# THE EFFECT OF DISTILLER'S GRAINS ON THE PREVALENCE AND CONCENTRATION OF *ESCHERICHIA COLI* O157 IN CATTLE

by

MEGAN E. JACOB

B.S., University of Wyoming, 2005
M.S., Kansas State University, 2007

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
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# Abstract

*Escherichia coli* O157 is a major foodborne pathogen that causes enteritis in humans ranging in severity from mild to bloody diarrhea to hemolytic uremic syndrome and even death. Cattle are asymptomatic carriers and fecal shedding of the organism is the major source of contamination of food and water for human infections. Distiller's grains (DG) are ethanol fermentation co-products that are valuable feed ingredients for use in cattle diets. Previous research suggests an association between feeding DG and an increased fecal shedding of *Escherichia coli* O157:H7. The objectives of the research were to evaluate fecal *E. coli* O157:H7 prevalence and concentration in cattle fed diets with and without DG, determine if the association was dependent on inclusion level or form (wet or dried), evaluate the association in populations of cattle at harvest, and evaluate a potential intervention strategy. Our results indicated that cattle fed DG had a higher prevalence and shed a higher concentration of *E. coli* O157 than cattle fed diets without DG. The relationship was not dependent on the DG form, however, it was affected by the inclusion level of DG in the diet. Cattle that were fed 40% DG had a higher *E. coli* O157:H7 prevalence than cattle fed control or 20% DG diets and cattle fed 20% DG had a prevalence that was not statistically different from control cattle. The same response was observed in a subpopulation of cattle, termed super-shedders, which shed *E. coli* O157:H7 at higher concentrations than the general population. At harvest, we did not find differences in *E. coli* O157:H7 or super-shedder prevalence between cattle fed diets with or without DG, however, study design limitations affected the power of the study. Finally, previous work had shown that cattle fed dry-rolled grains had a decreased prevalence of *E. coli* O157:H7 when compared to cattle fed steam-flaked grains. We evaluated the effect of feeding DG and dry-rolled corn (DRC), alone or in combination, and observed no difference in *E. coli* O157 prevalence between cattle fed either DG or DRC diets. In conclusion, DG supplementation increased the prevalence and concentration of *E. coli* O157:H7 in cattle.

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# Preface

*Escherichia coli* O157:H7 are Shiga toxin-producing bacterial foodborne pathogens that cause gastroenteritis, hemolytic uremic syndrome and occasionally death in people. Cattle serve as a reservoir for *E. coli* O157:H7 in which the organism can asymptomatically colonize the hindgut and be shed in the feces. Cattle feces are major source of contamination for food products, water, and the environment. The prevalence of *E. coli* O157:H7 in cattle is highly variable and has been associated with many factors including season, age, and diet. The type of grain, grain processing method, and proportion of grain to forage in cattle diets are associated with different *E. coli* O157:H7 prevalence estimates. In a preliminary study, we observed that cattle fed distiller's grains had higher *E. coli* O157 prevalence on one of two collection days than cattle fed control diets with no distillers grains; this relationship was also reported in two epidemiological based studies evaluating multiple management factors.

Distiller's grains are a co-product derived from the fermentation of cereal grains for ethanol production. During ethanol production, the starch portion of the grain is removed and the remaining nutrients, protein, fiber, and fat are concentrated approximately three-fold. The product is fed to cattle as a protein or energy source. Distillers grains can be fed in a wet or dehydrated (dried) form and can comprise anywhere between 10 and 50% of cattle diets, depending on the purpose and economics of the product. Because of the increased demand for fuel ethanol, the availability of these co-products has increased dramatically in recent years and they are a useful feed ingredient for cattle producers.

