 at levels below the threshold of detection (10^2) by this method, fecal slurry tubes were incubated for 6 hr at 37°C and then tubes were vortexed and one ml was transferred to another test tube containing 9.0 ml GNccv. This tube was incubated (37°C) for an additional 18-24 hrs and then plated onto CT-SMACNal[50]. Similar procedures were used to confirm *E. coli* O157 on these plates.

Direct streak of pre- and post-enrichment samples

Prior to enrichment, a loopful of GNccv-fecal slurry was streaked onto CT-SMAC in triplicate. This procedure was then repeated after tubes were incubated for 6 hr at 37°C (post-enrichment direct streak). Up to two sorbitol-negative colonies were tested for the confirmation of *E. coli* O157 for each method.

Standard IMS

This procedure was performed as described in experiment one.

Spiral plating

This procedure was performed on 150 samples collected during the first two weeks following inoculation. Prior to enrichment, a 100 µl aliquot from the GNccv-fecal slurry was spiral plated onto one CT-SMAC plate and one CT-SMACNal^{50} plate. The plating was done using a WASP 2 Spiral plater (Microbiology International, Frederick, MD). After overnight incubation at 37°C, sorbitol-negative colonies (if present) were counted using an aCOLyte SuperCount colony counter (Microbiology International). Two colonies from each plate were tested for indole production and latex agglutination for the O157 antigen. *Escherichia coli* O157 counts from spiral plates were expressed as CFU per g of feces.

Data Analysis

The Shapiro-Wilk test was used to determine if concentration data were normally distributed using Proc UNIVARIATE of SAS (SAS Version 9.1, Cary, NC). Pearson correlation coefficients were generated between the log-transformed concentration of *E. coli* O157 as determined by the gold standard method and the log-transformed MPN values for the IMS and post-enrichment direct streak methods. Correlations were determined using Proc CORR of SAS. Linear trendlines, equations and R-square values were determined with Microsoft Excel® (Microsoft Corporation, Redman, WA). For determining sensitivity and specificity estimates of methods to identify samples with high concentrations of *E. coli* O157, we used 3.0 and 4.0 $\log_{10}$ CFU per g of feces as threshold values to separate low (below threshold) and high (above threshold) concentrations. Sensitivity estimates were the proportions of truly positive samples (based on the gold standard) that tested positive (either overall, in a specified category, or above specified threshold values). Specificity estimates were calculated similarly based on truly

negative samples that tested negative. Exact 95% binomial confidence intervals (CI) were calculated for sensitivity and specificity estimates using the BETAINV function of Microsoft Excel®. Exact CIs are given in parentheses for all presented sensitivity and specificity estimates. Positive predictive values (PPV) were calculated as the proportion of test positive samples that were true positives.

RESULTS

Experiment One

Sample distribution

In total, 60 fecal samples were collected in the experiment, but one was excluded because there was less than 0.5 g of fecal material. Descriptive statistics for samples are shown in Table 1. *Escherichia coli* O157 was detected and quantified in all samples. The minimum and maximum concentrations of *E. coli* O157 were 2.5×10^1 CFU/g and 2.3×10^5 CFU/g, respectively. Concentrations of *E. coli* O157 were not normally distributed, however, log-transformed concentrations were not significantly different from normal ($P = 0.42$).

Direct streak of pre-enriched sample

Escherichia coli O157 was isolated from 37 of the 59 (62.7%) direct streak, pre-enriched samples when a maximum of two colonies present on CT-SMAC were evaluated per plate. Sensitivity estimates categorized by the concentration of *E. coli* O157 in samples, are shown in Figure 1. Using this method to identify samples with *E. coli* O157 concentrations above 3.0 $\log_{10}$ CFU/g, sensitivity and specificity estimates were 74.4% (58.8-86.5%) and 68.8% (41.3-

89.0%), respectively. Sensitivity and specificity estimates for this method to detect samples above 4.0 $\log_{10}$ CFU/g were 85.0% (62.1-96.8%) and 48.7% (32.4-65.2%), respectively.

Standard IMS

Using IMS, *E. coli* O157 was detected in 52 of 59 known positive fecal samples (sensitivity estimate 88.1% [77.1-95.1%]). Mean and SE of the gold standard concentration of *E. coli* O157 in samples testing positive and negative by this method were 3.62 ± 0.12 and 3.27 ± 0.29 CFU/g. Sensitivity and specificity estimates for identifying samples > 4.0 $\log_{10}$ CFU per g were 95.0% (75.1-99.9%) and 15.4% (5.9-30.5%), respectively.

Most probable number

Mean MPN values for the IMS-MPN and post enrichment direct streaking-MPN methods were 3.7 x 10^3 and 1.0 x 10^3 MPN/g, respectively. Log base 10 transformations were made on the MPN values, but normality was not present in either original or transformed concentrations of *E. coli* O157. However, linear relationships were evaluated in an attempt to examine the association of these methods with the gold standard (Figure 2). The gold standard was correlated ($P < 0.01$) with both IMS-MPN and post enrichment direct streaking-MPN methods. Additionally, the IMS-MPN method was correlated with the direct streak method ($r = 0.54$; $P < 0.01$).

Sensitivity and specificity of MPN methods were estimated by creating categories of $\log_{10}$ groups of *E. coli* O157 concentrations derived from linear equations, which were generated by plotting gold standard concentrations versus non-zero MPN values (Figure 2). If MPN values were zero, then zero was used. The IMS-MPN method identified samples with > 3.0 $\log_{10}$ CFU/g with sensitivity and specificity estimates of 90.7% (77.9-97.4%) and 25.0% (7.3-52.4%), respectively. At this concentration threshold, the post enrichment direct streaking-MPN method

had sensitivity and specificity estimates of 76.7% (61.4-88.2%) and 56.2% (29.9-80.3%), respectively. Using 4.0 $\log_{10}$ CFU/g as the threshold, sensitivity and specificity estimates for the IMS-MPN and post enrichment direct streaking-MPN methods were 40.0% (19.1-64.0%) and 92.3% (79.1-98.4%), and 35.0% (15.4-59.2%) and 100% (91.0-100%), respectively.

Experiment Two

Sample distribution

In this experiment, concentrations of *E. coli* O157 were handled as categorical data (Table 2). The gold standard detected *E. coli* O157 in 397 of 480 (82.7%) fecal samples collected. Based on the gold standard concentration or detection by enrichment, samples were categorized into one of eight categories: zero when samples were negative on all tests, <1 when samples were positive only by secondary enrichment, and 1-2, 2-3, 3-4, 4-5, 5-6 and 6-7 corresponding to the $\log_{10}$ *E. coli* O157 CFU/g.

Direct streak of pre- and post-enriched samples

For each method, the results were categorized as 0, 1, 2, or 3 *E. coli* O157 positive CT-SMAC plates. Distributions of samples with these responses for both pre- and post-enrichment direct streak methods, categorized by the gold standard concentration of *E. coli* O157, are shown in Figures 3 and 4. Sensitivity, specificity, and positive predictive values for pre- and post-enrichment direct streak methods identifying samples with high levels of *E. coli* O157 (threshold concentration values of 3.0 or 4.0 $\log_{10}$ CFU/g) are presented in Table 3.

Standard IMS

Escherichia coli O157 was detected using the standard IMS procedure in 353 of 480 total samples. This technique detected the organism in 342 of the 397 samples (sensitivity estimate

86.1% [82.4-89.4%]) found positive by the gold standard and in 11 of 83 samples that were negative by the gold standard. Sensitivity was greater than 90% when the concentration of *E. coli* O157 was greater than 2.0 $\log_{10}$ CFU/g (Figure 5). The method identified samples with high levels of *E. coli* O157 (3.0 $\log_{10}$ CFU/g) with a 97.3% (93.2-99.3%) sensitivity and a 37.0% (31.8-42.5%) specificity. At a threshold of 4.0 $\log_{10}$ CFU/g, sensitivity and specificity estimates were 96.3% (89.7-99.2%) and 31.2% (26.6-36.0%), respectively.

Spiral plating

The spiral plating technique was performed on 150 samples. However, only samples with gold standard counts (n = 138) were evaluated and samples with sorbitol-negative isolates picked from the CT-SMAC spiral plate, but not confirmed as *E. coli* O157 (n = 10) were considered to have a spiral plate concentration of zero. *Escherichia coli* O157 were detected and counted for 115 of 138 samples plated on CT-SMAC and 135 of 138 samples plated on CT-SMACNal^{50}. The linear relationship between log-transformed gold standard counts of *E. coli* O157 and log-transformed spiral plate counts of *E. coli* O157 is shown in Figure 6. Pearson correlation coefficients indicated that both $\log_{10}$ spiral plate counts on CT-SMAC (r = 0.43) and CT-SMACNal^{50} (r = 0.90) were significantly correlated with $\log_{10}$ gold standard concentration. If samples that did not have counts on the CT-SMAC spiral plate (n = 23) are removed, the correlation coefficient for that comparison improves (r = 0.66). The spiral plate CT-SMAC method identified samples with high concentrations of *E. coli* O157 defined as > 3.0 $\log_{10}$ CFU/g with sensitivity and specificity estimates of 79.0% (70.6-85.9%) and 63.2% (38.4-83.7%), respectively. Utilizing 4.0 $\log_{10}$ CFU/g as the threshold value, sensitivity and specificity estimates were 34.2% (23.9-45.7%) and 88.1% (77.1-95.1%), respectively.

DISCUSSION

Cattle shedding *E. coli* O157 at high concentrations may play an important role in transmission of the organism to people and other cattle. Methods targeted to detect these animals are more useful if estimates of test performance are available. For our experiments, we used feces from cattle that were experimentally inoculated with *E. coli* O157. This ensured presence of the organism in most samples, while retaining the complex nature of the microbial ecology that can present a challenge when detecting *E. coli* O157 from bovine feces. We felt this was a more appropriate model for evaluating the detection methods compared to using fecal samples inoculated with *E. coli* O157. However, the fecal shedding patterns of inoculated cattle in our studies may not be entirely reflective of populations of naturally shedding cattle.

In our study, we used approximately two grams of feces instead of a larger amount such as 10 g , which has been used previously (1,6). This smaller amount was selected for two reasons. First, using 10 g of feces for detection in very large numbers of animals may not always be economically or logistically feasible due to the increased cost of media and incubator space required. Additionally, acquiring 10 g of feces via rectal grab at any set time during the day is not always possible. Because our intention was to compare these methods as they would be used in large scale research or surveillance, the smaller sample seemed more applicable. Secondly, we wanted to evaluate all methods using identical specimens to ensure that variation in concentration within the feces collected from the animal did not represent an additional source of variation when comparing experimental methods to the gold standard. This variation has been shown previously by Naylor et al. (10) and Pearce et al. (12).

The sensitivity of detecting *E. coli* O157 by the standard IMS method in our study was similar to previous studies where sensitivity was enhanced with increasing concentrations of *E.*

coli O157 and a plateau effect was seen as concentrations approached 2.0 $\log_{10}$ CFU/g (6, 11). In experiment two of the current study, the standard IMS method detected *E. coli* O157 in 11 samples that were not detected by the gold standard. Three of these samples were from the same animal on three consecutive sampling dates, two were from the same animal in non-consecutive samples and the remaining six samples were from six different animals. All of these 11 samples were collected within a two-week period. These may be due to the presence of non-inoculated strains of *E. coli* O157 that were susceptible to nalidixic acid and therefore not detected by the gold standard method, or simply that the gold standard method did not detect inoculated strains with 100% sensitivity. This is a potential source of error when comparing enumeration procedures with a gold standard that may be imperfect, but in this study the effect was negligible.

Direct plating (prior to enrichment) is a common methodology used to quantify naturally occurring *E. coli* O157 in cattle feces (6, 7, 11). LeJeune et al. (6) employed a similar challenged animal model and found a strong correlation (r = 0.88) between their gold standard *E. coli* O157 concentrations and direct spread plating of diluted samples (300 µl) onto CT-SMAC. This correlation was determined using samples testing positive on CT-SMAC, representing 130 of 224 (58.0%) samples positive by the gold standard (6). In the current study, we used direct streaking instead of direct spread plating. In experiment one, with only one direct streak plate, we observed sensitivity estimates above 70% and specificity at or above 50% depending on the definition of high concentration (threshold). We pursued this method further in experiment two and added replications (three plates) in an attempt to improve sensitivity and specificity. With the additional plates, we observed relatively high sensitivity with comparable specificity. An added benefit to this method, is that it can be used for many applications by only changing the interpretation of the results. For instance, if the goal is to maximize specificity with less concern

to sensitivity then using a threshold value of 3.0 $\log_{10}$ CFU/g and using 3 positive plates as the criteria for high concentration could be used. However, if the goal was to optimize both sensitivity and specificity then using a threshold of 4.0 $\log_{10}$ CFU/g and 2+ positive plates as the criteria would be better. Other advantages to this method are that it does not require special equipment (IMS, spiral plating, PCR), the required culture materials are relatively inexpensive (bacterial loops, culture medium and Petri dishes) and it is a feasible method for handling large numbers of samples.

Immunomagnetic separation with MPN has been used in previous studies to enumerate *E. coli* O157 in cattle feces (2, 16, 17). Stephens et al. (16) compared the technique to a gold standard by utilizing fecal samples spiked with streptomycin resistant *E. coli* O157 strains and observed that IMS-MPN values were consistently lower than gold-standard values. In our study, the IMS-MPN method was positively correlated with the gold standard, but relatively low estimates of sensitivity or specificity may prevent this method from being successfully applied for distinguishing samples with high concentrations of *E. coli* O157. The correlation between gold standard and IMS-MPN concentrations was lower in our study than in a previous study (16). One key difference between this previous study and our study was the amount of sample used for each test. Stephens et al. (16) performed serial dilutions of 1 ml into 9 ml whereas in the current study, serial dilutions were performed with 0.2 ml into 1.8 ml. Even though both are a 1:10 dilution, this may impact results given samples were enriched after dilution. Refrigerating samples overnight before performing IMS on 10^{-3} and 10^{-5} may also have impacted our results in a negative fashion, but previous experiments have shown that storing fecal slurry samples at 2°C for up to 3 days did not affect counts of inoculated *E. coli* O157 (2). This procedure was implemented to reduce the number of IMS tests because this can become quite costly with

multiple tests per sample. The post enrichment direct streaking-MPN method also was correlated with the gold standard and the cost of materials needed for this method is low, but either sensitivity or specificity estimates were relatively poor depending on the threshold used to define a high concentration of *E. coli* O157.

Spiral plating is another popular method of enumerating *E. coli* O157 in feces (13, 15). While this method yielded accurate counts at higher levels (> 4.0 $\log_{10}$ CFU/g), the precision was reduced as concentrations decline below this threshold (15). It is important to note, that these previous studies utilize a different selective agar for spiral plating than that used in the current study. Categorizing samples into low and high *E. coli* O157 concentrations with the spiral plating method leads to compromises in either sensitivity or specificity depending on the threshold value utilized. Utilizing 3.0 $\log_{10}$ CFU as the threshold value for high concentration of *E. coli* O157 compromises specificity, whereas using 4.0 $\log_{10}$ CFU compromises sensitivity. The initial equipment costs of the spiral plating technique may be substantial and represent an additional disadvantage of the technique unless a laboratory already has the equipment available. The strong linear relationship between gold standard *E. coli* O157 concentrations and spiral plate concentrations on CT-SMACNal^{50} (Figure 6), show that spiral plating can be highly effective for enumeration. However, the use of resistant strains and selective agar is very advantageous given the microbial ecology of cattle feces, and the results from the CT-SMAC plates illustrates that the effectiveness of the method can be diminished substantially without the ability to select isolates based on resistance.

We evaluated multiple culture procedures that may be used for identifying high concentrations of *E. coli* O157 in cattle feces, and conclude that different methods could be useful depending on the objectives of testing and the resources available. When the intent is to

identify animals shedding high levels of *E. coli* O157 in a field situation, then predictive values, which indicate the probability of a high concentration given the test result, would be of primary interest. Thus, it is important to consider the population prevalence in addition to the diagnostic sensitivity and specificity of the testing scheme. For example, we have illustrated that decreasing test specificity, prevalence of high-shedders or both will lead to lower positive predictive values, which equates to an increase in the proportion of positive tests that are false positives (Table 3). Throughout this table, we included predictive value calculations at 10 and 20% prevalence since the observed study prevalence depended on the threshold concentration used to define a high concentration of *E. coli* O157 and the shedding patterns of inoculated cattle in our study may not be entirely reflective of populations of naturally shedding cattle. Of the methods that we evaluated, direct pre-enrichment streaks of 1:10 diluted feces in triplicate appeared to be the most useful with multiple potential applications for identifying fecal samples with high levels of *E. coli* O157. Regardless of the method utilized, the data generated on test sensitivity and specificity are essential for appropriate application of detection methods and for interpretation of testing results of *E. coli* O157 shedding patterns in cattle populations.

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TABLE 2.1 Distribution of *Escherichia coli* O157 concentrations in fecal samples in experiment one.

	Mean CFU/g	SD CFU/g	Log_{10} CFU/g	SD log_{10} CFU/g	No. of samples
Distribution					
1 – 2 log_{10} CFU/g	46	18	1.63	0.20	3
2 – 3 log_{10} CFU/g	497	281	2.62	0.28	13
3 – 4 log_{10} CFU/g	4,730	2,871	3.59	0.29	23
4 – 5 log_{10} CFU/g	28,561	16,337	4.40	0.22	18
5 – 6 log_{10} CFU/g	175,996	75,189	5.22	0.19	2
Overall	16,635	35,036	3.58	0.88	59

TABLE 2.2 Distribution of samples in experiment two by category of *Escherichia coli* O157 concentration as determined by the gold standard.

Category	No. of samples	Proportion of total
Not detected, 0 CFU/g	83	17.3%
< 1 $\log_{10}$ CFU/g	167	34.8%
1 – 2 $\log_{10}$ CFU/g	36	7.5%
2 – 3 $\log_{10}$ CFU/g	46	9.6%
3 – 4 $\log_{10}$ CFU/g	66	13.8%
4 – 5 $\log_{10}$ CFU/g	46	9.6%
5 – 6 $\log_{10}$ CFU/g	28	5.8%
6 – 7 $\log_{10}$ CFU/g	8	1.7%

TABLE 2.3 Sensitivity and specificity estimates with corresponding 95% exact confidence intervals (CI) for direct streak methods using one or more (1+), two or more (2+), or three (3) positive plates[2] to classify samples as positive at concentrations of greater than 3 or 4 $\log_{10}$ *E. coli* O157 CFU per g of feces. Corresponding positive predictive values (PPV) at the observed, 10% and 20% prevalence also are given[1].

	$\log_{10}$ CFU/g	Sensitivity % (CI)	Specificity % (CI)	PPV at study prevalence	PPV at 10% prevalence	PPV at 20% prevalence
Pre-enrichment						
≥1 positive plates	3	89.2 (83.0-93.7)	89.8 (86.0-92.8)	0.80	0.49	0.69
	4	96.3 (89.7-99.2)	78.1 (73.8-82.1)	0.48	0.33	0.52
≥2 positive plates	3	79.7 (72.3-86.0)	96.7 (94.2-98.3)	0.92	0.73	0.86
	4	87.8 (78.7-94.0)	85.7 (81.9-89.0)	0.56	0.41	0.61
3 positive plates	3	62.2 (53.8-70.0)	98.5 (96.5-99.5)	0.95	0.82	0.91
	4	68.3 (57.1-78.1)	89.7 (86.3-92.5)	0.58	0.42	0.62
Post-enrichment						
≥1 positive plates	3	96.6 (92.3-98.9)	61.4 (56.0-66.7)	0.53	0.22	0.39
	4	97.6 (91.5-99.7)	52.0 (47.0-57.0)	0.30	0.18	0.34
≥2 positive plates	3	91.9 (86.3-95.7)	68.7 (63.4-73.6)	0.57	0.25	0.42
	4	93.9 (86.3-98.0)	59.1 (54.0-63.9)	0.32	0.20	0.36
3 positive plates	3	77.0 (69.4-83.5)	75.6 (70.6-80.1)	0.59	0.26	0.44
	4	79.3 (68.9-87.4)	67.3 (62.5-71.9)	0.33	0.21	0.38

[1]Proportions of test positive samples that were positive at that concentration with the gold standard method; calculated at the observed prevalence and 10% and 20% prevalence of high *E. coli* O157 concentrations using sensitivity and specificity estimates from the study.
[2]At least one confirmed *E. coli* O157 colony when two colonies per plate were evaluated.

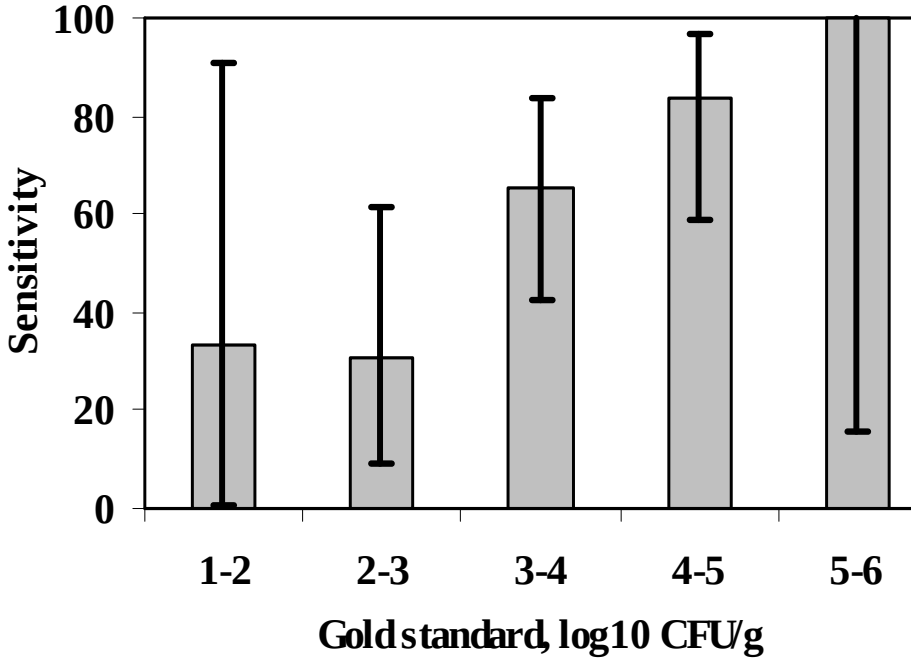

FIG. 2.1 Sensitivity estimates and corresponding 95% confidence intervals (error bars) of the pre-enrichment streak method categorized by the gold standard concentration (experiment one). Estimates are presented as the percent of samples with a confirmed *Escherichia coli* O157 colony on the pre-enrichment direct streak plate when only two colonies were tested.

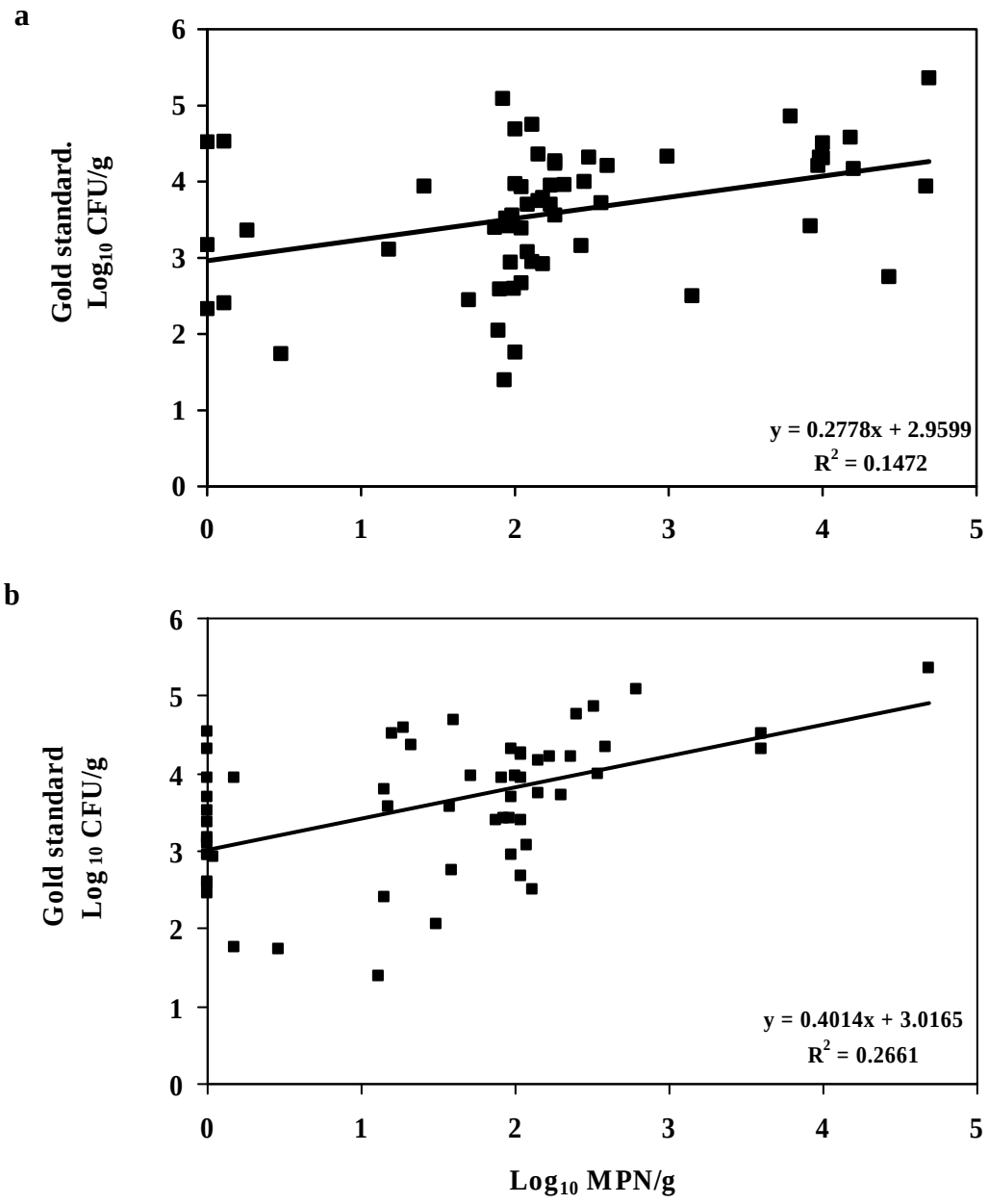

FIG. 2.2 Relationships between the log-transformed gold standard concentration of *Escherichia coli* O157 in fecal samples and the log-transformed MPN concentration of *E. coli* O157 in the same sample as determined by the IMS/MPN (a) method and the direct streak MPN (b) method. Equations and R-square values for linear trendlines are presented.