The objective of this research was to characterize the association between feeding cattle distiller's grains and the fecal shedding of *E. coli* O157:H7. Specifically, studies were conducted to evaluate *E. coli* O157:H7 prevalence and concentration in cattle fed diets with and without distillers grains, determine if the association was dependent on distillers grains inclusion level or form (wet or dried), evaluate the association in populations of cattle at harvest, and identify potential interactions with pre-harvest *E. coli* O157:H7 intervention strategies. The dissertation contains the following studies:

# CHAPTER 1 - Dietary Interactions and Interventions Affecting *Escherichia coli* O157 Colonization and Shedding in Cattle

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## Abstract

*Escherichia coli* O157 is an important foodborne pathogen affecting human health and the beef cattle industry. Contamination of carcasses at slaughter is correlated to the prevalence of *E. coli* O157 in cattle feces. Many associations have been made between dietary factors and *E. coli* O157 prevalence in cattle feces. Pre-harvest interventions, such as diet management, could reduce the fecal prevalence and diminish the impact of this adulterant. Dietary influences including grain type and processing method, forage quality, and distillers grains have all been associated with *E. coli* O157 prevalence. In addition, several plant compounds including phenolic acids and essential oils have been proposed as in-feed intervention strategies. The specific mechanisms responsible for increased or decreased *E. coli* O157 shedding or survival are not known but are often attributed to changes in hindgut ecology induced by diet types. Some interventions may have a direct bacterial effect. Frequently, results of studies are conflicting or not repeatable, which speaks to the complexity of the hindgut ecosystem, variation in animal feed utilization, and variation within feed products. Still, understanding specific mechanisms, driven by diet influences, responsible for *E. coli* O157 shedding will aid in the development and implementation of better and practical pre-harvest intervention strategies.

## Introduction

According to USDA Food Safety and Inspection Service (FSIS) estimates, more than 33 million pounds of beef products were recalled during 2007, and more than 7 million pounds were recalled in 2008 for possible *Escherichia coli* O157:H7 contamination (http://www.fsis.usda.gov/FSIS_Recalls/). These dramatic numbers indicate potential health implications for humans and economic repercussions for the beef industry. *Escherichia coli* O157, a Shiga toxin-producing serotype of *E. coli*, is an important foodborne pathogen associated with enteritis in thousands of people in the United States every year. In more severe cases, infection can lead to hemolytic uremic syndrome, and possibly death (Rangel et al., 2005). In cattle, a primary reservoir of *E. coli* O157, the organism colonizes the gut and is shed in the feces. Cattle feces are a major source of contamination of food products (Rangel et al., 2005). Because of the risk to human health and economic burden of recalls to the cattle industry, it is important to understand the ecology of *E. coli* O157 in cattle, as well as develop and implement strategies to reduce colonization and shedding.

*Escherichia coli* O157 generally colonizes the lower gastrointestinal tract of cattle, specifically the mucosal area of the terminal rectum (Naylor et al., 2003; Low et al., 2005). Calves are likely colonized early in life (Gannon et al., 2002), and the organism is generally considered part of the normal gastrointestinal flora of cattle and not associated with disease. Presence of *E. coli* O157 in cattle feedlots appears fairly ubiquitous (Sargeant et al., 2003), however, several variables including season, geographic location and diet have been associated with increased prevalence (Herriott et al., 1998; Bach et al., 2002; Dewell et al., 2005; Renter et al., 2008; Chase-Topping et al., 2008). Although many associations have been made, explanations or reasons why specific factors influence *E. coli* O157 presence in cattle remain largely unknown.

Because of its location in the gut, *E. coli* O157 colonization and survival are likely affected by gastrointestinal conditions including pH, concentration of volatile fatty acids (VFA), presence of competing organisms, and, possibly, other unknown factors. Diet, which impacts the gastrointestinal conditions, is frequently associated with *E. coli* O157 prevalence (Dargatz et al., 1997; Herriott et al, 1998; Callaway et al., 2003b; Fox et al., 2007; Jacob et al., 2008a). Often, results of studies evaluating associations between diets or diet components and *E. coli* O157 prevalence are conflicting or not repeatable, which speaks to the complexity of hindgut ecology

and mechanisms responsible for increased or decreased colonization and fecal shedding. Still, feeds and feeding management have been proposed as possible pre-harvest intervention strategies (Callaway et al., 2003a; Loneragan and Brashears, 2005; LeJeune and Wetzel, 2007). The primary objective of this review is to highlight dietary components or practices that have been associated with *E. coli* O157 prevalence in ruminants, primarily cattle, and provide insight into factors that may be beneficial in developing intervention strategies. Although feed additives currently in use or being developed including ionophores, probiotics, and sodium chlorate have been associated with *E. coli* O157prevalence, they are not included in this review.