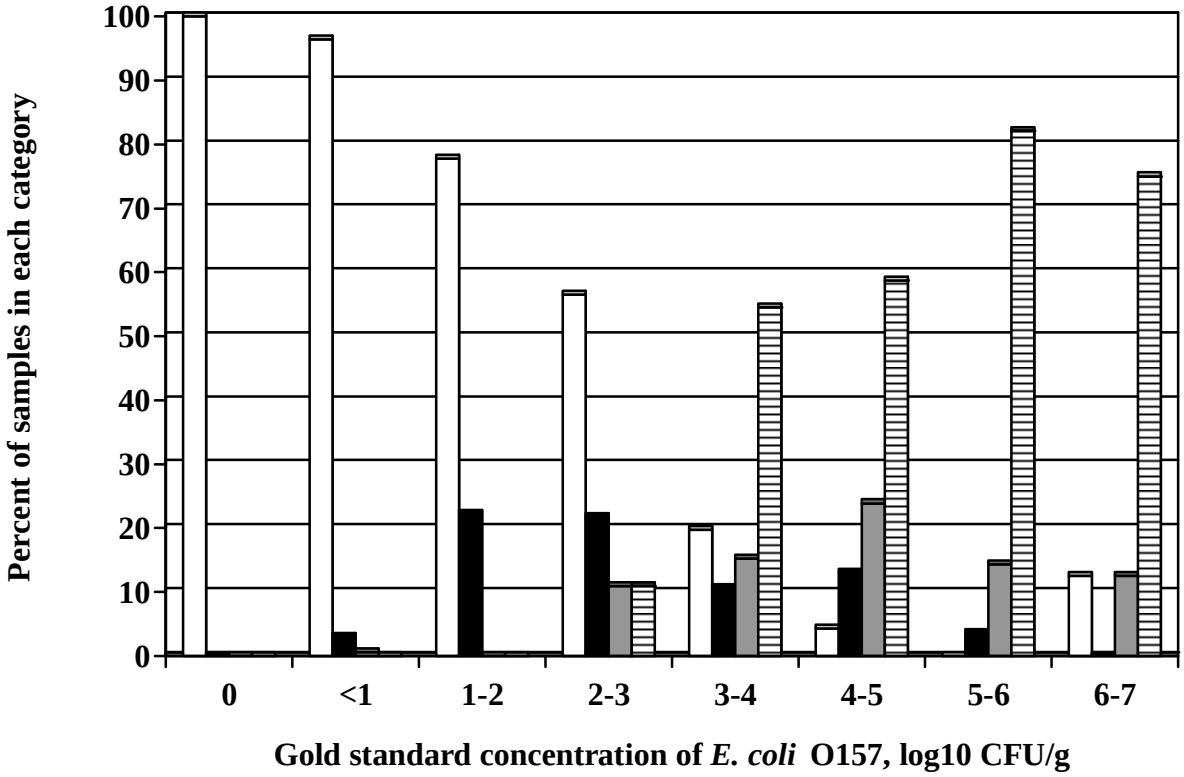

FIG. 2.3 Percent of samples with zero (white bars), one (black bars), two (grey bars), or three (hatched bars) of three pre-enrichment plates testing positive for *E. coli* O157 in experiment two. Percentages are categorized by the gold standard concentration (x-axis). A positive plate had at least one confirmed *E. coli* O157 colony when only two suspect colonies were tested.

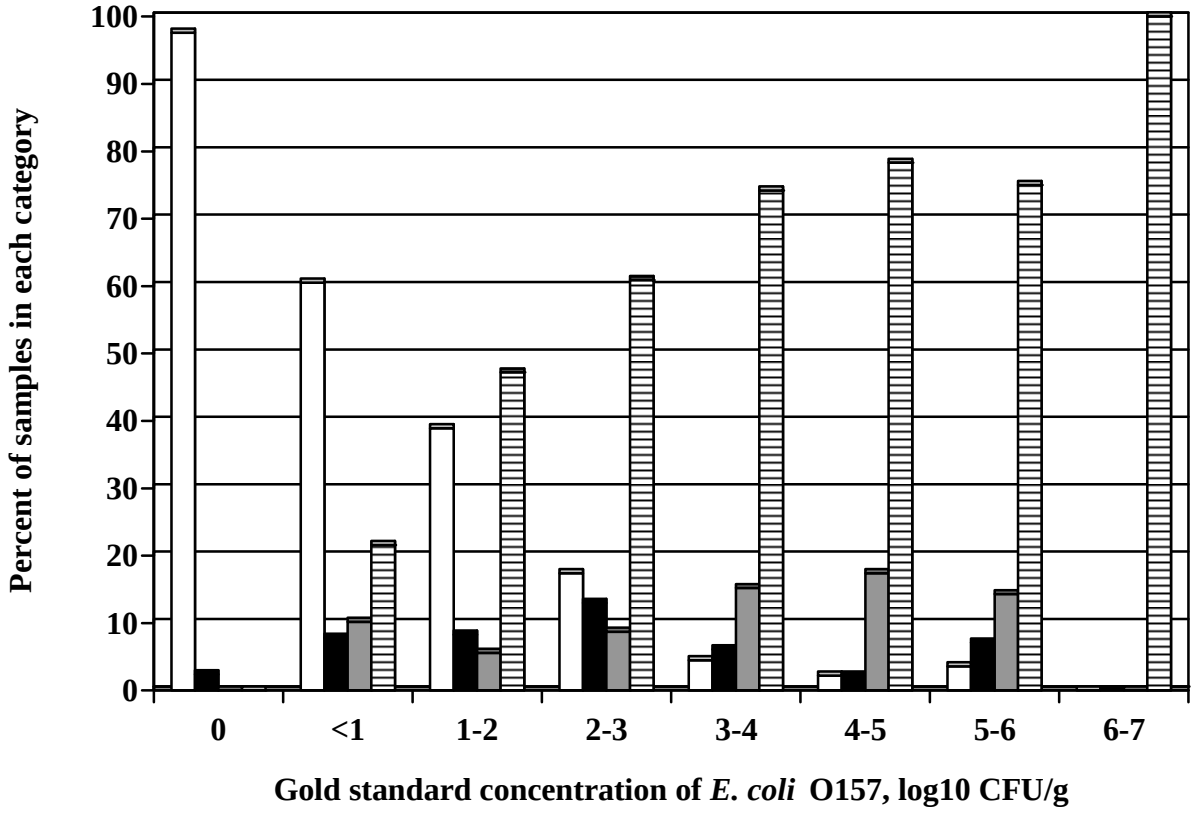

FIG. 2.4 Percent of samples with zero (white bars), one (black bars), two (grey bars), or three (hatched bars) of three post-enrichment plates testing positive for *E. coli* O157 in experiment two. Percentages are categorized by the gold standard concentration (x-axis). A positive plate had at least one confirmed *E. coli* O157 colony when only two suspect colonies were tested.

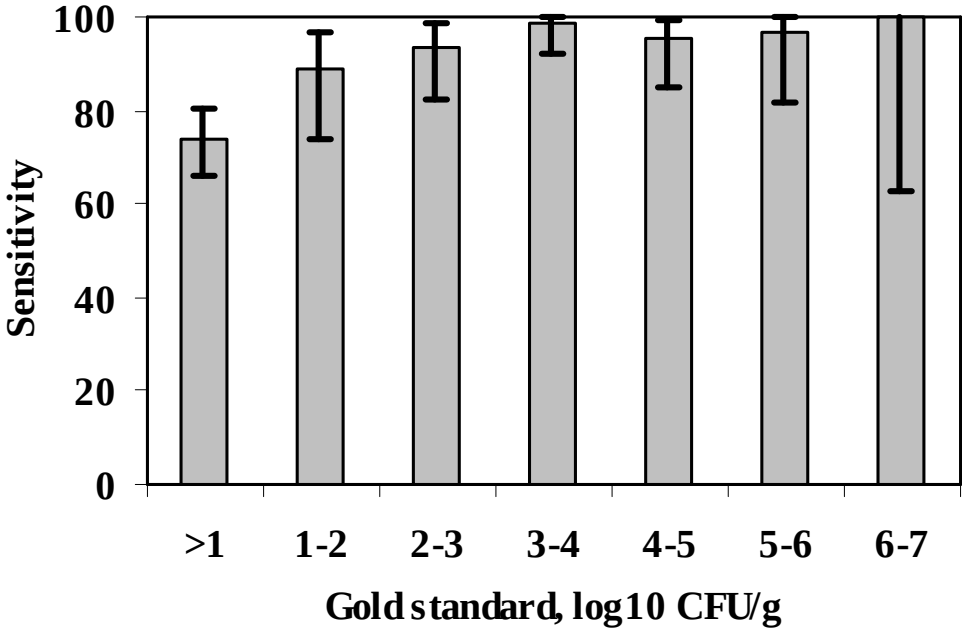

FIG. 2.5 Sensitivity estimates and corresponding 95% confidence intervals (error bars) for the standard IMS procedure categorized by the gold standard concentrations of *E. coli* O157 in experiment two. Estimates are presented as the percent of samples with a confirmed *E. coli* O157 colony when up to six colonies were tested.

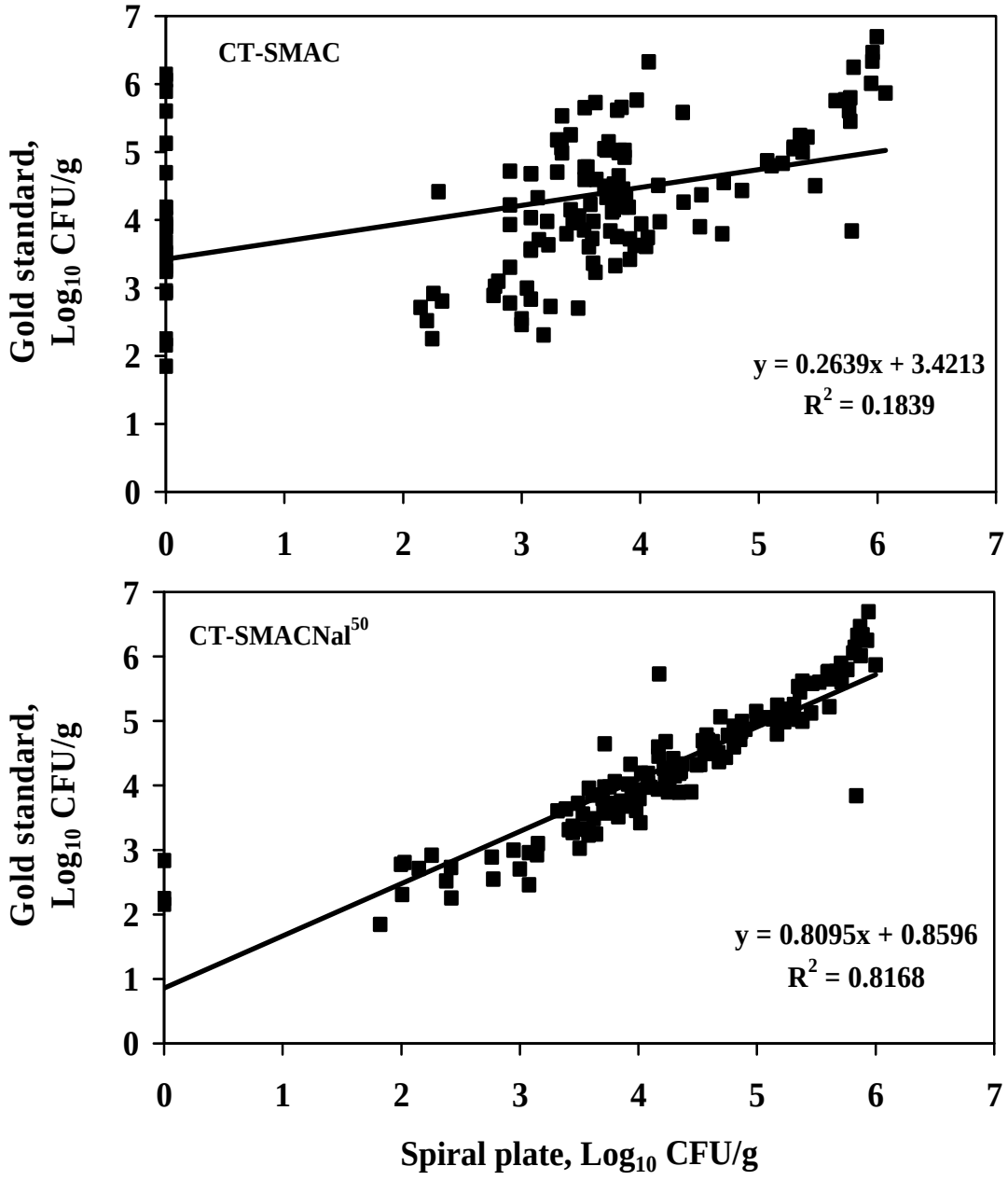

FIG. 2.6 Relationships between the log-transformed gold standard concentration of *E. coli* O157 in fecal samples (n = 138) and the log-transformed spiral plate concentration in the same sample when plated on CT-SMAC and CT-SMACNal^{50}. Equations and R-square values are presented for linear trendlines.

CHAPTER 3 - Associations between *Escherichia coli* O157 in feces at harvest and contamination of pre-intervention beef carcasses

Cattle shedding *E. coli* O157 at high levels in feces may increase overall fecal prevalence in cattle populations, and fecal prevalence at slaughter may be associated with carcass contamination. Our objectives were to assess associations at slaughter between high-shedding animals, fecal-positive animals and pre-evisceration carcass contamination, and to quantify effects associated with the probability of carcasses testing positive. Up to 32 animals were sampled from each of 50 truckloads of cattle arriving at a commercial abattoir in the Midwest U.S. during a 5-week summer period. Carcass swab samples collected pre-evisceration and fecal samples collected post-evisceration were matched within animals and analyzed for the presence of *E. coli* O157 using selective enrichment, immunomagnetic separation and selective media. In addition, a direct plating procedure was performed on feces to identify high-shedding animals. *Escherichia coli* O157 was isolated from 39 of 1,503 (2.6%) carcass samples in 15 (30%) truckloads, and from 127 of 1,495 (8.5%) fecal samples in 37 (74%) truckloads. Fifty-five (3.7%) high-shedding animals were detected from 26 (52%) truckloads. Truckload fecal (r_s = 0.61) and high-shedder (r_s = 0.68) prevalence were both significantly correlated with carcass prevalence. Multivariate analysis indicated that the probability of carcass contamination was significantly associated with proximity to a previous positive carcass, proximity to a previous high-shedding animal, and the *E. coli* O157 status of the ensuing carcass. We found that the probability of a pre-evisceration carcass testing positive varied tremendously (0.04 - 0.87) depending on the fecal and carcass status of other cattle within the truckload.

Key words*: Escherichia coli* O157, fecal prevalence, high-shedders, carcass prevalence

INTRODUCTION

Recent outbreaks of illness due to the consumption of contaminated vegetables and meat products demonstrate the continued importance of *Escherichia coli* O157:H7 as a foodborne pathogen. Cattle feces have been implicated as a principle source of food contamination *(16, 18)*. In the U.S. from 1982 to 2002, there were 350 reported outbreaks of *E. coli* O157:H7 comprising 8,598 cases of human infection, 1,493 hospitalizations, and 40 deaths *(23)*. The economic impact of this pathogen to the beef industry is also a concern. One U.S. trade association estimates that in the past 10 years, *E. coli* O157 has cost the beef industry $2.67 billion in loss of demand, product recalls, and research to reduce this pathogen both pre- and post-harvest *(2)*.

Contamination of beef products with *E. coli* O157 generally occurs during the slaughter process where feces and hides are considered the major sources of contamination *(16, 18)*. Elder et al. *(11)* found that lot prevalence of *E. coli* O157 on cattle hides and in cattle feces was positively correlated with lot prevalence of *E. coli* O157 on carcasses. This relationship was confirmed by Woerner et al. *(30)* who observed a higher carcass prevalence of *E. coli* O157 when the prevalence in pen-floor fecal samples collected three days prior to slaughter was greater than 20%. Thus, pens or lots of cattle with a large proportion of animals shedding *E. coli* O157 in feces may lead to an increased risk of carcass contamination.

Considerable research has been done to quantify fecal shedding of *E. coli* O157 and identify animals shedding high concentrations in feces (greater than 3 or 4 $\log_{10}$ CFU per gram). Methodologies evaluated include direct plating *(14, 17, 22, 27)*, spiral plating *(14, 25)*, the combination of immunomagnetic separation with most-probable number techniques *(12, 14, 28)*, and real-time PCR *(15)*. Within a population, most cattle shed *E. coli* O157 intermittently at

relatively low concentrations, but some may shed at high concentrations *(26)*. Enumerating or identifying high-shedding animals, has enabled more detailed assessments of cattle populations and the use of modeling techniques for assessing transmission patterns within populations *(20)*. Based upon mathematical modeling, cattle that shed at high concentrations are believed to transmit *E. coli* O157 to a greater number of cohorts than those that shed intermittently or at low concentrations *(20)*.

When a pen, lot, or truckload of cattle has a large proportion of high-shedding animals, it could be assumed that the overall fecal prevalence of *E. coli* O157 within that group of cattle and the probability of carcass contamination at slaughter will both be higher. However, the relationships between such effects have not been fully quantified despite previous studies addressing related issues *(9, 11, 20, 30)*. Quantifying associations among overall fecal shedding, high-shedding, and carcass contamination could enhance future risk assessments, surveillance, and research into potential mitigation strategies. Thus, our objectives were to quantify associations at slaughter between high-shedding animals, fecal-positive animals, and pre-evisceration carcass contamination within truckloads of finished cattle, and to quantify the effects associated with the probability of carcasses testing positive.

MATERIALS AND METHODS

Study population. We studied fifty truckloads of finished cattle (steers or heifers) from multiple sources that were slaughtered at a commercial abattoir in the Midwest U.S. Approximately 60 animals were slaughtered per hour at this abattoir. Samples were collected two or three days per week over a five week period in May – June 2007. We sampled a maximum of 32 animals per truckload based on an *a priori* calculation indicating this sample size would be sufficient to detect a positive load with five-percent prevalence within loads. In

loads having more than 32 animals, the first 32 animals were sampled, and in loads with less than 32 animals, all animals were sampled.

Sample collection. Fecal and carcass samples were matched within animals. Carcasses were swabbed using a Speci-Sponge (Nasco, Fort Atkinson, WI) soaked with 15 ml of buffered peptone water (BPW; Sigma-Aldrich, St. Louis, MO) and held with a nitrile glove (Kimberly-Clark, Roswell, GA). Prior to swabbing, excess BPW was expelled from the sponge by squeezing the sponge within the Speci-Sponge bag. Each carcass was swabbed after hide removal, but prior to evisceration or any postharvest interventions. The area (approximately 1,000 cm^2) on the right side of each carcass, from the tail head ventro-caudo-lateral over the rump and part of the round, was swabbed with the sponge using a back-and-forth motion. The sponge was then placed back into the Speci-Sponge bag containing BPW. After evisceration, feces were collected by a different member of the sampling team from intact rectums using a plastic spoon. The spoon and feces were then placed into a Whirl-pack bag (Nasco). Samples were transported in coolers with ice packs to the Kansas State University College of Veterinary Medicine Preharvest Food Safety Laboratory and stored under refrigeration (4°C) for processing within 48 h of collection.

Detection of *E. coli* O157. Carcass sponge samples were cultured for *E. coli* O157 using the procedure of Elder et al. *(11)* with minor modifications. Briefly, 90 ml of brilliant green bile broth 2% (BD, Sparks, MD) was added to each Speci-Sponge bag and then incubated for 10 h at 37°C. After enrichment, bags were shaken and 1 ml of broth was subjected to immunomagnetic separation (IMS) and plated onto sorbitol MacConkey agar (BD) containing cefixime (50 ng/ml)

and tellurite (2.5 µg/ml; CT-SMAC). Plates were incubated for 16 to 18 h at 37°C and then up to six non sorbitol-fermenting colonies were picked and streaked onto blood agar (Remel, Lenexa, KS) and incubated (37°C) overnight. Colonies from the blood agar were tested for indole production and latex agglutination of the O157 antigen (Oxoid Limited, Basingstoke, Hampshire, England). If these tests were positive, isolates were then tested by PCR for *eae, stx*1*, stx*2 and *hyl*A genes with the following assay conditions: an initial denaturation at 95°C for 3 min; 30 cycles of 95°C for 20 s, 58°C for 40 s, and 72°C for 90 s; and a final 5 min extension to completion at 72°C. Isolates were considered *E. coli* O157 if they had the *eae* and *hyl*A genes and at least one of the *stx* genes.

Fecal samples were removed from the Whirl-pack bags and approximately 1 g was added to a test tube containing 9 ml of gram-negative (GN) broth (BD, Franklin Lakes, NJ) containing cefixime (Sigma-Aldrich; 50 ng/ml), cefsulodin (Sigma-Aldrich; 10 µg/ml), and vancomycin (Sigma-Aldrich; 8 µg/ml; GNccv). Tubes were then vortexed for 1 min and the fecal slurry was streaked for isolation onto a CT-SMAC plate using a sterile bacterial loop. This direct plating technique, described by Sanderson et al. *(27)*, was used to detect animals shedding *E. coli* O157 at high levels ($> 5 \times 10^4$ CFU/g feces). Using this method, a fecal sample (animal) was considered to have high levels of *E. coli* O157 (high-shedder) when *E. coli* O157 was isolated and confirmed from the direct plate *(27)*. Test tubes containing the remaining fecal slurry were then incubated for 6 h at 37°C, vortexed, and 1 ml from each tube was subjected to IMS and plated onto a CT-SMAC plate. Isolation and identification of *E. coli* O157 from colonies on CT-SMAC from both procedures (direct and IMS) were performed as described above. Animals were considered high-shedders if *E. coli* O157 was isolated and identified by direct plating

(irrespective of IMS results); animals were considered fecal positive if *E. coli* O157 was isolated and identified by either direct plating or IMS.

Statistical analyses. Estimates of prevalence and 95% exact binomial confidence intervals were calculated for each outcome (carcass prevalence, fecal prevalence and high-shedder prevalence) using Microsoft® Office Excel 2003. At the truckload level, Spearman rank-order correlation coefficients (with Fisher's z transformation) were determined between the percentage of carcasses testing positive, the percentage of animals testing fecal positive, the percentage of high-shedding animals and the total number of animals in each truckload. Correlation coefficients, level of significance and 95% exact confidence intervals were determined using PROC CORR of SAS (Version 9.1; SAS Institute, Cary, NC).

Variables were created to represent the *E. coli* O157 status of carcasses, high-shedding, and feces of the penultimate (immediately preceding) carcass or animal, the antepenultimate (immediately preceding the penultimate) carcass or animal, and ensuing (immediately following) carcass or animal in the slaughter order within truckload. For appropriate carcasses (*i.e.*, beginning or end of a truckload), a null value was entered for these variables if there were no preceding or ensuing carcasses or animals within truckload. Another set of variables was created to represent proximity of the current carcass to the previous *E. coli* O157 positive carcass, previous high-shedding animal, and previous fecal-positive animal within truckload. These variables represented an ordinal response for the adjacent animal, two preceding, three preceding, four or more preceding, or no preceding positive tests. Linear contrasts (PROC GLIMMIX of SAS) were used to evaluate whether these variables impacted the probability of a

carcass testing positive in a linear manner and, if so, were treated as a continuous variable in other modeling procedures *(1)*.

Modeling procedures were performed using PROC GLIMMIX of SAS and included truckload as a random effect. Unconditional associations between the probability of carcass samples testing positive for *E. coli* O157 and other variables of interest were identified by logistic regression. Variables associated with the *E. coli* O157 status of carcass samples during this initial screening ($P < 0.2$) were added (manually) into a multivariate logistic regression model one-by-one in a forward selection process with a selection criterion of $P < 0.05$ *(10)*. To address potential collinearity, associations among factors associated with outcomes based on screening were examined, and factors were chosen for model inclusion based on the strength of association with the outcome variable. After the forward selection process was completed, remaining variables were re-offered one-by-one into the model. Once the final main effects were determined, interaction terms were assessed *(10)*.

RESULTS

In this study, we collected carcass samples from 1,503 cattle arriving in 50 truckloads. Eight fecal samples distributed among six loads were missing because rectums were void of feces. Overall, *E. coli* O157 was isolated from 39 of 1,503 (2.6%; CI 1.9 – 3.5%) carcass samples and 127 of 1,495 (8.5%; CI 7.1 – 10.0%) fecal samples (Table 1). Of the 127 fecal-positive animals, 55 (43.3%; CI 34.5 – 52.4%) were considered high-shedders. At the truckload level, at least one carcass tested positive in 15 of 50 (30.0%; CI 17.9 - 44.6%) loads. Thirty-seven of 50 (74.0%; CI 59.7 – 85.4%) loads had at least one fecal-positive animal and 26 of 50 (52.0%; CI 37.4 – 66.3%) loads had at least one high-shedding animal. Within truckload,

prevalence of carcass positives, fecal positives and high-shedding animals was 2.5%, 8.6% and 3.7%, respectively (Table 1).

The percentage of carcasses within truckload testing positive for *E. coli* O157 was significantly (*P*-values < 0.01) correlated with the percentage of fecal-positive cattle (r_s = 0.61; CI 0.40 – 0.76) and the percentage of high-shedding cattle (r_s = 0.68; CI 0.50 – 0.81). Additionally, the percentage of high-shedding cattle was correlated (r_s = 0.82; CI 0.71 – 0.90) with the percentage of fecal-positive cattle. The total number of animals on each truckload was not correlated (*P*-values > 0.15) with the percentage of carcasses testing positive (r_s = 0.20), the percentage of fecal-positive cattle (r_s = 0.04), or the percentage of high-shedding cattle (r_s = 0.11).

The proximity of the previous positive carcass, previous high-shedding animal, and previous fecal-positive animal to the current carcass of interest were all linearly associated (*P values* < 0.01) with the current carcass testing positive for *E. coli* O157 (Figure 1). Therefore, in subsequent analyses, these variables were modeled as continuous variables (Agresti, 1996). All variables evaluated were positively associated (OR > 1.0) with the probability of a carcass testing positive for *E. coli* O157, but the ensuing animal being fecal positive or the ensuing animal being a high-shedding animal did not meet the screening criteria (*P* < 0.2) and thus were not used when determining the multivariate model (Table 2). Many of the variables also were highly associated with each other (data not shown). The final multivariate model included three variables: proximity to the previous positive carcass, proximity to the previous high-shedding animal, and the *E. coli* O157 status of the ensuing carcass. All of these variables were positively associated with the probability of a carcass testing positive (Table 3). Using parameter estimates from the final model, a figure was constructed to illustrate how variables in the final model

impacted the probability of a carcass testing positive for *E. coli* O157 (Figure 2). The probability of a carcass testing positive increased in a linear manner with decreasing proximity to the previous carcass and high-shedder positive, and for any given level of these variables, the probability was greater if the ensuing carcass was positive. The overall probability was dependent upon all three effects and ranged from 0.04 to 0.87.

DISCUSSION

In this study, we quantified associations between high-shedding animals, fecal-positive animals, and pre-evisceration carcass contamination within truckloads of finished cattle at slaughter. We used truckload as the hierarchical unit for a group of animals instead of farm, lot, or pen, as in previous studies on *E. coli* O157 prevalence at slaughter *(3, 5, 11, 12)*. Often there are multiple truckloads from pens or lots of cattle, and transportation has been shown to increase the prevalence of *E. coli* O157 on cattle hides *(3)*. Therefore, we felt truckload was an appropriate measure for grouping cattle to assess prevalence and associations at harvest. Although the total number of cattle within truckloads varied, commingling of animals during transport was likely equivalent in large and small loads because large livestock trailers contain multiple compartments which segregate animals into smaller groups. The lack of significant correlations among prevalence estimates (fecal, carcass, high-shedder) and the number of animals in truckloads provides evidence that load size did not impact *E. coli* O157 prevalence. At the truckload level, prevalence of at least one positive pre-evisceration, pre-intervention carcass (30%) was slightly lower than previous reports on lots of cattle [87% of lots *(11)*, 47% of lots *(30)*]. However, it is difficult to directly compare prevalence estimates among studies due to potential differences in sampling strategies, microbial methods, study populations, and several

other factors that can impact point-in-time estimates of prevalence *(24)*. Nevertheless, we did find that our results for prevalence of truckloads with at least one positive fecal sample (74%) were similar to previous observations on lots of slaughtered cattle [72% of lots *(11)*, 73% of lots *(30)*].

At the individual animal level, we found an overall prevalence of *E. coli* O157 in fecal samples (9%) that was consistent with previous estimates at slaughter, which have ranged from 6 to 28% *(5, 11, 13, 19, 21)*. The method we used to identify high-shedding animals may misclassify individuals on occasion as the reported sensitivity and specificity for identifying samples with greater than 5×10^4 CFU/g of *E. coli* O157 are 82.6% and 92.3% respectively *(27)*. However, the prevalence of high-shedding animals in the current study was approximately 3.7%, which was equivalent to an estimate from a previous study in cattle at slaughter where a different direct plating technique was utilized (3.7%) *(19)*. Estimates of carcass prevalence prior to evisceration in the current study (approximately 3%) were lower than previously reported estimates of 10% *(3)*, 10% *(30)*, 15% *(4)*, 27% (*5*), and 43% *(11)*. Although direct comparisons are again difficult, one possible explanation for the lower prevalence may be differences in the site and total area of the carcass that was swabbed. We swabbed an area of approximately 1,000 cm^2 in one location on the carcass, whereas previous studies sampled multiple locations *(5, 11, 30)* or larger areas *(4)*. It is important to note that the studied abattoir employed multiple interventions to reduce bacterial contamination of carcasses in the production line after evisceration, and we only sampled carcasses prior to evisceration and interventions.

Given recent evidence that animals shedding high levels of *E. coli* O157 in feces can increase overall fecal prevalence and potentially carcass prevalence, the primary intent of our study was to quantify the relationships between carcass prevalence, and overall fecal and high-

shedder prevalence at harvest. Elder et al. *(11)* reported strong correlations (r_s = 0.58; P = 0.001) between carcass prevalence and preharvest prevalence (where fecal and hide results were interpreted in parallel). We also found strong correlations, both between high-shedder prevalence and carcass prevalence (r_s = 0.68), and between overall fecal prevalence and carcass prevalence (r_s = 0.61). Cobbold et al. *(9)* found that feedlot pens with a high-shedding animal had a higher pen prevalence of *E. coli* O157 compared to pens without a high-shedding animal. This is consistent with modeling efforts by Matthews et al. *(20)* who suggested that the transmission of *E. coli* O157 in cattle populations is primarily due to a few animals that are shedding at high levels. Additionally, Fegan et al. *(13)* sampled four unrelated groups of cattle at slaughter and isolated *E. coli* O157 from carcasses in only one group – the one with the highest count of *E. coli* O157 in feces and the highest fecal prevalence. Because high-shedder prevalence was associated with overall fecal prevalence, and animals in our study that were positive by the direct plating (high-shedder) method were considered fecal-positive, obvious collinearity existed between these measures as evidenced by the strong correlation between them (r_s = 0.82). Although the correlation between high-shedder prevalence and carcass prevalence was slightly stronger than the correlation between fecal prevalence and carcass prevalence, the broad overlap of confidence intervals indicate these measures were not different.

To further quantify the factors associated with the probability of a carcass testing positive, we used more precise variables and multivariate statistical methods. We found that several variables were unconditionally associated with carcass and fecal status of *E. coli* O157 at harvest, including relationships between outcomes measured on previous, current and subsequent animals in the slaughter order (Table 2). Obvious collinearity existed between some of the variables evaluated (data not shown) merely because they represented similar information (*e.g.*,

proximity to previous fecal-positive animal, and penultimate and antepenultimate animal being fecal positive). The variables that best explained the variability in carcass contamination within a truckload of cattle were the preceding carcasses being positive, preceding animals being high-shedders, and the ensuing carcass being positive (Table 3). Parameter estimates in the multivariate model were largest for the two carcass variables, which indicates that the probability of a pre-evisceration carcass testing positive for *E. coli* O157 was much higher when other nearby carcasses were contaminated. If carcasses are contaminated by a common source, it is likely to result in groups of consecutive positive carcasses *(13)*. Presence of a high-shedding animal previous to the current carcass may serve as the source for contamination, which then may be perpetuated down the production line *(7, 8)*. However, Barkocy-Gallagher et al. *(6)* did not observe discernable evidence of cross-contamination within lots of cattle when comparing genomic fingerprints of *E. coli* O157 isolates; though they did not evaluate adjacent carcasses or match pre- and postharvest samples to the same animal. Foodborne pathogens also may become aerosolized during the slaughter process (*29*) and subsequently contaminate other carcasses. If aerosolization of the pathogen did occur, it would likely occur during mechanical hide removal *(29)*, and thus preceding carcasses may be more likely to be exposed and contaminated then the carcasses of ensuing animals. However, this logic is not supported by data from our study as we found that the ensuing animal being a high-shedder was not associated with carcasses testing positive. Unfortunately we were unable to sample hides in this study, which may have allowed further insight into potential modes of transmission at slaughter.

In conclusion, we found that pre-evisceration carcass prevalence was highly correlated with overall fecal and high-shedder prevalence within truckloads of finished cattle. In addition, we quantified the effect of factors significantly associated with the probability of a carcass

testing positive for *E. coli* O157. The probability of a pre-evisceration carcass testing positive varied between 0.04 and 0.87 depending on the proximity to a previous positive carcass, proximity to a previous high-shedding animal, and the *E. coli* O157 status of the ensuing carcass.

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TABLE 3.1 *Descriptive statistics for samples collected and cultured for* Escherichia coli *O157*

Cumulative Results	Overall	95% Confidence Intervals
Number of cattle sampled	1,503	-
Number of truckloads sampled	50	-
Pre-evisceration carcass prevalence, %	2.6	1.9 – 3.5
High-shedder prevalence, %	3.7	2.8 – 4.8
Fecal prevalence, %	8.5	7.1 – 10.0

Results Within Truckloads	Mean	Standard deviation	Median	Range
Number of cattle	40.0	9.1	44.5	19 – 49
Number of cattle sampled	30.1	3.8	32.0	19 – 32
Pre-evisceration carcass prevalence, %	2.5	5.1	0.0	0.0 – 25.0
High-shedder prevalence, %	3.7	5.5	3.1	0.0 – 22.7
Fecal prevalence, %	8.6	10.6	3.7	0.0 – 58.1

TABLE 3.2 *Independent variables unconditionally associated with the isolation of* Escherichia coli *O157 from pre-evisceration carcass swabs*[a]

Variable, outcome	*P* value	Odds ratio (95% CI)
Animal being a high shedder, Yes or No	0.13	2.3 (0.8 – 6.5)
Penultimate[b] animal (within load) being a high-shedder, Yes or No	< 0.01	3.8 (1.5 – 9.8)
Antepenultimate[b] animal (within load) being a high-shedder, Yes or No	< 0.01	3.9 (1.5 – 10.1)
Proximity to previous high-shedding animal (within load)[c]	< 0.01	1.7 (1.3 – 2.1)
Ensuing animal (within load) being a high-shedder, Yes or No	0.44	1.6 (0.5 – 4.9)
Animal being fecal positive, Yes or No	0.07	2.1 (0.9 - 4.9)
Penultimate[b] animal (within load) being fecal positive, Yes or No	< 0.01	2.9 (1.3 – 6.4)
Antepenultimate[b] animal (within load) being fecal positive, Yes or No	< 0.01	3.6 (1.7 – 7.7)
Proximity to previous fecal positive (within load)[c]	< 0.01	1.5 (1.3 – 1.9)
Ensuing animal (within load) being fecal positive, Yes or No	0.21	1.7 (0.7 – 4.0)
Penultimate[b] carcass (within load) being positive, Yes or No	0.06	2.5 (1.0 – 6.6)
Antepenultimate[b] carcass (within load) being positive, Yes or No	0.05	2.7 (1.0 – 7.0)
Proximity to previous carcass positive (within load)[c]	< 0.01	1.5 (1.2 – 1.9)
Ensuing carcass (within load) being positive, Yes or No	0.06	2.5 (1.0 – 6.6)

[a] Analysis was performed with truckload as a random effect; "no" was used as the reference outcome for all "yes or no" outcomes.

[b] Penultimate and antepenultimate represent the carcass or animal immediately preceding or two preceding the carcass of interest, respectively.

[c] Odds ratio represents the increase in the odds of being carcass positive by decreasing this variable by 1 unit. Outcomes were classified as none (no previous positives), or as a positive 4 or more units preceding, 3 units preceding, 2 units preceding or immediately preceding.

TABLE 3.3 *Multivariate associations with carcasses testing positive for* Escherichia coli *O157*[1]

Variable, units or outcome	β	S.E.	*P* value[b]	Odds ratio (95% CI)
Intercept	-3.20	0.57	-	-
Proximity to previous high-shedding animal (within load)[c], 1 animal	0.47	0.12	< 0.01	1.6 (1.3 – 2.1)
Proximity to previous carcass positive (within load)[c], 1 carcass	0.52	0.12	< 0.01	1.7 (1.3 – 2.1)
Ensuing carcass (within load) being positive, Yes or No [d]	1.19	0.54	0.03	3.3 (1.1 – 9.4)

[a] Model included truckload as a random effect (parameter estimate = 0.003 and S.E. = 0.38)

[b] Type III *F*-statistic.

[c] Odds ratios represent the increase in odds of being carcass positive by decreasing the independent variable by 1 unit, where units were none (no previous positives), a positive 4 or more preceding, 3 preceding, 2 preceding or immediately preceding the carcass of interest.

[d] "No" was used as the reference outcome.

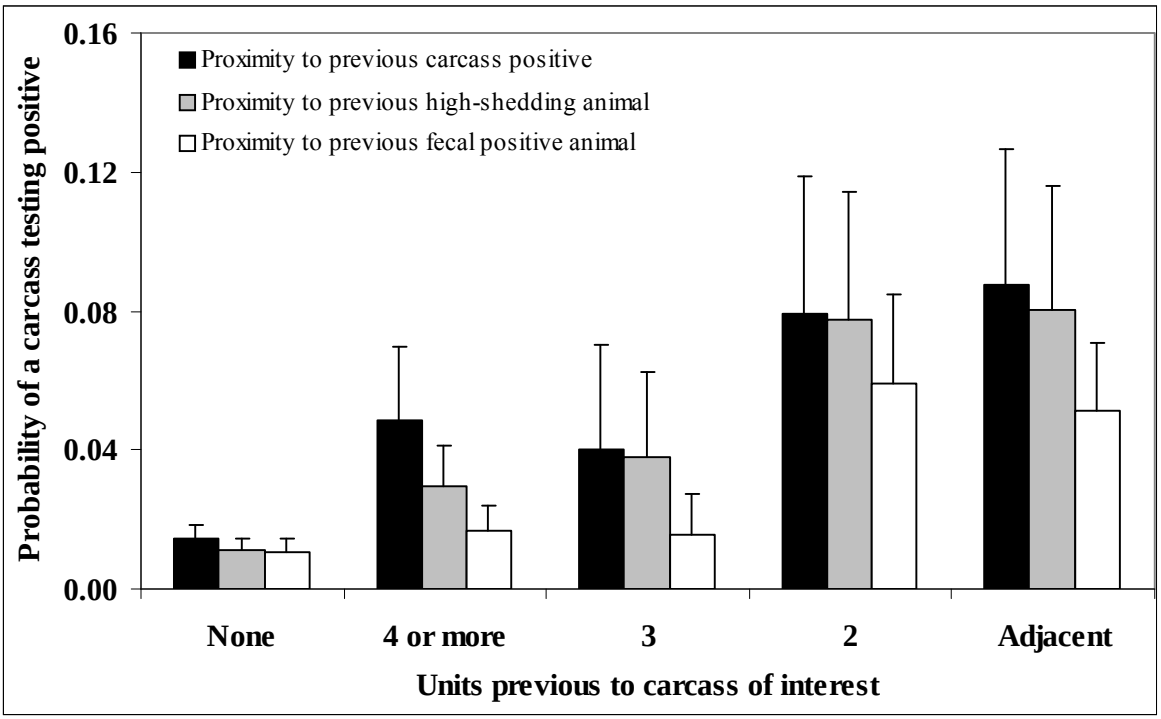

FIGURE 3.1 *Unconditional probability of isolating* Escherichia coli *O157 from carcass swab samples given the relative proximity to a previous positive carcass (black bars), high-shedding animal (grey bars), or fecal-positive animal (white bars). Probabilities were calculated using parameter estimates from logistic regression models utilizing truckload as a random effect. Error bars represent the standard error of probability estimates.*

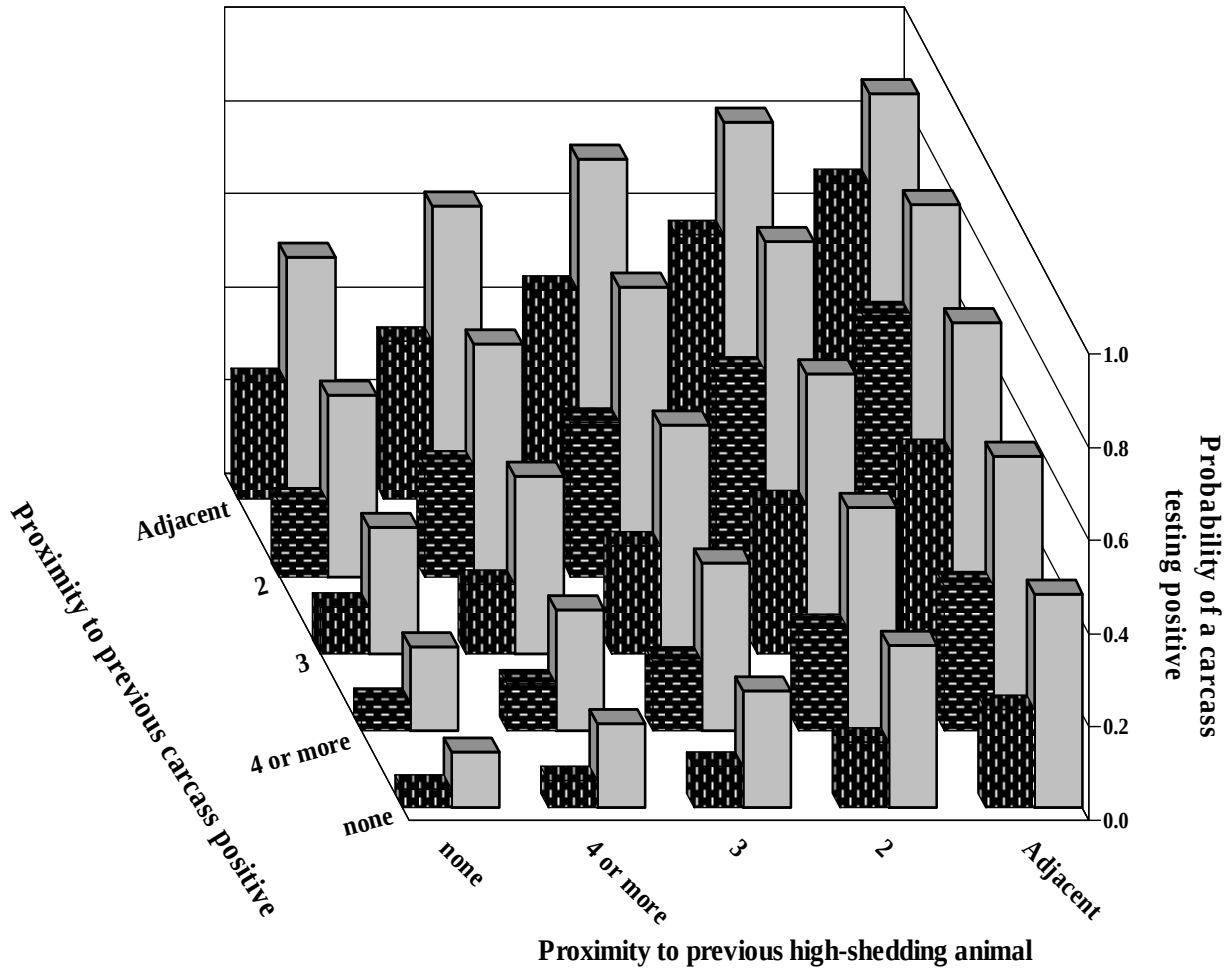

FIGURE 3.2 *Predicted probabilities of a pre-evisceration carcass swab testing positive for* Escherichia coli *O157 based on estimates from a multivariate logistic regression model. Hatched bars represent estimates if the ensuing carcass sample was negative for* E. coli *O157 and light bars represent estimates if the ensuing carcass sample was positive. The "none" designation represents the situation where no other positives were detected from previous samples within the sampled truckload.*

CHAPTER 4 - Effects of mucin and its carbohydrate constituents on *Escherichia coli* O157 in batch culture fermentations with ruminal or fecal microbial inoculum

In cattle, *Escherichia coli* O157 generally persists in the hindgut more often than in the rumen. We hypothesize that substrates in this region, particularly those contained in intestinal mucus, may offer a preferential energy source for *E. coli* O157. Therefore, our objective was to test the effects of mucin and its carbohydrate constituents on *in vitro* growth of *E. coli* O157 in ruminal or fecal microbial fermentation. Ruminal contents and feces were collected from a ruminally-cannulated donor steer fed a corn grain-based finishing diet. Ruminal contents were strained through 2 layers of cheesecloth, incubated at 39°C for 1 h and the floating hay mat was removed with a vacuum suction and the remaining was utilized as rumen microbial inoculum. Feces were suspended in physiologic saline to increase fluidity, blended and strained through two layers of cheesecloth. The resulting fluid was utilized as fecal microbial inocula. Fermentations (50 ml) were performed in serum bottles with a 2:1 mineral buffer to microbial inoculum ratio. Substrates (fucose, galactose, mannose, gluconic acid, galacturonic acid, glucuronic acid, glucuronic acid, gastric mucin, galactosamine, and glucosamine) were added at 500 mg/bottle. A mixture of five strains of nalidixic acid resistant *E. coli* O157 strains was added to each fermentation and concentrations were determined at 0, 6, 12 and 24 h of incubation. In ruminal fermentations, fucose, mannose, glucuronic acid, galacturonic acid, glucosamine, galactosamine and gastric mucin had no effect on *E. coli* O157 concentration compared to the control (no substrate added) fermentation. However, gluconic acid inclusion as a substrate increased *E. coli* O157 concentration at 24 h. In fecal fermentations, mannose,

galactose, gluconic acid, glucuronic acid, galacturonic acid, glucosamine and gastric mucin increased *E. coli* O157 growth compared to control at 24 h, while galactosamine and fucose did not. Gluconic acid was the most stimulatory substrate, increasing *E. coli* O157 by more than 1.0 log in ruminal fermentations and 2.0 log in fecal fermentations. In summary, availability of mucus constituents, particularly gluconic acid, may explain the higher prevalence of *E. coli* O157 in the hindgut compared to the rumen of the digestive tract.

Key words: *E. coli* O157, *in vitro* fermentation, growth, mucus, carbohydrate constituents

INTRODUCTION

Preharvest control of foodborne pathogens, such as *E. coli* O157, is important to both consumers and producers of food of animal or plant origin. *Escherichia coli* O157:H7 is a specific serotype of enterohemorrhagic *E. coli*, and illness caused by this pathogen is generally characterized by abdominal pain and hemorrhagic colitis (US FDA, 2001). In children and the elderly, *E. coli* O157 can cause a more severe form of the disease.