## Grain Type and Processing

Cattle are typically fed high energy grain diets to increase weight gain and efficiency of feed conversion; grain type in diets has been linked to *E. coli* O157 prevalence. Barley grain has been positively associated with *E. coli* O157 shedding in both observational and experimental studies (Dargatz et al., 1997; Buchko et al., 2000; Berg et al., 2004). Specifically, Berg et al. (2004) reported that cattle shed a higher concentration of *E. coli* O157 and had higher fecal pH when fed a barley grain diet compared with cattle fed a corn-based diet. Interestingly, concentrations of generic *E. coli* populations were higher in corn-fed cattle (6.2 log CFU/g) than in barley-fed cattle (5.6 log CFU/g), similar to the grain and forage effect reported by Diez-Gonzalez (1998). Although hypothesized to be a change in hindgut ecology, the specific mechanism responsible for increased *E. coli* O157 shedding in barley fed cattle is not known. Barley, which has lower starch content than other traditional cereal grains (Huntington, 1997), is more rapidly and completely digested in the rumen (Theurer, 1986; Ørskov, 1986) and results in less undigested starch for secondary fermentation in the large intestine. Therefore, cattle fed barley grain-based diets have an increased pH and decreased VFA concentrations in the hindgut. A study to evaluate the survival of inoculated *E. coli* O157 in fecal samples from cattle fed either barley or corn diets found few differences in the pathogen survival; however, pH and VFA concentrations were generally similar between the two diets before *E. coli* O157 disappeared (Bach et al., 2005b).

In addition to the hypothesized rationale of increased hindgut starch concentration in corn-fed cattle, one study evaluated effects of supplementing canola oil in barley- and corn-based diets because the total oil content between the two grain types is likely different and may impact

hindgut conditions (Bach et al, 2005a). Fats or oils could have a direct impact on *E. coli* O157 because fatty acids, particularly unsaturated fatty acids, have antibacterial activity (Galbraith and Miller, 1973). Fecal shedding of inoculated *E. coli* O157 was not different between diets in this study, and although fecal pH was lower and VFA concentrations were higher in corn-fed than in barley-fed cattle, there was no effect on shedding, fecal pH or VFA concentration with supplementation of canola oil (Bach et al., 2005a).

An association between *E. coli* O157 prevalence and diets containing cottonseed has also been inconsistent. Cottonseed, which has high oil content, could affect the hindgut ecosystem. Garber et al. (1995) reported a negative association between feeding whole cottonseed to heifers and fecal shedding of *E. coli* O157 in a case-control study; Hancock et al. (1994) reported similar findings. Others have shown no relationship between the two factors (Dargatz et al., 1997; Herriott et al., 1998; Buchko et al., 2000). Several other positive associations between feed types like corn silage (Herriott et al., 1998) and *E. coli* O157 shedding in cattle have been reported sporadically, but again, these observations are limited (e.g. Dargatz et al., 1997).

The processing method used to prepare cereal grains for cattle diets also affects substrate availability in the hindgut. Processing grains with heat, moisture, or mechanical treatment will increase starch degradation in the rumen, which in turn influences starch availability and fermentation in the lower gastrointestinal tract (Huntington, 1997). Fox et al. (2007) reported that grain processing method affected *E. coli* O157 prevalence in cattle. In that study, heifers fed steam-flaked grains (more completely digested in the rumen) had higher *E. coli* O157 prevalence than heifers fed dry-rolled grain diets (less completely digested in the rumen) on most sampling days. They hypothesized that dry-rolled grains provided more substrate (starch) to the hindgut, reducing pH and creating an inhospitable environment for *E. coli* O157. The authors measured fecal pH as an indicator for fermentation activity in the hindgut and found no significant difference between cattle fed the two types of processed grains (Fox et al., 2007). Depenbusch et al. (2008) also showed a trend of higher *E. coli* O157 prevalence in cattle fed steam-flaked grain diets compared with cattle fed dry-rolled grain diets for 30 days. In one of the two experiments, positive *E. coli* O157 samples were associated with greater fecal starch concentration; however, neither fecal starch nor fecal pH was associated with *E. coli* O157-positive samples in a second experiment (Depenbusch et al, 2008). An increased fecal starch concentration does not support the hypothesis that increased substrate negatively affects *E. coli* O157. In an observational study

of cattle in Midwestern feedlots, Dewell et al. (2005) found no significant effect of grain processing on *E. coli* O157 prevalence in cattle.

In an Australian study, steam-flaked sorghum or rolled barley resulted in increased fecal generic *E. coli* concentrations compared with diets with whole sorghum or barley (Gilbert et al., 2005). The whole grains were associated with higher fecal starch concentrations and higher fecal pH. Because fecal pH and starch concentration are not consistently associated with *E. coli* O157 (Depenbusch et al, 2008; Gilbert et al., 2005), neither is likely entirely responsible for the association of steam-flaked grains with higher *E. coli* O157 prevalence. Still, it does appear different processing methods (steam-flaking and dry-rolling) may affect *E. coli* prevalence and concentrations.

## Forage Quality

Similar to the difference reported between grain type and processing, differences in *E. coli* O157 prevalence have been reported in ruminants fed diets with different forage qualities. Kudva et al. (1995) reported that switching experimentally inoculated sheep from an alfalfa pellet diet to a low quality forage diets increased *E. coli* O157 shedding. In another study, *E. coli* O157 was inoculated in fecal samples from cattle fed straw, low-digestible grass silage, and highly digestible grass silage plus maize silage and the survival was analyzed (Franz et al., 2005). The authors reported a faster rate of decline in concentrations of *E. coli* O157 in low quality forages, associated with higher pH and fiber content, which contradicts the work of Kudva et al. (1995). Perhaps these differences could be explained by phenolic acids found in the different forage types (described later).

## Forage and Grain Diets

Studies evaluating effects of forage and grain diets on the fecal shedding of *E. coli* O157 in ruminants are perhaps the most numerous and conflicting (Table 1.1). Experimental inoculation studies of both sheep and cattle have shown that animals fed forage diets shed *E. coli* O157 in the feces for a longer duration than animals consuming grain-based diets (Kudva et al., 1997; Hovde et al., 1999; Van Baale et al. 2004). However, not all studies have found significant differences in *E. coli* O157 prevalence between these feed types (Tkalcic et al., 2000; Fegan et al., 2004). Additionally, a study evaluating survival of inoculated *E. coli* O157 in

manure samples from cattle fed hay- or silage-based diets had conflicting results, which were related to the duration the donor animals were on feed (Wells et al., 2005).

Generally, the rationale for a positive association is an increased ruminal and/or hindgut pH and decreased VFA concentrations associated with the forage diet, which contribute to a more hospitable environment for *E. coli* O157 survival and colonization. Van Baale et al. (2004) found an increased fecal and ruminal pH in calves fed forage diets, which complemented their *E. coli* O157 shedding results. This rationale is logical considering that when ruminants are fed grain diets, starch can be fermented in the rumen or pass through before secondary fermentation occurs in the cecum and colon, lowering pH and increasing VFA concentrations (Huntington, 1997). Russell et al. (2000) reported that grain feeding had a greater effect on fermentation and bacterial populations in the hindgut than in the rumen.

Interestingly, Diez-Gonzalez et al. (1998) reported significantly higher total *E. coli* concentrations in feces of cattle fed concentrate diets compared with feces from cattle fed forage diets. As expected, lower pH and higher VFA concentrations were observed in cattle fed grain than in cattle fed forage diets (Diez-Gonzalez et al., 1998). Similar findings with generic *E. coli* have been reported by others (Krause et al., 2003; Gilbert et al., 2005). The association between these generic *E. coli* and *E. coli* O157 populations is not known. However, Diez-Gonzalez (1998) reported that increased concentrations of acid-resistant *E. coli* were observed in cattle fed diets with grain than in cattle fed a diet with no grain. It has been suggested that *E. coli* O157 survival is favored in low pH and high VFA concentration conditions (Russell et al., 2000). Acid resistance has been shown to occur in *E. coli* O157 incubated in rumen fluid (Tkalcic et al., 2000). However, not all studies report differences in acid-resistance of *E. coli* O157 between grain- and forage-based diets (Hovde et al., 1999; Van Kessel et al., 2002; Grauke et al., 2003). Fu et al. (2003) speculated that *E. coli* O157 growth and acid resistance depend on both pH and VFA concentrations. Others have shown that under anaerobic conditions, short-chained fatty acids in human fecal samples can suppress *E. coli* O157 growth (Shin et al., 2002). More complete reviews on acid resistance and other forage feeding effects have been published previously (Russell et al., 2000; Callaway et al., 2003b). Nevertheless, the role of acid-resistance on *E. coli* O157 survival and prevalence is highly debated and still not well understood.