In cattle, a major reservoir, *E. coli* O157 is more prevalent in digesta of the lower gastrointestinal tract (cecum, colon, and rectum) compared to the rumen (Grauke et al., 2002; Naylor et al., 2003; Van Baale et al., 2004). Apparently, the conditions in the hindgut compared to the rumen, are more hospitable to the survival and growth of *E. coli* O157 (Fox et al., 2007). It is also possible that the lower gastrointestinal tract may provide substrates that selectively serve as nutrients for the growth of *E. coli* O157. Components of gastrointestinal mucus (fucose, galactose, mannose, N-acetylgalactosamine, N-aceylglucosamine, galacturonic acid, glucuronic acid, and gluconic acid) are known to perpetuate colonization of *E. coli* in the mouse intestine and stimulate *in vitro* growth of *E. coli* (Peekhaus and Conway, 1998; Montagne et al., 2000; Chang et al., 2004). Therefore, it was suggested that components of mucus secretions may serve as substrates or growth factors for *E. coli* O157 (Miranda et al., 2004).

In vitro fermentation with ruminal fluid or fecal inoculum historically has been used to assess gut microbial activities and interactions, and digestibility of specific substrates (El Shaer et al., 1987; Mould et al., 2005; Varadyova et al., 2005). Ruminal fluid inoculum also has been used to evaluate factors that impact *in vitro* growth and survival of *E. coli* O157 (Diez-Gonzales and Russell, 1997; Bach et al., 2003; Edrington et al., 2003; Annamalai et al., 2004; Edrington et al., 2006). Our objectives were to conduct *in vitro* ruminal or fecal microbial fermentation to

determine whether mucin or carbohydrate constituents of mucus influence *E. coli* O157 growth and assess how fermentation pH and volatile fatty acid concentrations may be related to *E. coli* O157 concentration.

MATERIALS AND METHODS

Escherichia coli O157 Inoculum Preparation

A mixture of five strains (01-2-1863, 01-2-7443, 01-2-10004, 01-2-10530, and 01-2-12329) of *E. coli* O157 was used for *in vitro* fermentation studies. These strains were isolated from feedlot pen fecal samples (Sargeant et al., 2003). The strains were made resistant to nalidixic acid (50 μg/ml; Nal^R) in the laboratory and stored in protect beads (CryoCare™, Key Scientific Products, Round Rock, TX) at -80°C. The major virulence genes (*eae, fliC, stx1* and *stx2)* and the genetic relatedness of the five isolates were determined by multiplex PCR (Fagan et al., 1999) and pulsed-field gel electrophoresis (Sargeant et al., 2006), respectively. Strains were considered distinct types based on differences in more than two bands (< 95% Dice similarity; Sargeant et al., 2006). To prepare the inoculum for the *in vitro* fermentation experiments, isolates of each strain from protect beads were streaked onto blood agar and incubated for approximately 16 h at 37°C. An individual colony of each strain was picked and inoculated into a separate bottle containing 100 ml Tryptic soy broth (TSB; Becton Dickinson and Co., Sparks, MD). Bottles were vortexed and incubated at 37°C for 18 h. After incubation, sub-samples (1 ml) from each bottle were pooled and vortexed to obtain the 5-strain mixture of Nal^R *E. coli* O157 for *in vitro* fermentations. The batch culture fermentations with ruminal or fecal microbial inoculum were performed three times with inocula collected and prepared for each replication.

Preparation of Ruminal and Fecal Microbial Inocula

Ruminal fluid and feces were collected from a ruminally-cannulated steer fed a high-grain diet consisting primarily of steam-flaked corn and alfalfa hay. The steer was housed at the Kansas State University Beef Cattle Research Center. Ruminal fluid was strained through 2 layers of cheesecloth to remove large feed particles and placed into a flask and capped with a butyl rubber stopper fitted with a Bunsen valve. Feces were collected via rectal palpation from the same steer and placed into a Whirl-Pak bag (Nasco, Ft. Atkinson, WI). Ruminal fluid and feces were then transported (approximately 5 km) to the Preharvest Food Safety Lab. Upon arrival, ruminal fluid was incubated for 1 h at 39°C and then the floating hay mat/foam fraction was removed by vacuum suction. Because the dry-matter content of feces was greater than that of ruminal fluid, Ringer's solution was added to the feces (6.0 ml/g) to prepare a fecal suspension (Mould et al., 2005). The resulting fecal slurry was then blended anaerobically in a Waring blender for 1 min and strained through 2 layers of cheesecloth to remove large particles and used as the fecal microbial inoculum.

In vitro fermentations

Batch culture fermentations were set up in 60-ml serum bottles (Wheaton Science Products, Millville, NJ) capped with butyl rubber stoppers fitted with Bunsen valves. Each bottle contained 50 ml of fermentation mixture composed of 33 ml of McDougal's buffer (McDougall, 1948) and 17 ml of the ruminal fluid or fecal microbial inoculum. The buffer and the microbial inoculum were added under a stream of flowing oxygen-free CO_2 gas (Hungate, 1966) to create and maintain an anaerobic environment within the bottles. Bottles were

numbered 1 to 40 and treatments were assigned in a completely randomized design with a 10 x 2 factorial treatment arrangement. Factor 1 was the type of substrate used and factor 2 was the inoculum (ruminal or fecal microbial inoculum). Substrates evaluated were added at 500 mg per bottle (50 ml) and consisted of control (no substrate), gluconic acid (Sigma-Aldrich, St. Louis, MO; product no. G9005), glucuronic acid (Sigma-Aldrich, product no. G8645), galacturonic acid (Sigma-Aldrich, product no. 48280), fucose (Sigma-Aldrich, product no. F2252), galactose (Sigma-Aldrich, product no. G0750), mannose (Sigma-Aldrich, product no. M6020), galactosamine (Sigma-Aldrich, product no. G0500), glucosamine (Sigma-Aldrich, product no. G4875) and commercially available gastric mucin (Sigma-Aldrich, product no. M2378). The 5 strain mixture of *Nal*^{R} *E. coli* O157, prepared as described above, was diluted 1,000-fold in buffered peptone water (Sigma-Aldrich) and 100 µl was inoculated into each fermentation. Serum bottles were incubated in an orbital shaking incubator (Gallenkamp, Leicester, UK) set at 70 rpm and 39°C.

Fermentation Sample Collection

Samples were collected at 0, 6, 12 and 24 h to determine fermentation pH, and concentrations of *Nal*^{R} *E. coli* O157 and volatile fatty acids (VFA). At each sampling time, bottles were swirled by hand and stoppers removed to place bottles under the flow of O_2-free CO_2 during removal of sample. An aliquot (100 µl) of each fermentation sample was pipetted into a 96-well (2.0 ml well capacity) assay block (Corning Inc., Corning, NY) for enumeration of *Nal*^{R} *E. coli* O157. Another aliquot of each fermentation sample (1 ml) was placed into a 5-ml vial containing 3 ml 25% metaphosphoric acid (Erwin et al., 1961). Vials were capped and inverted to mix the acidified sample and then frozen at -20 C for later VFA analysis.

Fermentation pH of each sample was recorded immediately with An ACCUMET model AR 10 pH meter (Fisher Scientific International, Pittsburgh, PA). The pH probe was placed in a 70% isopropyl alcohol solution for a minimum of 10 s and then rinsed in distilled water after each sample pH was measured.

Determination of Nal^R E. coli O157 Concentration

Serial dilutions of each sample were made in a 96-well assay block by transferring 100 µl into 0.9 ml buffered peptone water (Sigma-Aldrich). Typically, for 0 hr samples two ten-fold dilutions (10^{-1} and 10^{-2}) were performed and 100 µl of the original sample (10^{0}), and 10^{-1} and 10^{-2} dilutions were spread plated onto sorbitol MacConkey agar (Becton, Dickinson and Co.,) supplemented with cefixime (50 ng/ml), potassium tellurite (2.5 µg/ml) and nalidixic acid (50 µg/ml; CT-SMACnal). Six h samples were diluted to 10^{-4} and 100 µl of 10^{-1} through 10^{-4} dilutions were spread plated onto CT-SMACnal. Samples obtained at 12 and 24 h were diluted to 10^{-5} and 100 µl of 10^{-1} through 10^{-5} dilutions were spread plated onto CT-SMACnal. All dilutions were plated in triplicates and incubated at 37°C for 18 to 24 h. After incubation, sorbitol-negative colonies were counted to determine concentrations (CFU) per ml of fermentation (Van Baale et al., 2004) and concentrations were log (base 10) transformed for data analyses.

Volatile Fatty Acid Analyses

Concentrations of VFA in fermentation samples were determined using gas chromatography (Erwin et al., 1961). Only the major VFA (acetate, propionate, and butyrate) and their sum, reported as total VFA concentration, were used in the data analyses.

Statistical Analysis

Data from ruminal and fecal microbial fermentations were analyzed separately. All statistical analyses were performed with SAS Version 9.1 (SAS Institute, Cary, NC). The concentrations of $\log_{10}$ *Nal*^{R} *E. coli* O157, fermentation pH, and VFA concentrations were analyzed as repeated measures over time with PROC MIXED and included main effects of treatment (substrate) and sampling h, the interaction between treatment and h, and the random effect of replication of experiment. Least squares means were used to determine the level of significance between control and substrate treatments. Pearson correlation coefficients between *Nal*^{R} *E. coli* O157 concentrations, pH values, and VFA concentrations were determined by modeling these as dependent variables with treatment as an independent variable using PROC GLM, MANOVA/PRINTE option. Treatment was included when determining these correlation coefficients to assess correlations independent of treatment effects.

RESULTS

All five nalidixic acid adapted strains used in the study were positive for *eae* and *fliC* and except for one strain were negative for *stx*1 and positive for *Stx*2 genes (Figure 1). The *stx*1 positive strain (01-2-10530) was negative for the *stx*2 gene. The PFGE banding patterns indicated that all five strains were of distinct genetic types with < 95% Dice similarity (Figure 1).

Ruminal Fluid Fermentations

Treatment (substrate), sampling h, and the treatment × h interaction were significant ($P <$ 0.001) for *Nal*^{R} *E. coli* O157 concentration, fermentation pH, and VFA concentrations. Figure

2A, B, and C show the mean concentrations of *Nal*^{R} *E. coli* O157 for control and fermentations of all substrates with ruminal microbial inoculum over the 24-h incubation period. The mean concentrations of *Nal*^{R} *E. coli* O157 at 0 h (overall mean = 2.8 $\log_{10}$ CFU/ml) were similar across all treatments ($P > 0.20$). In control (no substrate) fermentations with ruminal microbial inoculum, mean and standard error of *Nal*^{R} *E. coli* O157 at 0, 6, 12 and 24 h of fermentation were 2.9, 2.7, 3.0 and 3.4 ± 0.3 $\log_{10}$ CFU/ml, respectively, and sampling h was a significant effect ($P < 0.01$) for *Nal*^{R} *E. coli* O157 concentration. At 12 h of fermentation, the concentration of *Nal*^{R} *E. coli* O157 in the control was not different ($P > 0.05$) from fermentations with mucin or carbohydrate constituents of mucin (Table 1). At 24 h of fermentation, the mean concentration of *Nal*^{R} *E. coli* O157 in fermentations with galactose was lower than the control and the mean concentration in gluconic acid fermentation was higher ($P < 0.01$) than the control and all other substrates except glucuronic acid (Table 1). Addition of fucose, mannose, galactosamine, gastric mucin, galactosamine or glucosamine as substrate had no effect on the *in vitro* growth of *E. coli* O157 during fermentation for 24 h.

Mean and standard error of pH in control fermentations with ruminal microbial inoculum at 0, 6, 12 and 24 h of incubation were 6.59, 7.03, 6.96 and 6.99 ± 0.09, respectively. Fermentation pH at 0 h was lower ($P < 0.01$) in fermentations with galacturonic acid compared to all other treatments except glucosamine. Changes in fermentation pH were evident over the 24-h fermentation period and were contingent upon the substrate (Figure 2D, E, and F). Compared to the control (no substrate), fermentation pH at 12 and 24 h were lower ($P < 0.01$) with fucose, galactose, mannose, galacturonic acid or glucosamine as the substrate (Table 1). Fermentation pH did not correlate ($P > 0.20$) with *Nal*^{R} *E. coli* O157 concentration in ruminal microbial fermentation (Table 2).

The mean and standard error of total VFA (acetate + propionate + butyrate) concentration at 0, 12 and 24 h were 26.3, 48.5 and 60.9 ± 5.7 m*M*, respectively, in ruminal microbial fermentations. Mean initial (0 h) total VFA concentrations were similar ($P > 0.20$) for all substrates and control fermentations. There were marked differences in mean concentrations of acetate, propionate, butyrate, and total VFA across substrates after 24 h of fermentation in ruminal microbial inoculum (Table 3). Acetate, butyrate and Total VFA concentrations at 24 h were greater for all substrate fermentations compared to the control, with the exception of galactosamine. In the case of propionate, concentrations for all substrates were higher than the control, except for galacturonic and galactosamine. Among VFA, only propionate concentration was positively correlated ($P = 0.05$) with *Nal*^{R} *E. coli* O157 concentrations at 12 h, but not at 24 h. No other significant correlations were observed in fermentations with ruminal microbial inoculum (Table 2).

Fecal Microbial Fermentation

In fecal microbial fermentations, substrate (treatment), sampling h and the treatment × h interaction were significant ($P < 0.001$) for fermentation pH and VFA concentrations, but the treatment × h interaction only tended ($P = 0.06$) to have an effect on *Nal*^{R} *E. coli* O157 concentration. However, substrate and h had significant effects ($P < 0.001$) on *Nal*^{R} *E. coli* O157 concentration. The concentrations of *Nal*^{R} *E. coli* O157 among treatments over the 24-h fermentation period in fecal microbial inoculum are shown in Figure 3. At 0, 6, 12 and 24 h, mean and standard error of *Nal*^{R} *E. coli* O157 concentrations in control fermentations were 2.8, 3.3, 2.9 and 2.75 ± 0.6 $\log_{10}$ CFU/ml, respectively. At 12 h, the concentrations of *Nal*^{R} *E. coli* O157 were greater for all substrates, except fucose, mannose, and galactosamine, than control (no substrate) (Table 1). Fucose and galactosamine fermentations remained statistically similar

to control fermentations even at 24 h with regard to *Nal*^{R} *E. coli* O157 concentrations while all other substrates yielded higher concentrations at the end of the fermentation period (Table 1). The concentration of *Nal*^{R} *E. coli* O157 in fermentations supplemented with gluconic acid was 1.5 and 2.0 $\log_{10}$ CFU/ml higher than control fermentations at 12 and 24 h, respectively.

In fecal microbial fermentations, mean pH and standard error at 0, 6, 12 and 24 h were 6.64, 7.13, 7.04 and 7.08 ± 0.06, respectively, in control fermentations. Interestingly, addition of galactosamine or galacturonic acid as the substrate resulted in lower ($P < 0.01$) mean fermentation pH at h 0 compared to control (Figure 3). Fermentation pH was lower in all treatments compared to control at 12 and 24 h (Table 1). Twelve-hour fermentation pH was positively correlated with 12 and 24 h *Nal*^{R} *E. coli* O157 concentrations, but 24-h fermentation pH was not correlated with *Nal*^{R} *E. coli* O157 concentration at 12 or 24 h (Table 2).

Mean total VFA concentrations at h 0 were similar ($P > 0.20$) across all substrates and control in fecal microbial fermentation. For control fermentations, the mean and standard error of 0, 12, and 24 h total VFA concentrations were 4.9, 10.1, and 11.9 ± 3.8 m*M*, respectively. Acetate and total VFA concentrations at 24 h were greater for all substrate fermentations compared to control (Table 3). However, propionate concentrations with galactosamine fermentations were similar to control ($P > 0.05$) and butyrate concentration with fucose fermentations also was similar to control. The concentrations of acetate and total VFA were negatively correlated with the concentration of *Nal*^{R} *E. coli* O157 (Table 2). Initial (0 h) VFA concentrations were not correlated ($P > 0.05$) with fermentation pH. At 12 h, acetate (r = -0.51), propionate (r = -0.66), butyrate (r = -0.27) and total VFA concentrations (r = -0.66) were negatively correlated ($P < 0.05$) with fermentation pH, but 24-h VFA concentration was not correlated ($P > 0.20$) with 24-h fermentation pH (data not shown).

DISCUSSION

Many food-producing animals are normal carriers of *E. coli* O157 (Beutin et al., 1993; Hancock et al., 1998; Zschock et al., 2000). The objective of this study was to evaluate whether mucin and its carbohydrate constituents may give *E. coli* O157 a selective advantage in the hindgut compared to the rumen of beef cattle. This may explain why the organism is able to establish and persist in the hindgut, but not in the rumen (Rasmussen et al., 1993; Brown et al., 1997; Grauke et al., 2002; Laven et al., 2003; Van Baale et al., 2004). Availability of dietary substrates in the hindgut for microbial fermentation is limited due to the vast microbial population in the rumen that depletes many of the sugars and nitrogen compounds in cattle diets. This reduction in substrates is advantageous to *E. coli* O157 because potentially inhibitory fermentative products will not be produced in large quantities in the hindgut. Organic acids (common fermentative products) are known to reduce viability and increase acid resistance of *E. coli* O157 (Diez-Gonzales and Russell, 1997; Jordan et al., 1999; Shin et al., 2002). The substrates evaluated in this study are components of mucus located either in the intestine (fucose, galactose, mannose, galactosamine and glucosamine; Montagne et al., 2000) or cecum (gluconic acid, glucuronic acid and galacturonic acid; Peekhaus and Conway, 1998). The D-isomers of gluconate, glucuronate, and galacturonate can be metabolized via the Entner-Duodoroff pathway of *E. coli* (Peekhaus and Conway, 1998). Additionally, fucose, glucuronic acid and galactose are components of colanic acid, an exopolysaccharide of *E. coli* O157, which enables the pathogen to survive under acidic conditions (Mao et al., 2006). To evaluate the influence of these compounds, we utilized an *in vitro* batch culture fermentation system with an initial *E. coli* O157 concentration of about 3.0 $\log_{10}$ CFU/ml. The fecal concentration of *E. coli* O157 in cattle range

from 0.6 to 7.0 $\log_{10}$ CFU/g, but most animals shed around 1.0 to 2.0 $\log_{10}$ CFU/g (Gyles, 2007). Though our initial *E. coli* O157 inoculum was 10-fold higher than fecal concentrations in most cattle it is still biologically relevant given the range of concentrations seen in cattle. *In vitro* fermentations with ruminal fluid and fecal inocula have historically been used to assess gut microbial activities and interactions, and digestibility of specific substrates (El Shaer et al., 1987; Mould et al., 2005; Varadyova et al., 2005). Ruminal fluid fermentation also has been used to evaluate factors that impact *in vitro* growth and survival of *E. coli* O157, such as ecological factors (Diez-Gonzalez and Russell, 1997), antimicrobials (Edrington et al., 2003, 2006), probiotics (Bach et al., 2003) and other bactericidal agents (Annamalai et al., 2004). Chaucheyras-Durand et al. (2006) evaluated the growth of *E. coli* O157 in both ruminal fluid and fecal microbial fermentations and reported that ruminal fluid, because of the resident microbial population, was inhibitory to *E. coli* O157. Our intention was to simulate the microbiological aspects of ruminal digesta and feces and evaluate compounds that commonly are found in mucus secretions in the hindgut and assess differences in Nal^R *E. coli* O157 concentrations, pH and VFA concentrations. Fecal microbial fermentation was used to represent hindgut fermentation (Rumney and Rowland, 1992; Mould et al., 2005). Ideally, we would have liked to directly compare the growth of Nal^R *E. coli* O157 in ruminal and fecal microbial inoculum to answer this question, but because of differences in dry-matter content, types and concentrations of microbes and nutrients, it is not meaningful to evaluate both fermentations on an equal basis. Therefore, we did not statistically compare the two fermentation inocula although they were from the same donor steer. The difference between the two fermentations is evident in the control fermentation based on the apparent difference in concentrations of fermentation products. The concentrations of acetate, propionate, butyrate, and the total VFA were much lower in fecal microbial

fermentation than ruminal microbial fermentation, and the difference was reflective of a smaller microbial population in the feces compared to rumen fluid. Apparently, addition of physiological saline to approximate the dry matter of the fecal microbial inoculum may have contributed to further reduction in the microbial population. However, addition of physiological saline to prepare a fecal suspension was needed to remove large particles and for the ease in setting up the fermentation. Because of the large number of comparisons evaluated, we used $P < 0.01$ as the level of significance to reduce type I error rate. Even with this conservative approach, we observed many differences between treatments with regard to the outcomes evaluated. However, some substrates did not elicit responses different than control, which suggests that some of the substrates may not have been fermented by the microbial population.

Many of the compounds evaluated impacted the growth of *Nal*^{R} *E. coli* O157 in ruminal or fecal microbial fermentations. Of the sugars (fucose, galactose and mannose), mannose and fucose showed moderate increase in *Nal*^{R} *E. coli* O157 concentration and decrease in fermentation pH compared to the control. However, in fermentations with ruminal microbial inoculum, galactose reduced *Nal*^{R} *E. coli* O157 concentration and pH below that of control, but other treatments, such as mannose, reduced pH in a similar manner without reducing *Nal*^{R} *E. coli* O157 concentrations. The reduction of *Nal*^{R} *E. coli* O157 by galactose in ruminal microbial fermentations was not observed in fecal microbial fermentations; in fact *Nal*^{R} *E. coli* O157 concentration was actually higher compared to control. It has previously been observed that in continuous culture of ruminal contents, an inoculated population of *E. coli* O157 was not reduced by fermentation pH, but competitive exclusion by other microbes caused a reduction in the population of *E. coli* O157 (Thran et al., 2003). This may explain the difference in response of *Nal*^{R} *E. coli* O157 to galactose in ruminal and fecal microbial inoculum, because galactose is

highly fermentable in the rumen by lactate-producing bacteria which have very rapid growth rates (Dennis et al., 1981), thus limiting availability of the substrate in batch culture fermentation.

Galactosamine fermentation did not result in higher concentrations of *Nal*^{R} *E. coli* O157 than control, but fermentations with glucosamine had higher *Nal*^{R} *E. coli* O157 concentrations than control in 12- and 24-h samples with moderate decreases in pH. Gastric mucin did not affect *Nal*^{R} *E. coli* O157 concentrations in ruminal microbial inoculum, but increased the concentration in fecal microbial inoculum. The difference in response of *Nal*^{R} *E. coli* O157 between fecal microbial and ruminal microbial inocula may be reflective of difference in mucinolytic activities. Because mucus is secreted in the hindgut and not in the rumen, the resident flora in the hindgut may contain higher numbers of bacteria capable of degrading mucin and releasing its carbohydrate constituents. The fact that complete gastric mucin was less stimulatory than some of the individual components suggests that it may be related to the rate of mucolytic activity or specific components of mucin are responsible for the stimulation of *E. coli* O157. Mucins serve as an important defense mechanism in the gastrointestinal tract to remove slow growing pathogens and support commensal bacteria (such as *E. coli*) of the gut (Deplancke and Gaskins, 2001). In germfree rats, lipopolysaccharide of an experimental *E. coli* strain was able to alter mucin glycosylation patterns to allow more favorable conditions for the growth and attachment of the organism (Enss et al., 1996). Because *E. coli* O157 is non-pathogenic to cattle, perhaps it may alter mucin chemistry to increase specific carbohydrate constituents allowing it to thrive in the hindgut.

Few substrates elicited significant responses in ruminal microbial inoculum, but gluconic acid increased the 24-h concentration of *Nal*^{R} *E. coli* O157 by 1.0 $\log_{10}$ CFU/ml in ruminal

microbial inoculum and 2.0 $\log_{10}$ CFU/ml in fecal microbial inoculum compared to control. Again, the increase may be reflective of the ability of *E. coli* O157 to utilize gluconic acid as an energy source. Given the ability of *E. coli* to utilize gluconic acid via the Entner-Duodoroff pathway (Peekhaus and Conway, 1998), it is possible that this compound may serve as a nutrient for *E. coli* O157 in the hindgut or other sites of the gastrointestinal tract where mucus is secreted. Additionally, this compound also is fermented by *Lactobacillus* sp. (Tsukahara et al., 2002), which may provide an explanation for the efficacy of *Lactobacillus-based* direct-fed microbials for reducing *E. coli* O157 prevalence in cattle or inhibiting *E. coli* O157 *in vitro* (Brashears et al., 2003; Chaucheyras-Durand et al., 2006; Peterson et al., 2007; Stephens et al., 2007). The reduction may be due to *Lactobacillus* competing or out-competing *E. coli* O157 for nutrients, such as gluconic acid.

The uronic acids (galacturonic and glucuronic acids) increased *Nal*^{R} *E. coli* O157 concentrations in fecal microbial inoculum, but galacturonic acid depressed pH more than glucuronic acid. However, galacturonic acid reduced *Nal*^{R} *E. coli* O157 concentrations in ruminal microbial fermentations. This reduction may be because initial pH was lower in fermentations with galacturonic acid compared to other treatments and control; however, this was true for both inocula. The difference in response of *E. coli* O157 to uronic acids in different inocula may suggest that substrate effects are indirect and actually are a function of how other microflora respond to the substrate.