## Distillers Grains

Because of increased availability due to increased ethanol production, distillers grains, an ethanol fermentation coproduct usually derived from corn, are included in cattle diets as a protein and energy source (Klopfenstein et al., 2008). After the starch from corn is fermented to ethanol, the remaining nutrients (protein, fiber, and fat) are concentrated approximately three-fold and fed to cattle in a wet or dehydrated form (Klopfenstein et al, 2008). Other cereal grains can be fermented in a similar manner. Several studies have demonstrated an association between feeding ethanol co-products (distillers or brewers grains) and *E. coli* O157 prevalence in cattle. In 2003, Synge et al., investigating management factors associated with *E. coli* O157 shedding, initially reported an association in Scottish cattle fed distillers grains. This observation was also seen in U.S. feedlots with brewers grains, a coproduct of the brewing industry (Dewell et al., 2005). Differences in the probability of detecting *E. coli* O157 in the terminal rectum of cattle fed varying levels of distillers grains were reported in a vaccine trial; however, the relationship was not linear (Peterson, et al., 2007). In a study aimed at evaluating the effect of feeding distillers grains on *E. coli* O157 shedding, cattle fed dried distillers grains with solubles (DDGS) at 25% of the final diet had a two-fold higher prevalence of the organism than cattle not fed DDGS (Jacob et al., 2008a). Likewise, a challenge model using calves orally inoculated with *E. coli* O157 and fed one of two diets, with or without 25% DDGS, found that calves fed distillers grains shed higher concentrations of *E. coli* O157 at the end of the study and had a higher concentration in gut contents at necropsy than calves in the control group (Jacob et al., 2008b). Persistence of the organism was also different in experimentally inoculated manure slurries from cattle fed varying levels of wet distillers grains with solubles (WDGS; Varel et al., 2008). In that study, *E. coli* O157 concentrations were greater for a longer duration in cattle fed 20 and 40% WDGS than in manure slurries from cattle fed 0% WDGS. Although the potential association between dietary distillers grains and *E. coli* O157 prevalence and/or persistence in cattle has been well described, statistically significant associations have not always been found (Jacob et al., 2009). Regardless, there is no published data to suggest distillers grains decrease the *E. coli* O157 prevalence or concentration in cattle.

The mechanism responsible for the trend of increased *E. coli* O157 when feeding distillers grains in cattle is not known. Similar to other dietary components, two general mechanisms have been proposed: 1) Distillers grains alter the hindgut ecology of cattle, making a more suitable environment for *E. coli* O157 or 2) A component of distillers grains stimulates

the growth of *E. coli* O157 (Jacob et al., 2008a). It is not unexpected that hindgut ecology changes when cattle are fed distillers grains. Klopfenstein et al. (2008) described the high ruminal escape property of protein in dried distillers grains diets, which could provide more protein in the hindgut and result in increased degradation and ammonia concentration. Also, the starch content of corn has been removed in distillers grains, which allows for less secondary fermentation compared with corn-based diets. In addition, distillers grains have previously been shown to alter rumen microbial populations (Fron et al., 1996). There is some evidence from *in vitro* ruminal fluid fermentations that *E. coli* O157 growth was actually stimulated compared with control fermentations; however, this was not observed in fecal fermentations, which may be expected if the site of action is the lower gut (Jacob et al., 2008a). Clearly, more research is needed before this association can be explained. Physiological factors beyond those altered by feeding distillers grains likely contribute to the discrepancy between some studies, and the increasing use of distillers grains is not solely responsible for *E. coli* O157 prevalence in cattle.