Almost all treatments had greater VFA concentrations than control after 24 h of fermentation. This was expected as the initial inoculum was diluted with buffer and/or saline and should have had relatively low concentrations of nutrients. In fecal microbial inoculum, acetate and total VFA concentrations were negatively correlated with *Nal*^{R} *E. coli* O157

concentrations. Because acetate is the predominant VFA, it is likely that the correlation between total VFA and *Nal*^{R} *E. coli* O157 was the result of the correlation between acetate and *E. coli* O157, especially since the acetate correlations are stronger. These correlations suggest that elevated acetate concentrations may inhibit or kill *E. coli* O157, but these correlations were not observed in ruminal microbial inoculum and it has been documented that *E. coli* O157 has cellular mechanisms to prevent the toxic accumulation of acetate within the bacterial cell (Diez-Gonzalez and Russell, 1997). The correlations may also be explained by competitive exclusion of *E. coli* O157 by acetate-producing bacteria present in fecal microbial inoculum (Wolfe, 2005). In contrast, higher VFA concentration and lower pH may be a result of *E. coli* O157 failing to grow and allowing other microbes to flourish. Based on these assumptions, it is difficult to assess if the substrates evaluated in our study that increase *E. coli* O157 concentrations have direct stimulatory effects on *E. coli* O157, indirectly affect the organism by altering or inhibiting other microflora, or both.

For most of the substrates evaluated, the effect of fermentation on *E. coli* O157 concentration was greater in fecal microbial inoculum compared to ruminal microbial inoculum, although we did not compare the two fermentations directly. The total number of bacteria present in the two inocula was likely greater for the rumen and few statistical differences were evident in fermentations with ruminal microbial inoculum suggesting that ruminal microorganisms may degrade these components and reduce their availability to *E. coli* O157.

IMPLICATIONS

Many of the substrates we evaluated stimulated *E. coli* O157, particularly in fecal microbial fermentation. Gluconic acid elicited higher concentrations of Nal^R *E. coli* O157 in ruminal microbial inoculum and had the highest response of any substrates in fecal microbial inoculum. These results suggest that gluconic acid may serve as a unique substrate available to promote growth of *E. coli* O157in the hindgut of beef cattle.

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Table 4.1 Concentrations of *Escherichia coli* O157 (log_{10} CFU/ml) and pH after 12 and 24 h of fermentation of mucin or its carbohydrate constituents with ruminal or fecal microbial inoculum.

	Ruminal microbial inoculum				Fecal microbial inoculum			
	12 h		24 h		12 h		24 h	
Substrate	*E. coli* O157	pH	*E. coli* O157	pH	*E. coli* O157	pH	*E. coli* O157	pH
Control	3.0^{ab}	7.0^{d}	3.4^{bc}	7.0^{d}	2.9^{a}	7.0^{e}	2.7^{a}	7.1^{f}
Fucose	2.8^{ab}	6.7^{bc}	3.4^{bc}	6.0^{a}	3.8^{abc}	6.4^{c}	3.4^{ab}	6.3^{ab}
Galactose	2.5^{a}	6.1^{a}	2.2^{a}	6.2^{ab}	3.9^{bc}	5.9^{a}	3.9^{bc}	6.2^{a}
Mannose	3.2^{ab}	6.1^{a}	3.5^{bc}	6.2^{ab}	3.7^{abc}	6.2^{b}	3.8^{bc}	6.3^{ab}
Gluconic acid	3.6^{b}	6.7^{cd}	4.4^{d}	6.9^{d}	4.4^{c}	6.7^{d}	4.7^{c}	6.8^{e}
Galacturonic acid	2.4^{a}	6.1^{a}	2.7^{ab}	6.3^{bc}	4.3^{c}	6.2^{bc}	4.5^{c}	6.4^{bc}
Glucuronic acid	3.5^{b}	6.8^{cd}	3.9^{cd}	6.8^{d}	4.0^{c}	6.6^{d}	4.3^{bc}	6.8^{e}
Gastric mucin	3.1^{ab}	6.9^{cd}	3.2^{bc}	6.8^{d}	3.9^{bc}	6.8^{d}	4.0^{bc}	6.8^{e}
Galactosamine	3.1^{ab}	6.8^{cd}	2.7^{ab}	6.8^{d}	3.0^{ab}	6.7^{d}	2.7^{a}	6.6^{d}
Glucosamine	3.4^{b}	6.5^{b}	3.4^{bc}	6.4^{c}	4.3^{c}	6.3^{bc}	4.3^{bc}	6.5^{cd}
SEM	0.3	0.1	0.3	0.1	0.6	0.1	0.6	0.1

$^{a, b, c, d, e}$ Means within a column not sharing a common superscript are significantly different ($P < 0.01$).

Table 4.2 Pearson correlation coefficients between *Escherichia coli* O157 concentrations and pH or VFA concentration after 12 and 24 h of fermentation in ruminal or fecal microbial inoculum.

	Ruminal microbial inoculum		Fecal microbial inoculum	
	E. coli O157	*E. coli* O157	*E. coli* O157	*E. coli* O157
Variable	12 h	24 h	12 h	24 h
12 h pH	-0.12	0.12	0.26†	0.29*
24 h pH	-0.09	0.15	0.11	0.17
12 h acetate	0.17	-0.02	-0.65**	-0.62**
24 h acetate	0.13	-0.01	-0.53**	-0.45**
12 h propionate	0.27†	0.09	-0.21	-0.19
24 h propionate	0.19	-0.02	-0.10	-0.03
12 h butyrate	-0.03	-0.05	-0.21	-0.24†
24 h butyrate	-0.09	-0.23	-0.24†	-0.26†
12 h total VFA	0.22	0.01	-0.51**	-0.48**
24 hr total VFA	0.16	-0.04	-0.41**	-0.33*

†, *, ** Correlations significant at $P < 0.10$, 0.05 and 0.01 levels, respectively.

Table 4.3 Concentrations (m*M*) of acetate (Ace), propionate (Pro), butyrate (But) and total volatile fatty acids (VFA) after 24 h of fermentation in ruminal or fecal microbial inoculum.

Substrate	Ruminal microbial inoculum				Fecal microbial inoculum			
	Ace	Pro	But	Total VFA	Ace	Pro	But	Total VFA
Control	38.9[a]	12.2[a]	9.8[a]	60.9[a]	7.5[a]	3.8[a]	0.7[a]	11.9[a]
Fucose	61.5[c]	48.0[f]	12.5[bc]	122.1[d]	39.3[c]	38.9[f]	1.6[ab]	79.7[e]
Galactose	65.9[cd]	32.8[d]	14.9[d]	113.6[cd]	38.4[c]	39.2[f]	1.9[b]	79.6[e]
Mannose	59.5[c]	33.4[d]	16.7[e]	109.6[bc]	34.5[c]	27.6[e]	2.1[bc]	64.3[cd]
Gluconic acid	49.5[b]	38.6[e]	13.1[bc]	101.2[b]	56.1[e]	10.5[bc]	2.3[bc]	68.9[cde]
Galacturonic acid	82.5[f]	14.9[ab]	12.6[bc]	110.0[bc]	61.9[g]	9.9[bc]	2.2[bc]	74.1[de]
Glucuronic acid	79.2[ef]	18.5[b]	12.4[b]	110.1[bc]	58.5[e]	9.9[bc]	2.1[bc]	70.5[de]
Porcine gastric mucin	72.7[de]	24.7[c]	14.0[cd]	111.4[bcd]	46.8[d]	21.0[d]	6.2[d]	74.0[de]
Galactosamine	43.9[ab]	11.9[a]	9.6[a]	65.3[a]	19.2[b]	7.7[ab]	8.4[e]	35.3[b]
Glucosamine	60.2[c]	23.6[c]	16.9[e]	110.7[b]	40.5[c]	14.0[c]	3.2[c]	57.7[c]
SEM	4.95	1.57	0.61	5.88	2.46	1.76	0.35	3.82

[a, b, c, d, e, f] Means within a column not sharing a common superscript are different ($P < 0.01$)

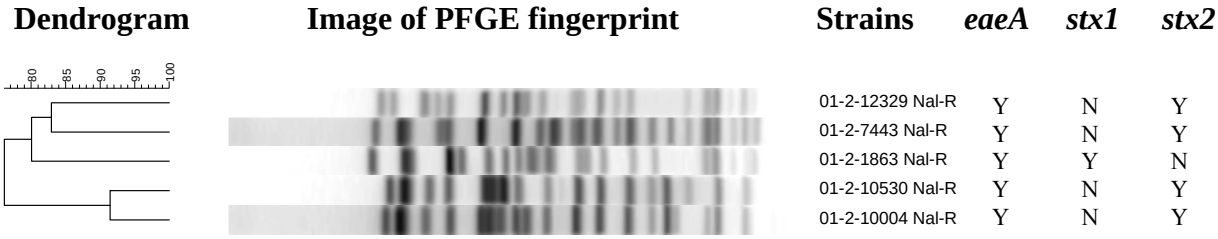

Figure. 4.1 Pulsed field gel electrophoresis typing and virulence gene profiles [Intimin (*eaeA*), Flagellum (*fliC*), Shiga-toxin 1 (*Stx1*), and Shiga-toxin 2 (*Stx2*)] of the nalidixic acid resistant (50 µg/ml) strains of *Escherichia coli* O157 used for *in vitro* fermentations.

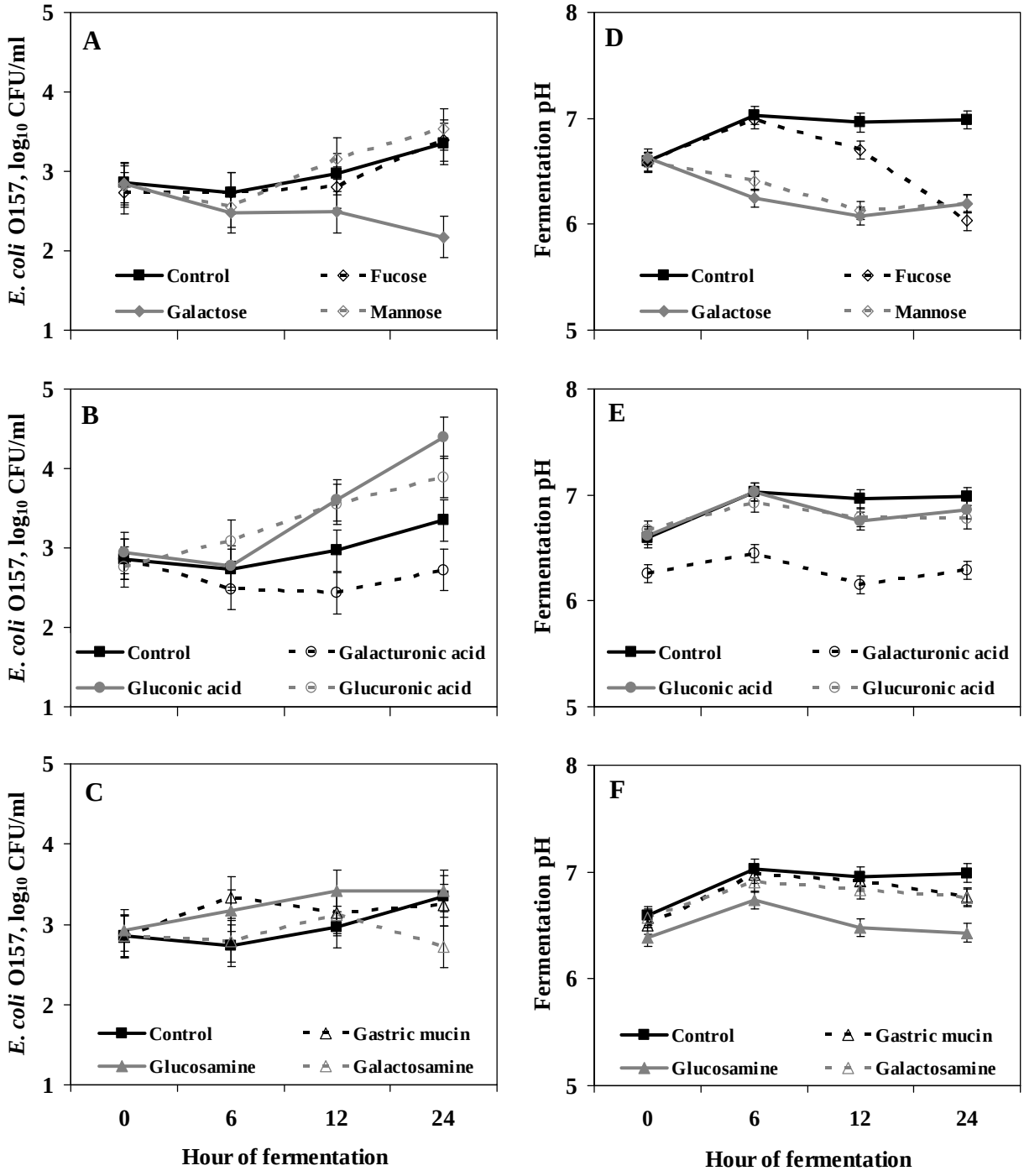

Figure 4.2 Least squares means of *Escherichia coli* O157 concentrations (A, B, C) and pH (D, E, F) after 0, 6, 12 and 24 h of fermentation with ruminal microbial inoculum. Error bars represent standard error of the mean.

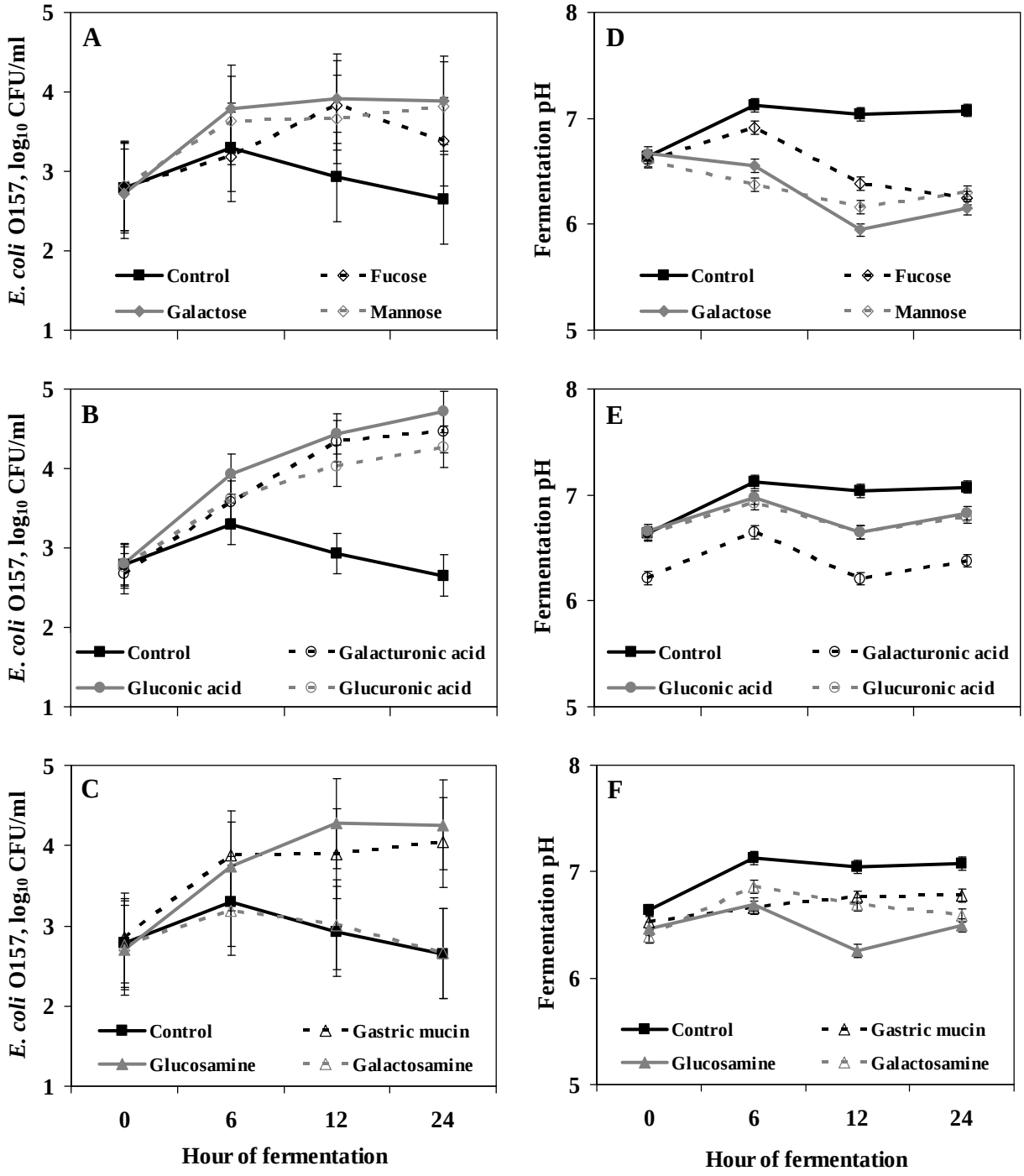

Figure 4.3 Least squares means of *Escherichia coli* O157 concentrations (A, B, C) and pH (D, E, F) after 0, 6, 12 and 24 h of fermentation with fecal microbial inoculum. Error bars represent standard error of the mean.

CHAPTER 5 - Dry-rolled or steam-flaked grain-based diets on fecal shedding of *E. coli* O157 in feedlot cattle

Hindgut is a major colonization site for *E. coli* O157 in cattle. In this study, diets were formulated to effect changes in hindgut fermentation to test our hypothesis that changes in hindgut ecosystem could have an impact on fecal shedding of *E. coli* O157. Feedlot heifers (n = 347) were pre-screened for prevalence of *E. coli* O157 by fecal and rectoanal mucosal swab (RAMS) cultures. A subset of 40 heifers identified as being positive for fecal shedding of *E. coli* O157 were selected, housed in individual pens, and randomly allocated to four dietary treatments. Treatments were arranged as a 2 x 2 factorial, with factor 1 consisting of grain type (sorghum or wheat) and factor 2 being method of grain processing (steam-flaking [SF] or dry-rolling [DR]). Four transition diets, each fed for 4 days, were used to adapt the animals to final diets that contained 93% concentrate and 7% roughage. The grain fraction consisted of dry-rolled sorghum (DRS), steam-flaked sorghum (SFS), a mixture of dry-rolled wheat and steam-flaked corn (DRW), or a mixture of steam-flaked wheat and steam-flaked corn (SFW). Wheat diets contained 52% wheat and 31% steam-flaked corn (DM basis). Fecal and RAMS samples were obtained 3 times a week to isolate (enrichment, immunomagenetic separation, and plating on selective medium) and identify (sorbitol negative, indole production, and agglutination test) *E. coli* O157. Data were analyzed as repeated measures of binomial response (positive or negative) on each sampling day. Method of processing (SF vs. DR), sampling day, and the grain type x day interaction were significant ($P < 0.05$), but not the method of processing x grain type interaction. Average prevalence from d 9 was higher ($P < 0.001$) in cattle fed SF grains (65%) compared to those fed DR grains (30%). Average prevalence in cattle fed sorghum (51%) or

wheat (43%) were similar ($P > 0.10$) on most sampling days. Results from this study indicate that feeding DR grains compared to SF grains reduced fecal shedding of *E. coli* O157. Possibly, DR allowed more substrate to reach the hindgut where it was fermented, thus making the hindgut inhospitable to the survival of *E. coli* O157. Dietary intervention to influence hindgut fermentation offers a simple and practical mitigation strategy to reduce prevalence of *E. coli* O157 in feedlot cattle.

Keywords: *E. coli* O157, finishing cattle, grain processing, preharvest intervention

INTRODUCTION

Escherichia coli O157 is an important human food-borne pathogen. The gastrointestinal tract of cattle is the major reservoir of the organism and cattle feces are the primary source of infection (Bach et al., 2002). Many preharvest intervention strategies aimed at reducing prevalence of *E. coli* O157 have been tested with varying levels of success (Callaway et al., 2003). Fecal shedding of *E. coli* O157 in cattle is reflective of the ability of the organism to persist in or colonize the gastrointestinal tract. Evidence suggests that the site of persistence or colonization is in the hindgut and not the rumen (Rasmussen et al., 1993; Brown et al. 1997; Grauke et al. 2002 and Van Baale et al. 2004). Although the reasons are not known, it is likely that the ecosystem of the hindgut is relatively more hospitable than the rumen. Therefore, we hypothesize that dietary factors that promote supply of substrates (starch, fiber, protein, or lipids) to the hindgut will have a significant effect on the ability of *E. coli* O157 to survive and colonize, and thereby influence shedding in feces. Our objective is to use processed grains to effect changes in hindgut fermentation that will have detrimental effects on the survival, growth and colonization of *E. coli* O157. Grains that are less extensively digested within the rumen present more starch to the hindgut, thereby increasing fermentative activity and acid production in the hindgut. Steam-flaking of grains has been shown to enhance ruminal starch digestion compared to dry-rolling, effectively reducing the amount of starch reaching the hindgut (Huntington, 1997). The objective of this study was to evaluate the effects of grain type (sorghum or wheat) and grain processing (dry-rolled or steam-flaked) in finishing diets on prevalence of *E. coli* O157 in cattle.

MATERIALS AND METHODS

Experimental Animals and Design

Animal management and handling procedures for this study were approved by the Kansas State University Institutional Animal Care and Use Committee. Feedlot heifers upon arrival at the Kansas State University Beef Cattle Research Center were ear tagged, vaccinated, implanted, and body weights recorded. Heifers (n = 347) were tested for prevalence of *E. coli* O157 by fecal and rectoanal mucosal swab (RAMS) cultures (Greenquist et al., 2005). Heifers positive for fecal shedding of *E. coli* O157 were retested within a week and 40 heifers (initial BW = 287 ± 5 kg) were selected for use in the study. Heifers were then grouped based upon pre-screen *E. coli* O157 positive samples, and within group were randomly assigned to 1 of 4 treatments. Treatments consisted of a 2 x 2 factorial arrangement with factor 1 being grain type (sorghum- or wheat-based diets) and factor 2 being the method of grain processing, steam-flaking (SF) or dry-rolling (DR). Within treatment, animals were randomly assigned to 1 of 2 barns and 1 of 20 individual pens within each barn.

A series of transition diets were used to adapt animals to high-concentrate finishing diets consisting of 81.4% (DM basis) dry-rolled or steam-flaked sorghum, or 52.0% (DM basis) dry-rolled or steam-flaked wheat (Table 1). Steam-flaked corn was added to wheat diets to achieve a similar forage: concentrate ratio among all diets. Each transition diet was fed for 4 days to achieve the final diet on d 16 of the study.

Intake and Feed Sampling

Feed samples were collected to determine DM (AOAC, 1990). Bunks were evaluated every morning at approximately 0700 h, and feed was delivered once daily at 0900 h. Heifers

were fed amounts sufficient to result in only traces of feed remaining on the following day, and any feed remaining from the previous day was weighed and DM was measured, to determine feed intake of each animal.

Fecal and RAMS Samples Collection and E. coli O157 Culture

Fecal and RAMS samples were collected from each heifer 3 times a week for 30 d (d 0, 2, 5, 7, 9, 12, 14, 16, 19, 21, 23, 26, 28 and 30). Fecal samples were deposited into Whirl-pack bags (Nasco, Ft. Atkinson, WI.) and placed on ice. Rectoanal mucosal swab samples were placed directly into test tubes containing 3 mL of gram-negative (GN) broth (Becton Dickinson Co., Franklin Lakes, NJ.) supplemented with cefixime, cefsulodin, and vancomycin (GNccv; Greenquist et al., 2005). Whirl-pack bags and RAMS test tubes were then transported on ice to the laboratory. Approximately 1 g of feces from each heifer was placed into a test tube containing 9 mL GNccv. Fecal and RAMS tubes were then vortexed for 1 m and incubated at 37°C for 6 h. Following enrichment, tubes were vortexed for 1 m and 1 mL from each tube was subjected to immunomagnetic separation (IMS; Dynal, Inc., New Hyde Park, NY.). Following the IMS procedure, samples were plated onto sorbitol MacConkey agar (Becton Dickinson Co.) containing cefixime (50 ng/mL) and potassium tellurite (2.5 μg/mL) (CT-SMAC). Plates were incubated overnight (16 to 18 h) and up to 6 sorbitol-negative colonies were grown on blood agar (Remel, Lenexa, KS.) for 12 to 18 h at 37°C. Cultures from blood agar were tested for indole production and latex agglutination for the O157 antigen (Oxoid Limited, Basingstoke, Hampshire, England). Species was confirmed by API (Rapid 20E; Biomerieux Inc., Hazelwood, MO.) on pre-screening samples and samples collected on d 0, 2, and 5.