## Dietary Interventions

### *Seaweed products*

There are a few reports on the ability of a commercially available brown seaweed product derived from *Ascophyllum nodosum* (Tasco-14$^{TM}$) to reduce *E. coli* O157 shedding in cattle. This product has been shown to improve carcass characteristics in slaughtered cattle (Braden et al., 2007). Using two pens of cattle in a commercial feedlot, Braden et al. (2004) found that feeding 2% Tasco-14$^{TM}$ reduced prevalence of *E. coli* O157 on hides and in fecal samples at slaughter when compared with the control group. The same product was used in a study with calves experimentally inoculated with *E. coli* O157:H7 (Bach et al., 2007). Pens of inoculated calves were fed a control diet or the seaweed at different levels (10 or 20 g/kg diet) for 7 or 14 days. Over the sampling period, mean *E. coli* O157:H7 concentrations and the frequency of obtaining a positive sample were lower from animals fed Tasco-14$^{TM}$ at 10 and 20 g/kg for 14 and 7 days, respectively, compared with animals fed the control treatment (no Tasco-14$^{TM}$) or Tasco-14 $^{TM}$ at 20 g/kg for 14 days. The mechanism for a decrease in fecal *E. coli* O157 shedding in cattle administered Tasco-14$^{TM}$ is not known but is hypothesized to be a direct microbial effect (Braden et al., 2004). This was supported by the work of Bach et al. (2007) who

found no changes in VFA concentrations or pH in fecal samples from the four treatments. This seaweed product may have potential as a pre-harvest intervention strategy.

### *Phenolics/essential oils*

Plants commonly synthesize phenolic compounds for defense against microorganisms and predators. The antimicrobial nature of these compounds is believed to be enzyme inhibition by oxidized compounds or interactions with proteins that have not been described (Cowan, 1999). There is some evidence to suggest that phenolic compounds can be inhibitory to *E. coli* O157. Survival of *E. coli* O157 inoculated into cattle fecal samples decreased, particularly when higher concentrations (0.5%) of *trans*-cinnamic and *para*-coumaric acids were applied to the samples (Wells et al., 2005). Only one of these compounds, *trans*-cinnamic acid, affected fecal pH (Wells et al., 2005), so it is not known whether inhibition was pH mediated or the result of a direct microbial effect. Tannins, a more complex phenolic compound observed in hydrolyzable and condensed types (Cowan, 1999), have also been evaluated for efficacy as an inhibitory substance for *E. coli* O157. In-vitro incubations showed that both tannin types decreased the growth rate of *E. coli* O157 in pure culture and were bactericidal, more so with the hydrolyzable type, to *E. coli* O157 (Min et al., 2007). In addition, the hydrolyzable tannin was shown to reduce fecal *E. coli* concentration in an *in vivo* experiment with cattle fed hay diets (Min et al., 2007).

It has been known for some time that essential oils, which are also phenolic compounds, obtained from plants have antibacterial properties that can inhibit foodborne pathogens in pure culture (Dabbah et al., 1970; Burt and Reinders, 2003; Burt, 2004). Often, these oils are more effective against gram-positive organisms (Dabbah, et al., 1970; Fisher and Phillips, 2006), although application of some plant oils has been shown to reduce coliform counts in stored manure (Varel and Miller, 2001). Although the specific mechanism of action is compound-dependent, the ability of oils to disrupt membranes and ion concentrations generate their antibacterial properties (Cowan 1999; Burt, 2004). Nannapaneni et al. (2008) demonstrated the susceptibility of *E. coli* O157:H7 isolates to two of seven orange essential oils by agar-disk diffusion. Recently, *in vitro* ruminal fluid fermentations with varying concentrations of orange peel or dried orange pulp, which contain essential oils, were shown to decrease inoculated *E. coli* O157:H7 (Callaway et al., 2008). The concentration of citrus oil reaching the lower gut, the

colonization site for *E. coli* O157, is unknown. However, because the organism can also be in the rumen (Laven et al., 2003) feeding orange pulp products may be a useful in-feed intervention strategy. Feeding citrus products, primarily dried pulp, to cattle as a source of energy is common in citrus-producing regions like Florida (Wing, 2003). Further work is needed to assess *in vivo* efficacy of these products; however, if proven to work they are potentially useful, particularly in diets of beef and dairy cattle in regions where the coproducts are available.