Fecal pH

Fecal pH was measured in samples collected on d 9, 16, 23 and 30 of the study. Approximately 5 g of feces from each animal were suspended in 25 mL of distilled water inside a 50-mL polypropylene tube (Becton Dickinson and Co.). The tube was vortexed to make a fecal suspension before pH was measured with an ACCUMET model AR 10 pH meter (Fisher Scientific International, Pittsburgh, PA.).

Statistical Analyses

Mean DM values for each dietary ingredient were calculated and used to determine daily DM intake for each animal. Intake expressed as average daily DM intake from d 9 to the end of the study was analyzed using the mixed procedure of SAS (SAS Version 9.1; Cary, NC) utilizing grain type, grain processing and the interaction as fixed effects, and barn as a random effect.

The prevalence of *E. coli* O157, expressed as a binomial response (positive or negative) on each sampling day, was analyzed with animal as the experimental unit. Cattle were considered positive if *E. coli* O157 was detected by either fecal or RAMS culture. Samples collected on d 0, 2, 5 and 7 were not included in the analysis because cattle were being fed transition diets consisting of less than 60% sorghum or less than 40% wheat. Data were analyzed using the glimmix procedure of SAS (SAS Version 9.1; Cary, NC) in a split-split-plot design with the whole-plot factor being pre-screen positive sample group in a randomized complete block design with barn as block. Grain-type, grain processing method and the two-way interaction comprised the sub-plot and the sub-sub-plot was repeated measures over sampling days utilizing first order autoregression.

Fecal pH data were analyzed with the Mixed procedure of SAS (SAS Version 9.1; Cary, NC) in a split-plot design with the whole plot factors being grain type, grain processing method,

and the two-way interaction, with repeated measures as the subplot. Barn was included as a random effect in this analysis.

RESULTS

One heifer in the steam-flaked sorghum treatment developed was removed from the study on d 5 of the study due to lameness.

Dry-Matter Intake

Mean daily DM intakes from d 9 to the end of the study for heifers fed steam-flaked sorghum, dry-rolled sorghum, steam-flaked wheat and dry-rolled wheat diets were 6.12 ± 0.38, 6.79 ± 0.36, 6.14 ± 0.38 and 7.21 ± 0.36 kg, respectively. Grain processing impacted ($P = 0.026$) DMI, but grain type and grain type x grain processing interaction did not ($P > 0.10$). Dry-matter intake of heifers fed diets with dry-rolled grains (7.00 ± 0.26 kg) was greater ($P = 0.026$) than in heifers fed diets with steam-flaked grains (6.13 ± 0.27 kg).

Prevalence of E. coli O157

Mean prevalence of *E. coli* O157 in all heifers across all sampling days was 50.0%. Analysis of prevalence data began on d 9 when animals were on the third transition diet. Mean prevalence of *E. coli* O157 from d 9 in heifers fed the SFS, DRS, SFW, and DRW diets were 73, 30, 58 and 29%, respectively. Grain-type did not impact ($P = 0.29$) prevalence of *E. coli* O157, but the grain type x sampling day interaction was significant ($P = 0.04$). It should be noted that the grains (sorghum and wheat) were included in diets at different levels to avoid potential ruminal digestive disorders associated with wheat-based diets. On d 12 and 19 of the study, prevalence of *E. coli* O157 was greater ($P < 0.05$) in heifers fed sorghum-based diets compared

to heifers fed wheat-based diets, but the difference was not significant on other sampling days (Figure 1).

Grain processing method (steam-flaking vs dry-rolling) affected ($P < 0.001$) prevalence of *E. coli* O157 in the heifers and the processing method x sampling day interaction was not significant ($P = 0.45$). Mean prevalence from d 9 in heifers fed dry-rolled grain diets (29.5%) was lower ($P < 0.001$) than prevalence in heifers fed steam-flaked grain diets (64.7%). Prevalence of *E. coli* O157 was higher ($P < 0.10$) in heifers fed steam-flaked grain diets compared to dry-rolled grain diets on all sampling days except d 9, 12, and 14 (Figure 2).

Fecal pH

Fecal pH was measured on d 9, 16, 23 and 30 as a potential indicator of hindgut fermentative activity. Mean fecal pH over all 4 sampling days in heifers fed the steam-flaked sorghum, dry-rolled sorghum, steam-flaked wheat or dry-rolled wheat diet was 6.47 ± 0.07, 6.53 ± 0.07, 6.53 ± 0.07 and 6.58 ± 0.07, respectively. Grain type, grain processing and their interaction had no effect on fecal pH; however, sampling day ($P = 0.02$) and grain type x sampling day interaction ($P = 0.01$) affected fecal pH. On d 9 of the study when animals were fed the third transition diet, fecal pH was lower ($P = 0.01$) in cattle fed sorghum diets (6.38 ± 0.08) compared to cattle fed wheat diets (6.56 ± 0.08), but this difference was not apparent on any other sampling days.

DISCUSSION

Preharvest intervention strategies to eliminate or reduce *E. coli* O157 in beef cattle have typically been evaluated using experimentally inoculated cattle (Callaway et al., 2002; Van Baale

et al., 2004; Bach et al., 2005), experimental trials utilizing large numbers of animals to compare prevalence of natural infection (Berg et al., 2004; Brashears et al., 2003), or observational studies (Garber et al., 1995; Dargatz et al., 1997; Herriott et al., 1998). Potential limitations to these types of studies include the need to euthanize experimentally inoculated animals, the cost incurred with sampling large numbers of animals, and external factors which are difficult to evaluate effectively in observational studies. For this study, we pre-screened a population of cattle, and selected a subpopulation that was positive for fecal *E. coli* O157 to impose treatments. This model proved useful in that prevalence was high enough to detect statistical differences among treatment groups with only 10 animals per treatment. We included RAMS method because RAMS has been shown to be more sensitive than fecal testing in detecting animals that are positive for *E. coli* O157 (Greenquist et al., 2005).

Fecal shedding of *E. coli* O157 is reflective of the ability of the organism to persist or colonize the gastrointestinal tract. Published data suggest that the hindgut may be the major site of *E. coli* O157 persistence. Naylor et al. (2003) suggested that the primary site of *E. coli* O157 colonization was the rectum, specifically the region approximately 2 to 5 cm proximal to the rectoanal junction. In studies with experimentally inoculated cattle (Grauke et al., 2002 and 2003; Van Baale et al., 2004) or prevalence data in gut contents collected from necropsied or slaughtered cattle (Laven et al., 2003; Van Baale et al., 2004), *E. coli* O157 was detected more frequently in the cecum and colon than in the rumen. Possibly, the conditions in the cecum and colon (higher pH, lower VFA concentrations, absence of ciliated protozoa, slower rate of passage of digesta, etc., compared to the rumen) are more hospitable to the survival and growth of *E. coli* O157.

Sorghum and wheat grains were chosen for this study because their ruminal digestibilities are substantially different, thus differing in the amount of starch reaching the hindgut (Huntington, 1997). Wheat diets, either steam-flaked or dry-rolled, in our study contained only 52.0% wheat, and steam-flaked corn was added to achieve similar forage: concentrate ratio among all diets. Because wheat has one of the fastest rates of ruminal starch digestion (Stock and Britton, 1993) with increased propensity to induce metabolic disorders, such as subacute acidosis, feeding high levels of wheat in cattle diets may increase the likelihood of digestive disorders (Axe et al., 1987; Stock and Britton, 1993).

In the current study, DMI was affected by grain processing method. Intakes were lower in cattle fed diets with steam-flaked grains. This observation is consistent with those presented in a review by Owens et al. (1997) in which steam-rolling sorghum, wheat or corn decreased ($P < 0.05$) daily DMI of feedlot cattle compared to dry-rolling the grains. Dry matter intakes of wheat-based diets have been reported to be lower than DMI of sorghum-based diets (Owens et al., 1997). In our study, we did not observe differences in DMI among heifers fed sorghum or wheat diets. Differences in DMI of diets containing different grain types or grain processing methods are likely a combination of differences in metabolizable energy and ruminal degradation of starch yielding differences in ruminal acid concentrations (Owens et al., 1997).

Previous studies have shown that cattle diets containing grains with lower ruminal starch degradation are associated with lower prevalence of *E. coli* O157 (Buchko et al., 2000; Berg et al., 2004). This may be due to a larger amount of starch being fermented in the hindgut, ultimately yielding higher organic acid concentrations and lower pH in the region of the gastrointestinal tract that has been identified as the primary colonization site of *E. coli* O157 (Grauke et al., 2002; Van Baale et al., 2004). Although starch fermentation was not measured in

this study, we believe that increasing hindgut fermentation of starch and consequent acid production will create inhospitable conditions for *E. coli* O157. Short-chain fatty acids (acetic, propionic, butyric) have been shown to suppress and inhibit growth of *E. coli* O157 at pH values of 6.0 and 5.5, respectively (Shin et al., 2002). Van Kessel et al. (2002) evaluated differences in pH and microbial populations in cannulated steers with starch hydrolysate infused into the rumen or abomasum. They reported that infusing starch into the abomasum decreased pH, and increased total anaerobic bacteria, total aerobic bacteria, and generic *E. coli* in the hindgut compared to infusing starch into the rumen. Increased availability of substrate in the hindgut increases the accumulation of organic acids (VFA) and reduces pH (Van Kessel et al., 2002). In our study, differences in fecal pH were not consistently detected among dietary treatments. It is possible that fecal pH may not truly reflect the pH of cecum or colon. In addition to grain processing, higher intake of grains allows more starch to reach the hindgut (Owens et al., 1997). The higher intake of DR grains compared to SF grains observed in our study would further enhance the effects of grain processing on *E. coli* O157 in the hindgut.

It is documented that dry-rolled grains have lower ruminal degradation of starch compared to steam-flaked grains, thus presenting more starch to the hindgut and possibly increasing fecal starch content (Zinn et al., 1995; Huntington, 1997). Previous studies have shown that fecal pH of cattle fed steam-flaked grains was higher ($P < 0.05$) than that of cattle fed dry-rolled grains (Oliveira et al., 1995; Barajas and Zinn, 1998). In these studies, cattle fed steam-flaked grains also had reduced fecal starch compared to cattle fed dry-rolled grains. Berg et al. (2004) observed lower ($P < 0.01$) fecal pH and lower ($P < 0.05$) prevalence of *E. coli* O157 and in cattle fed corn compared to cattle fed barley. Because barley is more digestible than corn

in the rumen, corn diets would present more starch to the hindgut and increase organic acid production, thus reducing pH and potentially reducing survivability of *E. coli* O157.

IMPLICATIONS

Prescreening and selecting cattle positive for shedding of *E. coli* O157 is a useful model to evaluate potential pre-harvest intervention strategies. Because grain processing influences site and extent of starch digestion in ruminants, it offers a simple and effective method of targeting specific regions of the gastrointestinal tract to affect changes in fermentation (acid production, pH, microflora, etc.). Utilizing grains processed by methods that are known to increase the amount of starch reaching the hindgut and enhance fermentation may be useful in reducing *E. coli* O157 in cattle if fed prior to slaughter.

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Table 5.1 Ingredient composition of experimental diets (% DM basis) and days fed.

Diet	Transition 1	Transition 2	Transition 3	Transition 4	Final diet
Days fed	0-3	4-7	8-11	12-15	16-30
Sorghum diets					
Sorghum[1]	48.4	56.6	64.9	73.1	81.4
Alfalfa hay	40.0	31.8	23.5	15.3	7.0
Corn steep liquor	5.5	5.5	5.5	5.5	5.5
Soybean meal	1.8	1.8	1.8	1.8	1.8
Premix[2]	4.3	4.3	4.3	4.3	4.3
Wheat diets					
Wheat[1]	31.4	36.5	41.7	46.8	52.0
Steam-flaked corn	18.8	21.9	25.0	28.1	31.2
Alfalfa hay	40.0	31.8	23.5	15.3	7.0
Corn steep liquor	5.5	5.5	5.5	5.5	5.5
Premix[2]	4.3	4.3	4.3	4.3	4.3

[1]Steam-flaked or dry-rolled as appropriate for treatments.

[2]Formulated to provide 0.7% Ca, 0.7% K, 0.3% NaCl, 0.1 mg / kg cobalt, 10 mg / kg copper, 0.5 mg / kg iodine, 60 mg / kg manganese, 0.3 mg / kg selenium, 60 mg / kg zinc, 0.05 g / ton melengestrol acetate, 30 g / ton monensin, and 9 g / ton tylosin in the final diet (DM basis).

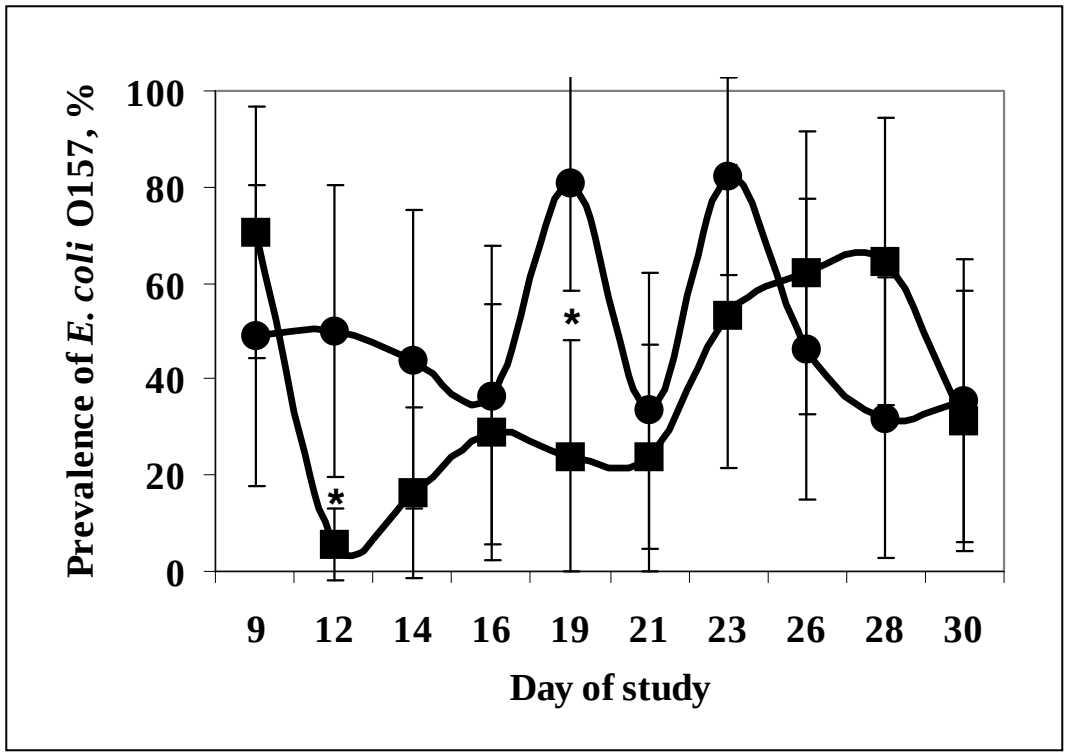

Figure 5.1 Least squares means (from logistic regression) for prevalence of *E. coli* O157 from d 9 of the study in heifers fed sorghum (circles; n = 19) or wheat (squares; n = 20) diets. Error bars represent SEM for the main effect on each sampling day. Main effect of grain type, $P = 0.29$. Grain type x sampling day interaction, $P = 0.04$. * = Means were statistically different ($P < 0.05$).

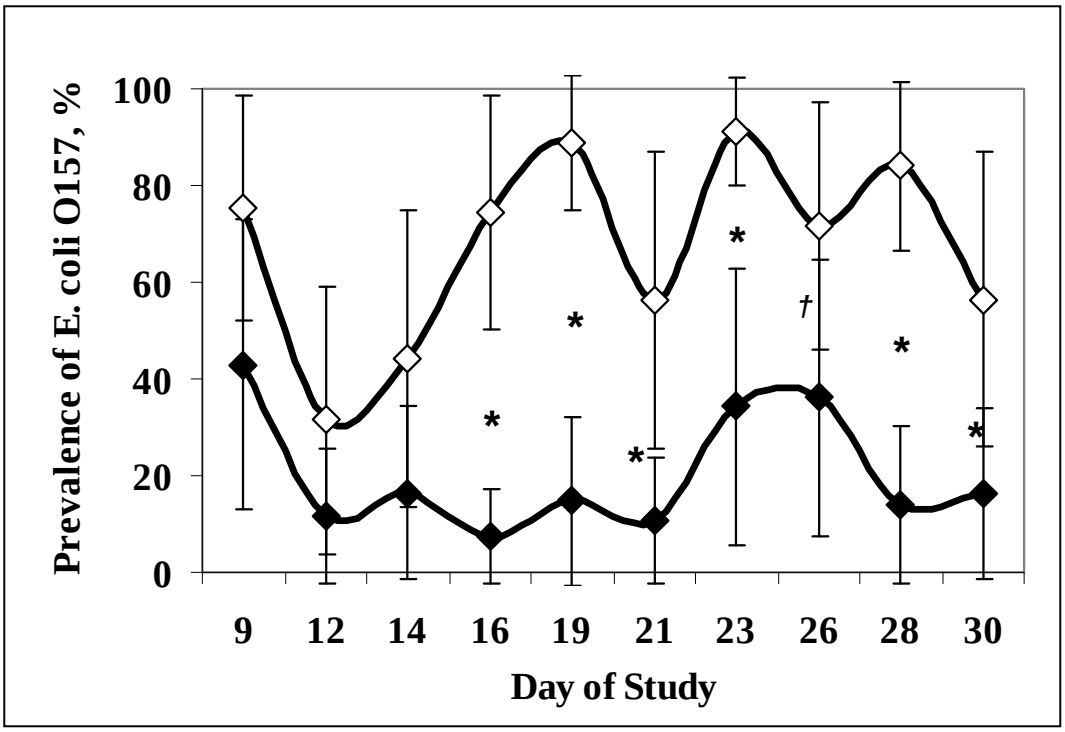

Figure 5.2 Least squares means (from logistic regression) for prevalence of *E. coli* O157 from d 9 of the study in heifers fed diets with dry-rolled grains (closed symbols; n = 20) or steam-flaked grains (open symbols; n = 19). Error bars represent SEM for the main effect on each sampling day. Main effect of grain processing method, $P < 0.001$. Processing method x sampling day interaction, $P = 0.45$. * = Means were statistically different ($P < 0.05$). † = Means were statistically different ($P < 0.10$).

CHAPTER 6 - The Effect of Competitive Exclusion *Escherichia coli* cultures on *E. coli* O157 Growth in Batch Culture Ruminal or Fecal Microbial Fermentation

The purpose of this study was to determine the efficacy of competitive exclusion (CE) *Escherichia coli* cultures (ATCC 202018 and 202019) to reduce *E. coli* O157 in batch culture fermentations with ruminal or fecal microbial inoculum derived from a cannulated feedlot steer fed a corn grain-based, high-grain diet. A non-CE (NCE) *E. coli* strain (ATCC 25922) was included as a control. Anaerobic fermentations (50 ml) were set up in serum bottles with 2:1 buffer to microbial inoculum ratio and with or without gluconic acid, a component of intestinal mucus, inclusion to stimulate the growth of *E. coli* O157. A five-strain mixture of *E. coli* O157 made resistant to nalidixic acid (50 μg/ml; Nal^R) was added to each fermentation and concentrations of Nal^R *E. coli* O157 were determined after 0, 6, 12 and 24 h of fermentation. In ruminal microbial fermentation, the CE treatment had a lower ($P < 0.01$) concentration of Nal^R *E. coli* O157 compared to the NCE control at 24 h, regardless of gluconic acid inclusion. In fecal microbial fermentation, the CE treatment did not affect Nal^R *E. coli* O157 concentrations in fermentations without gluconic acid, but with gluconic acid inclusion, Nal^R *E. coli* O157 concentrations were lower in the CE treatment compared to NCE controls at 12 and 24 h. In conclusion, *in vitro* fermentations with ruminal or fecal microbial inoculum could be used to assess the efficacy of CE cultures to reduce *E. coli* O157.

Escherichia coli O157:H7, a food-borne pathogen, continues to be of major public health concern in the United States. Ruminants are a major reservoir of this organism and adulterated ground beef was initially identified as the culprit for transmission to humans. More recently, outbreaks have been associated with the consumption of contaminated produce [5, 26]. Successful intervention strategies are used on beef hides and carcasses at slaughter to reduce the risk of the pathogen entering the food chain [16]. However, interventions at abattoirs do not preclude fecal contamination of water and fresh produce [31]. Therefore, efforts have been made to reduce the shedding of *E. coli* O157:H7 in beef cattle prior to the harvest [4, 12, 18-19].

The utilization of probiotic, also called direct-fed microbials or competitive exclusion (CE) cultures, as a preharvest intervention strategy appears to be a promising approach [8]. Probiotics have an indirect effect by altering microbial populations and products of fermentation in the gastrointestinal tract or have direct inhibitory or competitive effect on *E. coli* O157 and thus, may reduce the ability of *E. coli* O157 to survive in the gut and shed in the feces [8, 17, 18-19]. Although the terms probiotics and CE are used interchangeably, CE cultures are generally intended for microorganisms originating from an animal of the targeted host species [2]. n contrast, probiotic cultures can originate from any source and do not have to be even of an animal origin.

Zhao et al. [37] evaluated 1,200 bacterial isolates from feces and gastrointestinal tissues of cattle known to be culture negative for fecal *E. coli* O157 and reported that seventeen *E. coli* isolates had *in vitro* inhibitory effects on *E. coli* O157:H7. In the same study, two calves were inoculated with all seventeen strains to document their nonpathogenicity and four strains were selected based on recovery from inoculated calves. Three *E. coli* strains were then patented for their anti-*E. coli* O157 activity and were selected for further testing [9]. Cattle challenge studies

have confirmed the potential application of these *E. coli* strains to reduce prevalence of *E. coli* O157 in cattle [30, 38]. Possible mechanisms to explain anti-*E. coli* O157 activity include production of colicins, which are antimicrobial proteins that are inhibitory to other enteric bacteria [8, 10, 21, 37] and prevent tissue injury in epithelial cell monolayers induced by attaching and effacing bacteria [29]. Competitive exclusion *E. coli* cultures have been used to reduce infections caused by enterotoxigenic *E. coli* [34]. Competitive exclusion with nonpathogenic *E. coli* appears to be a logical option to reduce the prevalence of *E. coli* O157.

Other research efforts have focused on the potential of lactic acid bacteria, *Lactobacillus acidophilus* or other species, to reduce the prevalence of *E. coli* O157 in feedlot cattle [3, 24, 35]. Possibly, an increase in concentrations of volatile fatty acids, primarily acetate, may be related to the reduction in *E. coli* O157 [7, 22]. Competitive exclusion with nonpathogenic *E. coli* appears to be another option to reduce the prevalence of *E. coli* O157 [30, 38]. Zhao et al. [37] evaluated 1,200 bacterial isolates from feces and gastrointestinal tissues of cattle culture negative for fecal *E. coli* O157 and reported that several generic *E. coli* isolates had bactericidal effects on *E. coli* O157:H7. Possibly, the strains produced bacteriocins that inhibited growth *in vitro* [21, 37], prevented tissue injury in epithelial cell monolayers induced by attaching and effacing bacteria [29], and exhibited the potential to displace *E. coli* O157 and other serotypes of Shiga-toxigenic *E. coli* in experimentally challenged cattle [37-38].

In vitro fermentations with ruminal fluid or fecal suspension appear to be a simple and inexpensive approach to evaluate the efficacy of probiotic bacteria to inhibit growth of *E. coli* O157 [7]. Therefore, our objective was to evaluate the efficacy of two CE cultures of *E. coli* strains (ATCC 202018 and 202019) [9] to reduce *E. coli* O157 *in vitro* utilizing microbial

inoculum from ruminal fluid or feces of beef cattle. Gluconic acid, a component of intestinal mucus, stimulatory to *in vitro* growth of *E. coli* O157 [6, 13] was included in the fermentation.

Materials and Methods

Experimental design. The experiment was a completely randomized design with 3 x 2 x 2 factorial arrangement of treatments. Factor 1 was CE treatment: no added cultures (control), CE cultures of *E. coli* strains (ATCC 202018 and 202019; approx. 3×10^8 CFU of pooled cultures/ml of fermentation), or a negative control of a non-CE (NCE) strain of *E. coli* (ATCC 25922; approx. 2×10^8 CFU/ml of fermentation). Factor 2 was the addition of 0 or 500 mg gluconic acid to the fermentation (50 ml), and factor 3 was the type of microbial inoculum (ruminal or fecal). Fermentations were performed in 60-ml serum bottles (Wheaton Science Products, Millville, NJ) fitted with butyl rubber stoppers and Bunsen valves. Two fermentation bottles were set up for each CE treatment × gluconic acid × inoculum combination (24 bottles total). The experiment was performed a total of three times on separate days.