## Mechanisms and Implications

It is generally accepted that most cattle shedding *E. coli* O157 do so at a concentration of $<10^3$ CFU/g feces; however, there appears to be extreme individual animal variation, with some shedding the organism at much higher levels (e.g. $>10^4$ CFU/g feces; Low et al., 2005; Chase-Topping et al., 2007). These animal variations, particularly when animals shed a large concentration of *E. coli* O157, likely contribute to overall group prevalence (Matthews et al., 2006) but have not always been linked to diet and are not well understood. Other factors including season have frequently been associated with *E. coli* O157 prevalence. These factors show that the biological relationship between *E. coli* O157 and the ruminant reservoir is likely more complex than diet influences alone. However, because feed components continue to be associated with prevalence, understanding these interactions may allow us to exploit the mechanisms for potential pre-harvest intervention. It is difficult to assess the specific effect of different diet components on *E. coli* O157 growth and colonization in vivo. The true prevalence of *E. coli* O157 independent of any diet influence in cattle is not known so differences attributed to one component increasing the shedding cannot easily be distinguished from another component decreasing shedding.

The difference in prevalence observed between different diets is often attributed to changes in hindgut ecology, primarily pH and VFA concentrations; although in the case of phenolic and seaweed interventions it may be direct microbial effects. The pH and VFA concentrations throughout the rumen and intestine are directly related to feed composition; however, studies evaluating dietary influences on *E. coli* O157 rarely report these values. Even when reported, results are not always consistent and provide only a generalized hypothesis. One reason for the inconsistencies in pH and VFA concentration data may be the inherent differences in component utilization between animals. Ørskov (1986) reports a difference in starch

fermentation of 35% between two sheep fed an identical corn diet. Another reason for inconsistencies is the variability in nutrient composition between feed products including silage where the starch content is influenced by inclusion level and plant maturity (Huntington, 1997). Finally, dietary influences are sometimes reported using generic *E. coli* populations as a model for *E. coli* O157. There are inconsistencies in the response of *E. coli* O157 and more generic *E. coli* populations to pH and dietary influences. When used as a model, these results are difficult to interpret and suggest that *E. coli* may not always be equivalent for assessing an *E. coli* O157 response to diet influences (Grauke et al., 2003).

One proposed mechanism for *E. coli* O157 inhibition in the hindgut is an increase in secondary starch fermentation (Fox et al., 2007). Starch content can vary by grain type and processing method, but passage rate and consumption also contribute to the amount of starch initially fermented in the rumen (Huntington, 1997). Generally, a large percentage of starch (80-95%) is fermented in the rumen, and a considerable portion of the remaining starch undergoes digestion in the small intestine (Huntington, 1997). Still, starch that escapes the rumen and small intestine can undergo secondary fermentation in the large intestine, similar to ruminal fermentation, and result in changes in pH and VFA concentrations (Ørskov et al., 1970). The effect of starch or glucose infused both ruminally and/or abomasally into steers was shown to lower cecal and fecal pH compared with controls; however, total anaerobic and aerobic counts were higher (Van Kessel et al., 2002). There was no statistically significant difference in total *E. coli* counts. Diets with higher starch contents generally decrease the concentration of acetate while increasing propionate and butyric acid concentrations (Ørskov et al., 1970). Still, more specific effects of these specific short-chain fatty acids are not well described. In pure culture, propionate was shown to reduce viability of *E. coli* O157 at 37°C (McWilliam Leitch and Stewart, 2002). Antimicrobial activity toward *E. coli* O157 in this study was actually greater for lactate, another organic acid derived from glucose. Sensitivity of *E. coli* O157 to lactate has been described elsewhere (Jordan et al., 1999). In addition, Krause et al. (2003) showed that lactic acid bacteria showed results similar to *E. coli*, increasing in concentration in grain-fed cattle. The effect of these results, specifically as they relate to *E. coli* O157 is not known, however, lactic acid bacteria have been shown to have anti-*E. coli* O157 effects (Brashears et al., 2003).