Preparation of ruminal and fecal microbial inocula. Ruminal contents and feces were collected from a ruminally-cannulated steer fed a corn grain-based, high-grain diet housed at the Kansas State University Beef Cattle Research Center. Ruminal contents were collected through the ruminal cannula, strained through four layers of cheesecloth, placed in a flask capped with a butyl rubber stopper fitted with Bunsen valve, and transported to the Preharvest Food Safety Lab (approximately 5 km). The ruminal fluid was then incubated (39°C) for 1 h and the hay mat/foam fraction that rose to the top was removed by vacuum suction. The remaining fluid was mixed (2:1 buffer to inoculum ratio; vol:vol) with McDougall's buffer [20] and used as ruminal

microbial inoculum. Feces were collected via rectal palpation from the same steer, placed in a Whirl-pak bag (Nasco, Ft. Atkinson, WI) and transported to the laboratory. McDougall's buffer was added to feces in a 3:1 buffer to feces ratio (vol:wt) and then stomached for 1 min to mix the buffer and feces. The fecal slurry was then subjected to low-speed centrifugation (RPM = 4,000; K factor = 20,744) for 10 min to remove large particles and the resulting supernatant was used as fecal microbial inoculum.

Preparation of CE or NCE cultures. Frozen CE (ATCC 202018 and 202019) and NCE (ATCC 25922) cultures stored on Trypic Soy Agar (Becton-Dickinson, Sparks, MD) slants were streaked onto blood agar plates (Remel, Lenexa, KS) and incubated at 37°C overnight. Single colonies were inoculated into 10 ml Tryptic Soy Broth (TSB, Becton-Dickinson) and incubated for 12 h at 37°C. Following incubation, 1 ml of this culture was pipetted into 100 ml of TSB and incubated for 6 h at 37°C. Equal parts of two CE cultures were pooled to constitute CE treatment for use in appropriate fermentations. Non CE or pooled cultures of CE were diluted and spread plated on MacConkey agar (Becton-Dickinson) immediately prior to addition to *in vitro* fermentations to determine concentrations of added CE or NCE culture. The final concentrations of CE or NCE bacteria used in fermentations are shown in Table 1.

***Escherichia coli* O157 preparation.** A 5-strain mixture (01-2-1863, 01-2-7443, 01-2-10004, 01-2-10530, and 01-2-12329) of *E. coli* O157 was used in this study. The strains were originally isolated from feedlot pen fecal samples [28]. Based on multiplex PCR assay [11], all strains had the *eae* gene, and all except one had the *stx2* gene but not the *stx1* gene. The *stx2* negative strain, 01-2-1863, was PCR positive for the *stx1* gene. In the laboratory, strains were made resistant to

nalidixic acid (*Nal*^{R}, 50 µg/ml) and stored in protect beads (Cryo-Vac®, Key Scientific Products, Round Rock, TX) at -80°C until used in this study. Resistance to nalidixic acid did not change PCR results of the virulence genes in the strains (data not shown). The preparation for inoculation into fermentations was made by streaking each strain from protect beads (Key Scientific Products) onto blood agar (Remel, Lenexa, KS) and incubating overnight at 37°C. For each strain, an individual colony was picked and inoculated into a separate bottle containing 100 ml TSB (Becton-Dickinson). After inoculation, the bottles were vortexed and incubated at 37°C for 18 h. Following incubation, 1 ml from each bottle was pooled and vortexed to achieve the 5-strain mixture used *in vitro* fermentations. For each replication of the experiment, a fresh culture preparation of the 5-strain mixture was used.

Fermentation sample collection. The concentrations of inoculated strains of *Nal*^{R} *E. coli* O157 in fermentations were determined at 0, 6, 12 and 24 h. Bottles were swirled, stoppers were removed and bottles were placed under flowing oxygen-free CO_2 gas [15] to maintain anaerobic conditions during sampling. One ml of sample was pipetted into a 96-well assay block (Corning Inc., Corning, NY). Serial dilutions were then made by transferring 100 µl into 0.9 ml buffered peptone water (Sigma-Aldrich, St. Louis, MO). Appropriate dilutions were plated in triplicate on sorbitol MacConkey agar (BD) supplemented with cefixime (50 ng/ml), potassium tellurite (2.5 µg/ml) and nalidixic acid (50 µg/ml; CT-SMACnal). Plates were incubated at 37°C for 18 to 24 h. After incubation, colonies were counted to determine concentrations (CFU/ml) and were log (base 10) transformed for data analysis.

Statistical analysis. The effects of CE *E. coli* treatments on *Nal*^{R} *E. coli* O157 in ruminal or fecal microbial fermentations were analyzed separately. Concentrations (Log_{10} CFU/ml) of *Nal*^{R} *E. coli* O157 were analyzed as repeated measures over time (0, 6, 12 and 24 h) using the PROC MIXED procedure of SAS (Version 9.1; SAS Institute, Cary, NC) and included main effects of CE treatment, gluconic acid inclusion and hour of sampling. All two- and three-way interactions were included in the model. This experiment was repeated three times; therefore, the random effect of replication was included in the statistical model. Two-way comparisons were assessed with LSMEANS.

Results

Ruminal microbial fermentation. The concentration of *Nal*^{R} *E. coli* O157 was greater ($P <$ 0.01) in fermentations with gluconic acid compared to no gluconic acid at 12 and 24 h of fermentation, but not at 0 and 6 h ($P > 0.3$; Fig. 1A). In fermentation with ruminal microbial inoculum, the interaction between CE cultures and gluconic acid inclusion was not significant ($P = 0.61$) for *Nal*^{R} *E. coli* O157 concentration, suggesting that the CE treatment effect was similar whether *E. coli* O157 growth was stimulated by gluconic acid or not. The interaction of CE treatment and sampling h tended ($P = 0.09$) to affect the concentration of *Nal*^{R} *E. coli* O157. The concentrations of *Nal*^{R} *E. coli* O157 were not different ($P > 0.20$) between control, CE and NCE treatments at 0, 6 or 12 h, but at 24 h, mean *Nal*^{R} *E. coli* O157 concentrations were lower ($P <$ 0.01) in fermentations with CE treatment compared to both control and NCE treatments (Fig. 1B).

Fecal microbial fermentation. In fermentation with fecal microbial inoculum, the concentration of *Nal*^{R} *E. coli* O157 was affected ($P < 0.01$) by the interaction between CE or NCE cultures and gluconic acid, therefore, these factors were assessed simultaneously. Initially (0 h), mean *E. coli* O157 concentrations were similar ($P > 0.20$) across all treatments and control, but 6 h concentrations were greater ($P < 0.01$) in fermentations given gluconic acid, regardless of CE or NCE treatment (Fig. 2). At 12 h, a similar increase was observed, except that *Nal*^{R} *E. coli* O157 concentrations in fermentations given the CE cultures and gluconic acid were lower ($P < 0.01$) than control with gluconic acid or NCE treatment with gluconic acid, and greater than ($P < 0.05$) all treatments (control, CE, NCE) without gluconic acid (Fig. 2). The increase observed at 12 h continued for control fermentations with gluconic acid and NCE treated fermentations with gluconic acid, but not with CE treated fermentations with gluconic acid. The control treated fermentations with gluconic acid and NCE treated fermentations with gluconic acid had higher concentrations of *Nal*^{R} *E. coli* O157 at 24 h compared to the CE treated fermentations with gluconic acid ($P < 0.05$) and all fermentations (control, CE, NCE) that did not have gluconic acid. All fermentations not having gluconic acid and CE treated fermentations with gluconic acid all had similar ($P > 0.05$) *Nal*^{R} *E. coli* O157 concentrations at 24 h, and lower ($P < 0.01$) concentrations than control and NCE treatments with gluconic acid (Fig. 2).

Discussion

Our intention was to evaluate efficacy of *E. coli* CE cultures in an *in vitro* fermentation system and determine if efficacy was different when *E. coli* O157 growth was stimulated with gluconic acid inclusion. We utilized an additional strain of non CE *E. coli* to serve as a control in this study. The ability of this strain to competitively exclude *E. coli* O157 has not been

documented, but previously it has been utilized in fermentation studies to evaluate probiotics *in vitro* [33], competitive exclusion agents [25], and in studies using simulated gastric fluid acid to assess acid tolerance [36]. Gluconic acid is a common constituent of intestinal mucus [23]. Because *E. coli,* including *E. coli* O157, are able to metabolize gluconic acid via the Entner-Duodoroff pathway [23], this compound may serve as a selective or growth factor to *E. coli* O157 in ruminal or fecal fermentation. We hypothesize that this may partially explain why *E. coli* O157 is more prevalent in the hindgut of cattle [1, 14, 32] compared to the rumen, which is devoid of mucus production. Our study showed higher concentrations of Nal^R *E. coli* O157 in fermentations with gluconic acid compared to without gluconic acid, thus providing credence to our hypothesis.

In ruminal microbial fermentation, at 24 h, the CE treatment had lower Nal^R *E. coli* O157 concentrations than NCE and control, regardless of gluconic acid supplementation. However, in fecal microbial fermentation without gluconic acid supplementation, Nal^R *E. coli* O157 concentrations were relatively stable throughout the 24-h incubation, and CE treatment had no effect on the concentration of Nal^R *E. coli* O157. In fecal microbial fermentations with gluconic acid inclusion, the CE treatment was able to prevent the growth stimulation of *E. coli* O157 by gluconic acid yielding lower concentrations at 12 and 24 h compared to NCE treatment and control. This finding suggests that in the hindgut, the competitive exclusion by *E. coli* cultures may be more efficacious with high concentrations of *E. coli* O157. A CE product containing strains of *E. coli* may be more advantageous than other microbial species because *E. coli* can compete for nutrients such as gluconic acid, since metabolic pathways are similar to those of *E. coli* O157.

The effectiveness of CE cultures to reduce *E. coli* O157 concentrations was more evident in fermentations with ruminal microbial inoculum compared to the fecal microbial inoculum. In calf challenge studies utilizing these strains and additional generic *E. coli* strains, *E. coli* O157 was not isolated from the rumen at necropsy in probiotic treated calves (n =6), but was isolated from the cecum and colon of one of these calves [37]. In our *in vitro* study, the CE cultures were only efficacious in the fecal microbial inoculum when *E. coli* O157 growth was stimulated by gluconic acid. This is a potential limitation to the *in vitro* approach in that nutrients which would normally be readily available in the live animal may be depleted quickly in batch culture fermentation. The *in vitro* approach does, however offer a simple method to evaluate the CE cultures and future experiments may be useful to determine the exact mode of action of these CE strains.

In conclusion, CE cultures reduced Nal^R *E. coli* O157 concentration in ruminal microbial fermentation at 24 h, but in fecal microbial fermentation differences between CE and controls were only evident when *E. coli* O157 was stimulated by gluconic acid.

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Table 6.1 Concentration of competitive exclusion (CE) and non-competitive (NCE) strains of *Escherichia coli* used for treatments in batch culture fermentation.

Replication	CE *Escherichia coli* cultures			NCE *Escherichia coli* culture (ATCC 25922) inoculated, CFU/ml of fermentation
	ATCC 202018 CFU/50 µl of culture	ATCC 202019 CFU/50 µl of culture	Pooled cultures of ATCC 202018 and ATCC 202019 inoculated[1], CFU/ml of fermentation	
1	1.2×10^9	1.5×10^9	5.2×10^7	1.4×10^7
2	1.1×10^8	1.2×10^8	4.4×10^6	1.9×10^6
3	3.5×10^{10}	1.4×10^8	7.0×10^8	4.4×10^8
Average	1.2×10^{10}	5.9×10^8	2.5×10^8	1.5×10^8

ATCC = American type culture collection.

[1]Equal parts of the two CE cultures were mixed and 100 µl of the mixture was pipetted into each fermentation bottle**.**

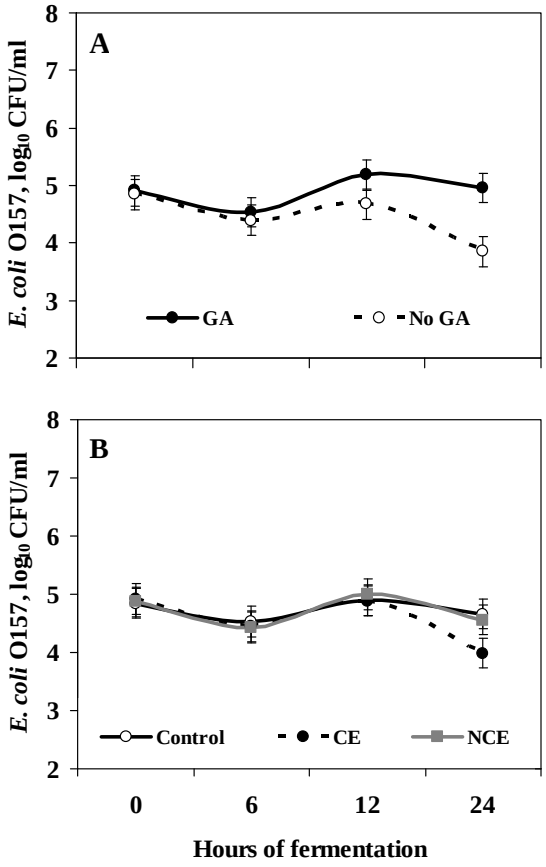

Fig. 6.1 Least squares means of *Escherichia. coli* O157 concentrations at 0, 6, 12 and 24 hours of fermentation in ruminal microbial inoculum with or without gluconic acid (A) and with control, competitive exclusion, (CE), and non competitive exclusion cultures (NCE) (B). Error bars represent standard error of the mean. The interaction between gluconic acid supplementation and competitive exclusion culture treatments was not significant ($P = 0.61$).

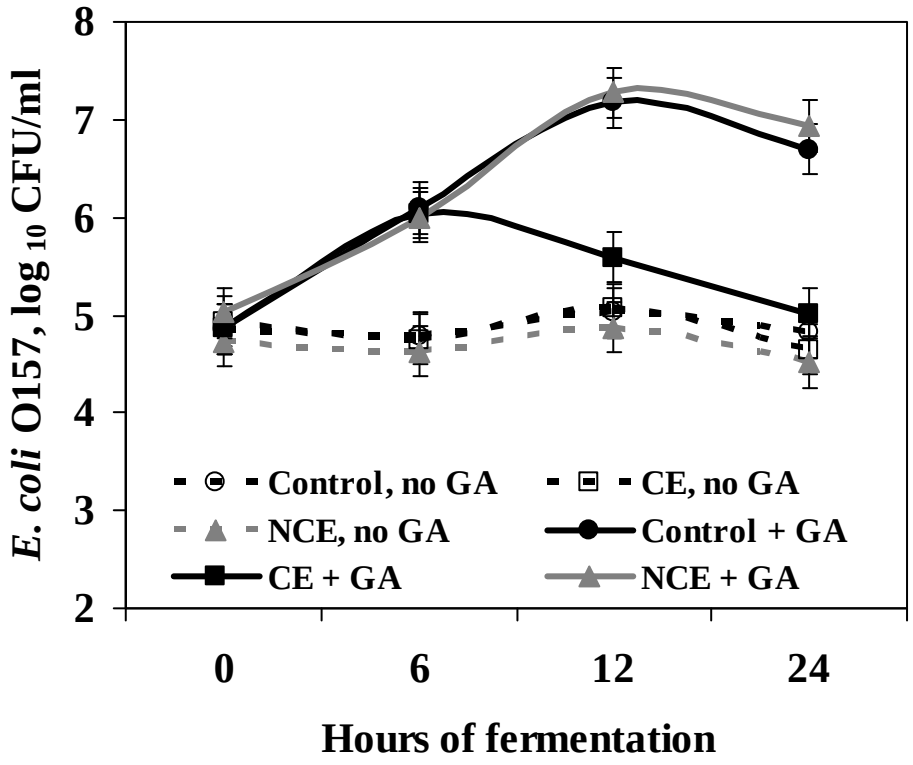

Fig. 6.2 Least squares means of *Escherichia coli* O157 concentrations after 0, 6, 12 and 24 hours of fermentation in fecal microbial inoculum. Error bars represent standard error of the mean. The interaction between competitive exclusion treatments and gluconic acid supplementation (GA) was significant ($P < 0.01$).

CHAPTER 7 - Efficacy of *Escherichia coli* O157:H7 siderophore receptor/porin proteins-based vaccine in feedlot cattle naturally shedding *Escherchia coli* O157

We evaluated the efficacy of an anti-*Escherichia coli* O157 siderophore receptor and porins (SRP) proteins-based vaccine in feedlot cattle naturally shedding the organism. Vaccine, at 1,500 or 1,000 μg protein per animal, was injected subcutaneously 21 days apart. The control was injected with sterile saline emulsified with an adjuvant. The SRP vaccine at 1,500 μg of dose reduced the prevalence of *E. coli* O157, the number of days cattle tested culture positive, and the number of days cattle were identified as high-shedders compared to the control. A similar trend was seen with 1,000 μg dose, but differences were not significant.

Key words: Pre-harvest, *E. coli* O157, vaccine

1. Introduction

Many human foodborne illnesses are caused by pathogens commonly harbored by food animals. *Escherichia coli* O157 is one of the major pathogens frequently isolated from cattle feces, which is a major source of contamination of beef carcass at harvest, produce and water. In addition to the human health concerns, the pathogen has major economic implications in the cattle industry. Costly recalls of beef products and loss of consumer confidence associated with outbreaks of foodborne illnesses affect profitability of cattle producers at many levels of production. In the past 10 years, *E. coli* O157 has been estimated to cost the beef cattle industry $2.67 billion [1]. Methods to intervene and reduce the opportunity of this pathogen to enter the food chain have been tested and implemented both at pre- and post-harvest stages.

A relatively new vaccine technology developed by Epitopix (Wilmar, MN) targets the pathogenic gram negative bacterial requirement for iron. The vaccine specifically targets siderophore receptor and porin proteins (SRP) of specific bacterial species or serotypes to disrupt their iron transport system, which ultimately causes death of the organism [2]. Preliminary experiments have shown that SRP vaccines reduce fecal shedding of *Salmonella* Newport and *E. coli* O157 in experimentally infected mice [2]. In two experiments involving experimentally inoculated cattle, an *E. coli* O157 SRP vaccine reduced fecal shedding of the inoculated strain of *E. coli* O157 [2-3]. Because of the efficacy of the *E. coli* O157:H7 SRP vaccine in cattle challenged with *E. coli* O157, the objective of the current experiment was to evaluate the efficacy in feedlot cattle naturally shedding *E. coli* O157.

2. Materials and methods

2.1 Vaccine preparation

The *E. coli* O157:H7 siderophore receptor and porin (SRP®) vaccine composition was produced under good laboratory practices under USDA regulatory compliance at Epitopix manufacturing facility (Willmar, MN). The vaccine strain was isolated from the feces of a steer in a commercial feedlot and was identified by somatic and flagellar serotyping and by PCR for major virulence genes. The strain was positive for *eae* and *hlyA* and negative for *stx1* and *stx2*. Seed stocks of the isolate were prepared and stored at -80C until used for vaccine production. Breifly, the steps in the vaccine manufacturing process included scale up from the working seed to commercial scale fermentation under conditions of iron-restriction. After fermentation, the cells expressing SRPs on the outer membrane were collected by tangential flow filtration and disrupted by homogenization. The disrupted bacterial suspension was then differentially solubilized using a biodegradable anionic surfactant. During this process the siderophore receptor and porin proteins in the solubilized bacterial suspension form insoluble aggregates that were collected by continuous flow high speed centrifugation. The harvested protein was resuspended in Tris buffer pH 8.5 and washed/concentrated by tangential flow diafiltration against physiological saline. The final dialyzed/concentrated SRP suspension was stored at 4 C until use. The electrophoretic profile of the vaccine composition, examined by SDS-PAGE, showed proteins of molecular weights of approximately 90 kDa, 86 kDa, 81 kDa, 78 kDa, 66 kDa, 56 kDa, 38 kDa, 37 kDa, and 29 kDa (Data not shown).

The aqueous suspension containing the SRP proteins was emulsified in a commercial adjuvant (Emulsigen, MVP Laboratories, Ralston, NE). The placebo for the control group was physiological saline emulsified with the same adjuvant at the same ratio. Two bottles labeled A and B (vaccine and placebo) were provided to the study personnel so that the all personal were

blind to the vaccine treatment. One bottle held the vaccine which contained the siderophore receptor and porin proteins while the other bottle held the placebo.

2.2 Experimental animals and approach

In the initial screening phase of the study, a population of approximately 600 feedlot heifers, housed in the Kansas State University Beef Cattle Research Center, was screened for *E. coli* O157 shedding in the feces. Feces were collected via rectal palpation or grabbed during defecation from individual animals restrained in a hydraulic working chute. Cattle positive for fecal shedding of *E. coli* O157 (n = 82) were re-sampled three or four days later to confirm shedding. At re-sampling, fecal samples and rectoanal mucosal swab (RAMS) samples were collected to identify animals that were shedding the organism (positive or negative), shedding the organism at high concentration ($> 10^3$) or positive for RAMS suggesting possible colonization of *E. coli* O157 at the terminal rectum [4]. The RAMS, collected prior to the fecal sample, was obtained by swabbing the inside of the rectum approximately 2 to 5 cm proximal to the anus [5]. Fecal samples were collected after RAMS samples to reduce potential fecal contamination of the swab. Sixty cattle were selected from the subpopulation of 82 for use in the experimental phase of the study. Fifty of the 60 cattle were fecal positive for *E. coli* O157 on two sampling occasions and the remaining 10 cattle were fecal positive on one occasion. Cattle were stratified based on results of fecal shedding of *E. coli* O157 in screening and re-screening samples and randomly allotted, within strata, to one of three treatment groups (20 animals/treatment). The stratification ensured that a similar number of high-shedding animals were allotted to each treatment. Treatments consisted of: 1) 3 ml sterile physiological saline emulsified with the adjuvant (placebo); 2) *E. coli* O157 SRP vaccine containing 1,000 μg of emulsified protein antigen (2 ml vaccine), or 3) *E. coli* O157 SRP vaccine containing 1,500 μg of emulsified protein

antigen (3 ml vaccine). Vaccines and the placebo were injected subcutaneously on days 0 and 21. Cattle were housed in one of three barns each containing 20 individual pens. Cattle were allocated to pens in treatment blocks within barn to eliminate sharing of waterers across treatments and reduce animal to animal contact across treatments. The assignment of treatments to barns and pens is shown in Fig. 1. Waterers were cleaned three times weekly to reduce the potential of these as a transmission source. Cattle were fed a standard feedlot receiving diet (Table 1) once daily. All animal procedures were performed in accordance with the Kansas State University Institutional Animal Care and Use Committee.

2.3 Sample collection during experimental phase

Fecal and RAMS samples were collected twice a week for the first three weeks (days 0, 4, 7, 11, 14 and 18), three times a week for the next five weeks (days 21, 25, 27, 29, 32, 34, 36, 39, 41, 43, 46, 48, 50, 53 and 55), and then once during the final week (day 57) to monitor shedding of *E. coli* O157. Samples were obtained by removing the animals from the individual pens and moving the animals through processing facilities where animals could be restrained for sampling. Blood samples were collected via jugular venipuncture on day 0, 7, 14, 21, 29, 36, 43, 50 and 57 of the experiment to monitor antibody titers against *E. coli* O157 siderophore receptor/porin proteins.

2.4 Isolation of E. coli O157 from feces collected during screening

In the initial screening of cattle for *E. coli* O157 in the feces, approximately 1 g of feces was placed in to a test tube containing 9 ml Gram-Negative (GN) broth (BD, Franklin Lakes, NJ.) containing cefixime (Sigma-Aldrich, St. Louis, MO; 50 ng/ml), cefsulodin (Sigma-Aldrich; 10 μg/ml) and vancomycin (Sigma-Aldrich; 8 μg/ml; GNccv). Test tubes were placed in a cooler and transported to the Kansas State University College of Veterinary Medicine Preharvest

Food Safety Laboratory (approximately 5 km). Upon arrival in the laboratory, tubes containing fecal samples in GNccv broth were vortexed for 1 min and incubated at 37°C for 6 h. Following the enrichment, tubes were vortexed for 1 min and 1 ml from each tube was subjected to immunomagnetic separation (IMS; Dynal, Inc. New Hyde Park, NY), and plated onto sorbitol MacConkey agar (BD) containing cefixime (Sigma-Aldrich; 50 ng/ml) and potassium tellurite (Sigma-Aldrich; 2.5 µg/ml) (CT-SMAC). Plates were incubated overnight (16 to 18 h) and up to six sorbitol-negative colonies were transferred on to blood agar (Remel, Lenexa, KS) for 12 to 18 h at 37°C. Blood agar colonies were tested for indole production, latex agglutination for the O157 antigen (Oxoid Limited, Basingstoke, Hampshire, England), and species were confirmed by API (Rapid 20E; Biomerieux, Inc., Hazelwood, Mo).

2.5 Isolation of E. coli O157 from feces or RAMS during re-screening and experimental phase

Following the initial screening, cattle that tested positive were re-screened to confirm the presence of *E. coli* O157. In re-screening, RAMS samples were collected and placed into a test tube containing 3 ml GNccv broth and transported to the laboratory in a cooler. In the lab, RAMS samples were vortexed for 1 min, and enriched for 6 h at 37°C. Isolation and identification of *E. coli* O157 from RAMS samples were similar to that fecal samples at screening described above. For fecal samples collected at re-screening and the experimental phase, approximately 2 g of feces of each sample was placed into a pre-weighed test tube containing 18 ml GNccv broth. Tubes were placed in a cooler and transported to the laboratory. In the laboratory, tubes were weighed again to determine the weight of the fecal sample. The procedures for isolation and identification of *E. coli* O157 were as described above. Also, an additional quantification procedure was used just prior to enrichment to identify cattle shedding high-concentrations (> 10^3 CFU/g) of *E. coli* O157 in the feces (Fox et al., 2007). Briefly, tubes

containing approximately 2 g of feces in 18 ml GNccv were vortexed for 1 min, and then a loopful of fecal suspension (10 µl; broad tip of a sterile calibrated bacterial loop; Fisher Scientific, Palatine, IL)) was streaked for isolation onto CT-SMAC plates, in triplicate, and incubated for 16 to 18 h at 37°C. From direct streaked CT-SMAC plates, up to two sorbitol negative colonies (from each plate) were transferred to blood agar plates and evaluated for indole production, latex agglutination for the O157 antigen (Oxoid Limited) and then the species was confirmed by API (Rapid 20E). Three assessments of prevalence were used in this study. Fecal positive cattle had *E. coli* O157 isolated from either direct plating or IMS procedure. If *E. coli* O157 was isolated from the RAMS sample, then the animal was considered RAMS positive and the third measure was to combine the fecal and RAMS tests and interpret results in parallel, i.e. if the animal was positive in at least one of these tests then it was considered positive. The last method is referred to as "combined" in this paper. Cattle were considered high-shedders of *E. coli* O157, if the organism was isolated and identified from two or three of the direct streak plates [6].

2.6 Anti-SRP antibody titer determination

An ELISA was used to determine serum antibody responses to SRP vaccination. Ninty-six-well microtiter plates (Maxisorp plates, Nalge Nunc International, Rochester, NY) were coated with *E. coli* O157 SRP extracted antigens (Lot #20070307), at 250 ng protein per well in carbonate coating buffer (Sodium carbonate 1.59 g/L, Sodium bicarbonate 2.93 g/L, pH 9.6), covered with lids, and incubated overnight at 4°C. The following day, coating buffer was removed, plates were patted dry and 200 µl of blocking buffer, 1% poly vinyl alcohol (Sigma-Aldrich) in phosphate buffered saline (PVA/PBS), was added to each well. Plates were covered and incubated for one to two hours at 37°C. Plates were emptied, patted dry and serially diluted

serum samples (four-fold dilutions from 1:100 through 1:102,400 prepared in 1% PVA/PBS were added in duplicate to the plates. The plates were covered and incubated for one hour at 37°C. The plates were then washed three times with 0.05% tween/PBS wash buffer in an ELISA plate washer (Biotek ELx405, address). One hundred µl of horseradish peroxidase conjugated sheep anti-bovine IgG H&L (Kirkegaard and Perry Labs. Inc., Gaithersburg, Md) was diluted 1:1,600 in 1% PVA/PBS containing 1% sheep serum and added to each well of the microtiter plate. Plates were covered and incubated for one hour at 37°C. After washing same as above), the plates were developed by adding 100 µl per well of 2-component ABTS® Peroxidase Substrate (2,2'-azino-di-3-ethylbenzthiazoline-6-sulfonate) (Kirkegaard & Perry Laboratories), at a concentration of 0.3 g/L in a glycine/citric acid buffer. The plates were incubated at room temperature until the absorbance 405/490 nm of the positive control wells were between 0.8 and 1.2. The absorbance of the plates was read in an ELISA plate reader (BioTek ELx405, Winooski, Vermont).

The positive control serum used was from a calf hyperimmunized with *E. coli* O157 SRP antigen. Sample titers were evaluated using positive control serum to determine a cut-off. Titers were defined as the reciprocal of the dilution of the sample whose value was equal to 50% of the cut-off.

2.7 Statistical analyses

Prevalence of *E. coli* O157 in feces or RAMS in the three treatment groups on day 0 of the experimental phase was analyzed using a simple chi-square test in PROC FREQ of SAS (Version 9.1, SAS Institute, Cary, NC). The prevalence of *E. coli* O157 in fecal, RAMS or combined (fecal or RAMS) samples during the experimental phase of the study, was analyzed using logistic regression in PROC GLIMMIX of SAS. The dependent variable was the animal

culture positive or negative for *E. coli* O157 in a given sampling week, thus a binomial distribution. Initially, the fixed effects of treatment, week and the treatment × week interaction were included in the logistic regression models. This was done to evaluate the significance of the interaction. Final logistic regression models included treatment and week as main effects, a random effect of barn, and repeated measures on animal to represent the lack of independence of collecting multiple samples from animals. Reported prevalence estimates were calculated from the logistic regression output by taking the antilog of the parameter estimate and dividing by one plus the antilog of the parameter estimate.

The number of days cattle tested positive for *E. coli* O157 in fecal, RAMS, or fecal or RAMS combined were analyzed with a non-parametric, permutation test performed with PROC MULTTEST of SAS. For these analyses, treatment was the class variable and contrast statements were used for two-way comparisons of treatments. A similar analysis was performed to determine difference between treatment groups with regard to the number of days cattle were shedding the pathogen at high concentration. The outcome for each animal was the number of days it was identified as a high-shedding animal.

Anti-SRP titers were log transformed and analyzed using PROC MIXED of SAS with treatment, week and the treatment × week interaction as main effects. Repeated measures on animals were accounted for in this model. Geometric means were calculated as the antilog of least squares means for data presentation.

3. Results

3.1 Anti-SRP antibody titers in placebo or SRP vaccinated cattle

Anti-SRP antibody titers were determined for animals weekly. On d 0 of the experimental phase, antibody titers were similar ($P > 0.70$) among all three treatment groups. However, in all ensuing weeks, anti-SRP antibody titers were greater ($P < 0.01$) in both vaccine treatments compared to the placebo (Fig. 2). Titers were similar between 2 ml and 3 ml vaccine treatments for most weeks, but on week two ($P = 0.06$) and week four ($P = 0.02$), titers were greater in the 3 ml vaccine group.

3.2. Prevalence of E. coli O157

The prevalence of *E. coli* O157 on day 0 of the experimental phase was 20.0% in fecal and 10.0% in RAMS samples and was similar ($P > 0.18$) among treatment groups. Preliminary logistic regression models revealed that the treatment × week interaction was not significant for any of the measures of prevalence (Fecal, $P = 0.97$; RAMS, $P = 0.94$; Fecal or RAMS, $P = 0.91$) and was removed from the final model to facilitate model convergence. Overall fecal prevalence estimates (across all sampling weeks) of *E. coli* O157 in cattle receiving placebo, 2 ml vaccine, and 3 ml vaccine were 24.6, 18.1, and 13.2%, respectively. Prevalence in feces was statistically different ($P = 0.02$) between placebo and 3 ml vaccine treatments, while the 2 ml vaccine treatment was similar to both the placebo and 3 ml vaccine groups ($P = 0.21$ and 0.26, respectively; Fig. 3). Prevalence in RAMS samples in cattle receiving placebo, 2 ml vaccine and 3 ml vaccine were 18.5, 16.4 and 10.4, respectively. Prevalence in RAMS was statistically different ($P = 0.04$) between placebo and 3 ml vaccine treatments, with the 2 ml vaccine treatment again being intermediate (Fig. 3). When fecal and RAMS results were combined, prevalence estimates of *E. coli* O157 for cattle receiving placebo, 2 ml vaccine, and 3 ml vaccine

was 33.7, 29.1, and 17.7%, respectively, and was statistically different between placebo and both vaccine treatments ([2 ml vaccine, $P = 0.02$; 3 ml vaccine, $P < 0.01$] Fig. 3).

3.2 Days culture positive for E. coli O157

In the experimental phase of this project, cattle tested positive for *E. coli* O157 on an average of 2.7 sampling days in feces, 2.4 sampling days in RAMS and 3.5 sampling days in combined samples. In the analysis of number of days cattle tested positive, we observed that the mean number of days cattle tested fecal positive or fecal or RAMS positive (combined) for *E. coli* O157 was statistically greater ($P < 0.05$) in placebo compared to 3 ml vaccine treatment (Fig. 4). However, this difference was not observed in the number of sampling days cattle tested RAMS positive ($P = 0.17$). In cattle given 2 ml vaccine, the mean number of sampling days positive for *E. coli* O157 by fecal or RAMS (combined) tests was statistically similar to both the placebo ($P = 0.76$) and the 3 ml vaccine-treated cattle ($P = 0.23$; Fig. 4).

3.3 Days positive for fecal shedding of E. coli O157 at high concentration

In the experimental phase of this study, 13 cattle tested positive for shedding *E. coli* O157 at high concentrations ($>10^3$ CFU/g). In the 3 ml vaccine treatment group, two animals tested positive for high-shedding on one sampling day and in the 2 ml vaccine treatment group, four animals tested positive for high-shedding on one sampling day. However, in the placebo treatment group, three animals were high-shedders on one sampling day, two animals were high-shedders on two sampling days and two animals were high-shedders on three sampling days. The mean number of sampling days cattle tested positive as high-shedders was statistically greater ($P = 0.02$) in placebo-treated cattle compared to 3 ml vaccine-treated cattle and tended to be greater ($P = 0.07$) placebo-treated cattle compared to 2 ml vaccine-treated cattle (Fig. 5).

4. Discussion

Because of the importance of *E. coli* O157 as a foodborne pathogen and the correlation between fecal prevalence and subsequent carcass contamination [7], a reduction in preharvest prevalence or elimination of the organism altogether will likely reduce the risk of outbreaks of human foodborne illnesses. Also, because cattle manure is implicated as a source of contamination for waterborne *E. coli* O157 [8-9] and *E. coli* O157 in produce [10], reducing prevalence and/or concentration of *E. coli* O157 in cattle feces prior to harvest may reduce these sources of illness in humans.

The use of vaccine to reduce *E. coli* O157 fecal shedding is not a novel pre-harvest strategy. Mice vaccinated against O-specific polysaccharide derived from *E. coli* O157 lipopolysaccharide and Shiga toxin 1B subunit derived from *Vibrio cholera* resulted in a the production of complement-dependent antibodies against *E. coli* O157 and neutralization antibodies against Stx1 [11]. An intimin vaccine derived from transgenic plant cells and delivered orally to mice, reduced the number of days mice shed *E. coli* O157 following experimental challenge with the pathogen compared to control [12]. In another study, an intimin vaccine given to pregnant dams induced production of anti-intimin titers in both serum and colostrum, and piglets from these dams that were experimentally inoculated with *E. coli* O157, had reduced colonization and intestinal damage from the inoculated *E. coli* O157. Potter et al. [13] developed a vaccine which targeted type III secretion system proteins of *E. coli* O157. Initial data showed efficacy of this vaccine in both challenged and naturally shedding cattle [13]. However, when this vaccine was tested in Canadian feedlots, no statistical association was observed between vaccine treatment and pen prevalence of *E. coli* O157 [14]. Immunizing

animals against a siderophore receptor (IroN) has previously shown to have a protective effect against extraintestinal pathogenic *E. coli* [15]. Previous results in our laboratory has demonstrated efficacy of the anti-*E. coli* O157 SRP vaccine in a experimentally challenged calves [3].

We observed that vaccination with either 1,000 or 1,500 μg of SRP protein antigen resulted in a 10-100 fold increase in anti-SRP antibody titers compared to the placebo. However, previous research has shown that vaccinating calves with EspA, a type III secreted protein and colonization factor for *E. coli* O157, generates an antigen-specific response, but did not protect the calves from being colonized with *E. coli* O157 [16]. Similar results were observed by vaccinating calves with intimin and enterohemorrhagic *Escherichia coli* factor for adherence (Efa-1) [17]. In our study, the 3 ml vaccine treatment effectively reduced prevalence of *E. coli* O157, the number of days cattle tested positive, and the number of days cattle were identified as high-shedders compared to the placebo. We were interested in identifying cattle shedding the pathogen at high concentrations because modeling efforts by Matthews et al. [18] revealed that 80% of natural transmission of *E. coli* O157 in a cattle population is attributed to 20% of infections in which animals are shedding the organism at abnormally high concentrations ($>10^4$ or 10^5 CFU/g). Reducing the number of animals shedding at high concentrations would be an important outcome of pre-harvest intervention strategy.

Overall prevalence by sampling day (16%) in a subset screened for fecal shedding of *E. coli* O157 was lower than expected. A study utilizing the similar natural prevalence model was conducted one year earlier in the same feedlot facility and observed an average *E. coli* O157 prevalence of 50% [19]. Utilizing animals that are naturally shedding the pathogen is an effective way to evaluate interventions without the costs associated with euthanizing

experimentally challenged animals or with large numbers of samples in epidemiological investigations [19]. Additionally, this approach may have more external validity for evaluating vaccine efficacy compared to animals that are challenged with a laboratory strain of *E. coli* O157. Because prevalence was low, model convergence became an issue in data analyses, thus data were analyzed as repeated measures in animals over weeks instead of sampling days. This increased prevalence (30%) and allowed us to analyze the data. We expect any bias introduced by this analysis to be non-differential between treatment groups, but we also examined the mean number of days cattle tested positive to confirm the results observed with prevalence.

We acknowledge that the assignment of treatments to pens in blocks may have compromised the independence of experimental units, but we felt this was necessary as the facilities were not designed for the evaluation of infectious agents and we needed to accurately represent the treatment assignments. Based on the treatment assignment, we would expect any factors which may potentially bias results, such as sharing of waterers, pen drainage and fence-line contact to impact treatments equally.

In summary, we found that the *E. coli* O157 SRP vaccine at 1,500 μg of protein antigen per animal reduced the prevalence of *E. coli* O157, the number of days cattle tested positive, and the number of days cattle were identified as high-shedders. A similar numerical trend was also seen with 1,000 μg of antigen per animal per administration, but the differences were not significant.

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Table 7.1

Ingredient composition and nutrient content of the experimental diet.

Ingredients:	Percent of diet dry matter
Steam-flaked corn	50.3
Alfalfa hay	40.0
Corn steep liquor	4.0
Vitamin/trace mineral premix	5.7
Nutrients:	
NEm	1.83 Mcal/kg
NEg	1.21 Mcal/kg
CP	14.5%
Ca	0.70%
P	0.33%
Ca:P	2.1:1

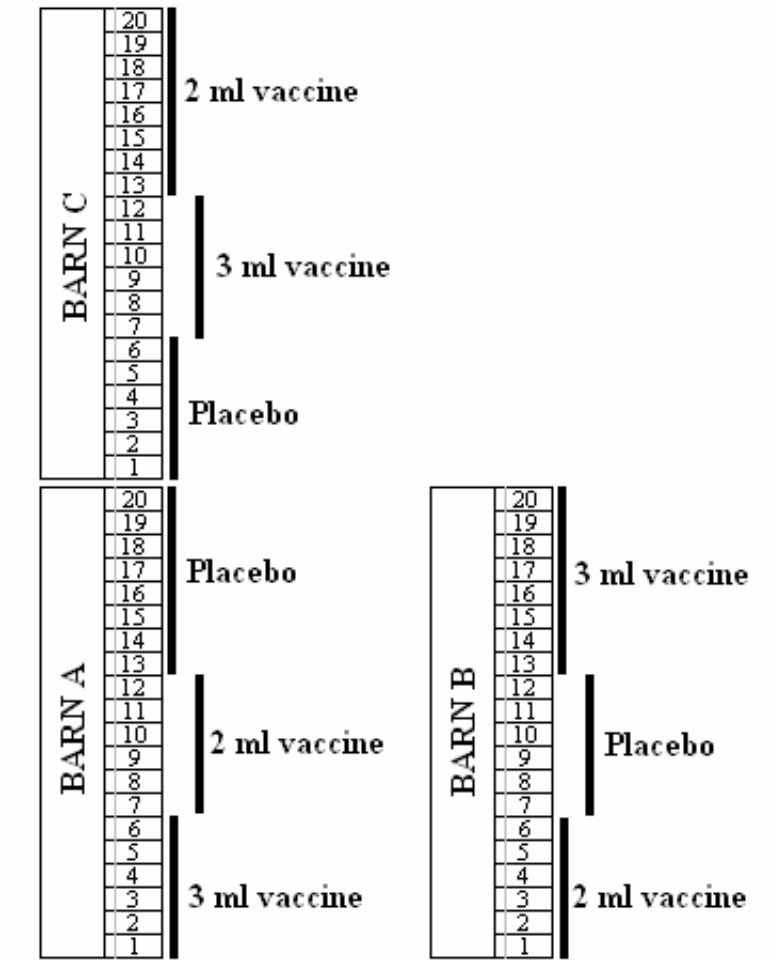

Fig. 7.1 Layout of barns, pens and treatment assignments.

Waterers were shared between pairs of adjacent pens.

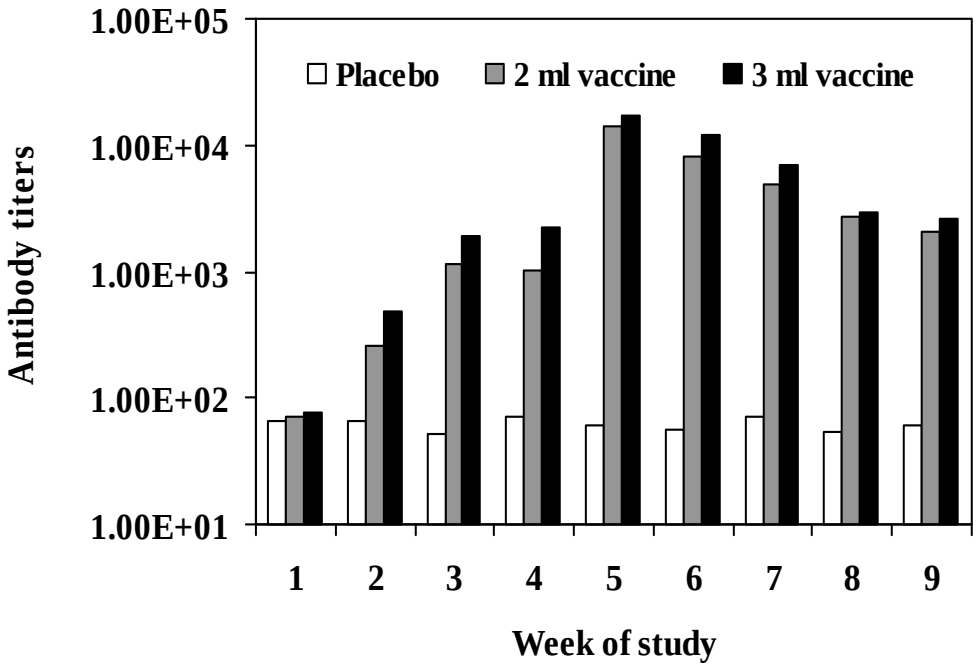

Fig. 7.2 Anti-SRP antibody titers from serum collected from cattle given placebo or *E. coli* O157 SRP vaccine. Statistical analysis was performed on log transformed titer values which were statistically greater ($P < 0.01$) for vaccinated cattle compared to placebo cattle for all weeks of the study except week 1.

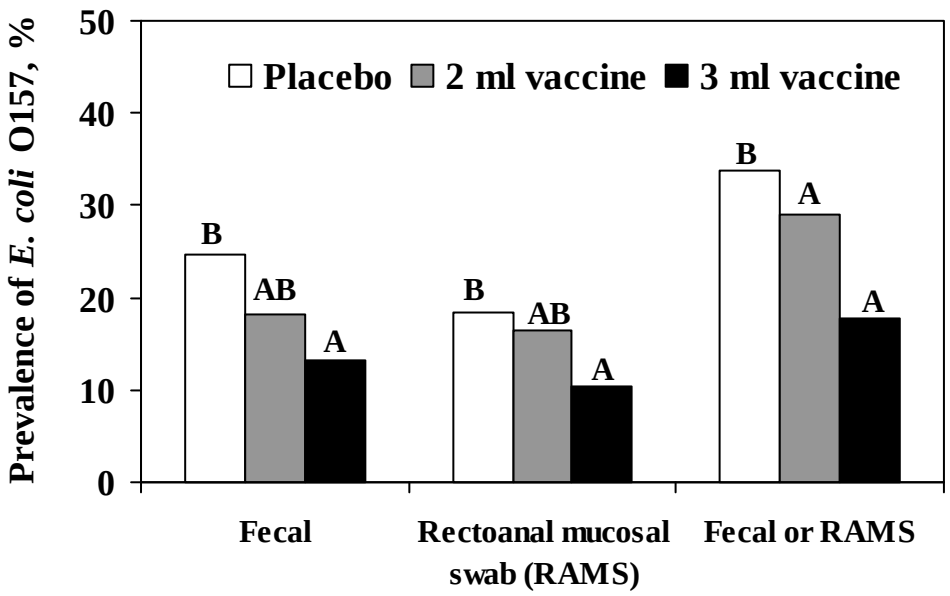

Fig. 7.3 Prevalence of *Escherichia coli* O157 in fecal, rectoanal mucosal swab or combined tests. Samples for placebo or vaccine- treated feedlot cattle. Bars not sharing a common letter are different ($P < 0.05$).

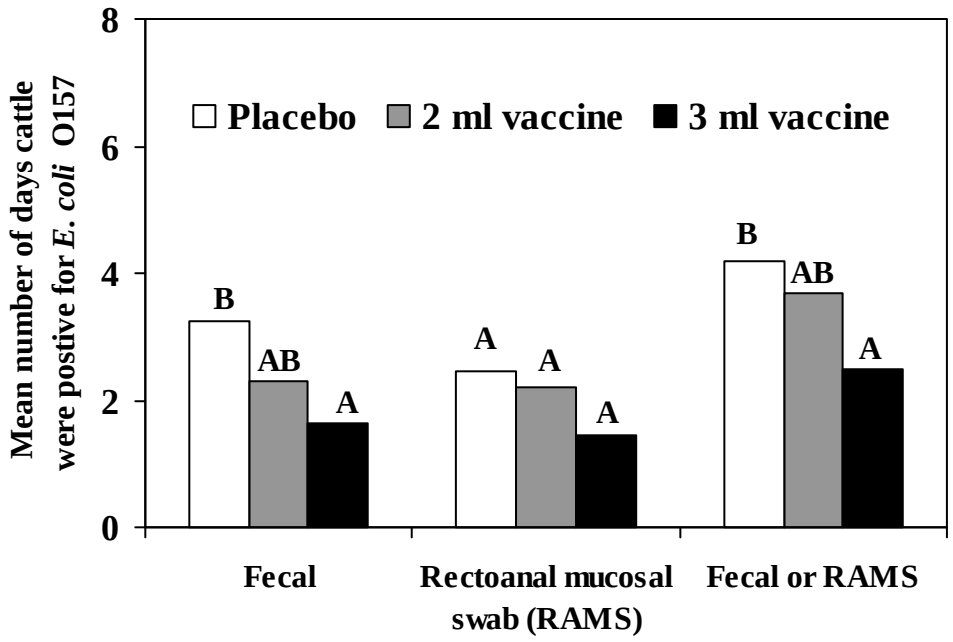

Fig. 7.4 Mean number of days cattle tested positive for *Escherichia coli* O157 by fecal, rectoanal mucosal swab culture, or combined tests for placebo and vaccine treated feedlot heifers. Cattle were sampled a total of 22 days in this experiment. Bars not sharing a common letter within sample type are different ($P < 0.05$).

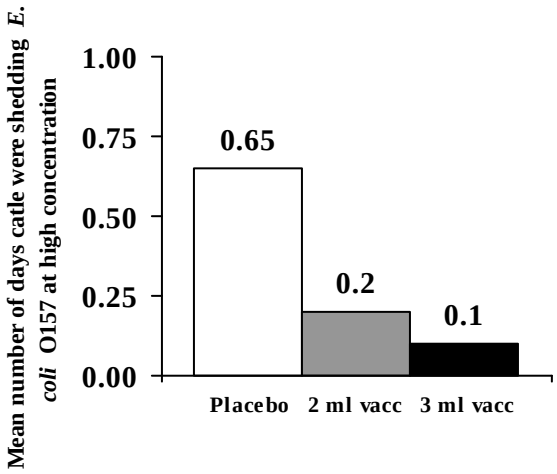

Fig. 7.5 Mean number of days cattle were shedding *Escherichia coli* O157 at high concentrations in the feces. Cattle were tested on a total of 22 days in this experiment. Means were different between placebo and 3 ml vaccinated group ($P = 0.02$) and tended ($P = 0.07$) to be different between placebo and 2 ml vaccinated group, but not between 2 ml and 3 ml vaccinated groups ($P = 0.86$).