Few studies relate dietary fiber content to *E. coli* O157 shedding in cattle, and fiber content is often not measured in fecal samples. However, dietary fiber is known to alter physiology and stimulate growth of bacteria in the human colon (Cummings and Stephen, 1980). Possibly, increased fiber content stimulates increased mucus production in the hindgut. Using in vitro models, Fox et al. (2008) showed that mucus components, particularly gluconic acid, stimulated *E. coli* O157 growth. Higher fiber content was associated with *E. coli* O157 decline in inoculated fecal samples from dairy cattle fed different forage diets (Franz et al., 2005). Additionally, Lema et al. (2002) reported that lambs inoculated with *E. coli* O157 and fed 5% dietary acid detergent fiber (ADF) shed a significantly higher concentration than animals with a higher dietary ADF percentage (10-35%). In both studies, increasing fecal pH values were seen with higher fiber content samples. Higher fiber content may be confounded by other dietary influences such as starch because of diet composition. The association between *E. coli* O157 and distillers grains, with concentrated fiber components and decreased starch content, is just one example of this complexity. Feeding high lipid content may also alter the hindgut ecosystem and affect *E. coli* O157; however, studies assessing various oils (canola and cottonseed) have generally shown no association. The lipid content in distillers grains is also concentrated, but again, these results are likely confounded by other factors (starch, fiber, etc.). Interestingly, essential oils, including those from citrus products have some direct antimicrobial property, have shown some efficacy, and may be a useful intervention strategy.

In conclusion, to better understand effects of diet on *E. coli* O157 colonization and shedding in cattle, more specific work to confirm and identify differences beyond pH and VFA concentrations is needed. Many inconsistencies regarding dietary influences on *E. coli* O157 are reported in the literature; however, variability in nutrient composition, animal utilization, and processing methods influence these physiological conditions and make repeatable results challenging. Although difficult, work to distinguish between confounding factors such as fiber and starch may add clarity to any potential mechanism associated with increased *E. coli* O157 colonization. In addition, the organism-host relationship is likely far more complex than dietary influences alone, and the response to dietary changes of other microbial populations, possibly other foodborne pathogens, is not known. Still, if simple mechanisms can be exploited or existing or new compounds with direct anti-*E. coli* O157 activity can be developed, practical pre-

harvest intervention strategies to reduce the economic and human health burden of this organism can be implemented.

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**Table 1.1 Effects of feeding grain- or forage-based diets on *Escherichia coli* O157 and generic *E. coli* shedding or survival in ruminant feces.**

| *Diet* | *Organism* | *Study design* | *Results* | *Reference* |
|---|---|---|---|---|
| 100% Grass vs.50% corn and 50% alfalfa | *E. coli* O157 | Sheep: experimental inoculation | Increased length of shedding in forage-fed | Kudva et al., 1997 |
| 62% barley + 19% corn, 90% corn, 100% alfalfa, and 100% hay | *E. coli* O157 | Cattle: experimental inoculation | Increased length of shedding in hay-fed | Hovde, et al., 1999 |
| 1.9 kg Bermuda grass + 3.8kg concentrate mix vs. 3.8 kg Bermuda grass + 1.9 kg concentrate mix | *E. coli* O157 | Cattle: experimental inoculation | No difference in fecal shedding | Tkalcic, et al., 2000 |
| 90% grain + 10% silage vs. 50% alfalfa hay + 50% grass hay | *E. coli* O157 | Cattle: experimental inoculation | No difference in fecal shedding | Grauke, et al., 2003 |
| Grass-fed vs. Lot-fed | *E. coli* O157 | Cattle: observation of natural prevalence | No difference in prevalence or concentration | Fegan, et al., 2004 |
| 85% forage + 15% grain vs. 15% forage + 85% grain | *E. coli* O157 | Cattle : experimental inoculation | Increased length of shedding in forage-fed | Van Baale, et al., 2004 |
| 100% hay vs. 88% corn silage + 9% cracked corn | *E. coli* O157 | Cattle: inoculation of feces | Death rate dependent on animal time on feed | Wells, et al., 2005 |
| No grain, 60% rolled corn, 80% rolled corn | Generic *E. coli* | Cattle: observation of natural *E. coli* population | Higher concentration in grain-fed | Diez-Gonzalez, et al., 1998 |
| 100% forage (grass) vs. 70% rolled sorghum + 30% grass | Generic *E. coli* | Cattle: observation of natural *E. coli* population | Higher concentration in grain-fed | Krause et al., 2003 |
| Roughage (+/-50% molasses) vs. 80% grain | Generic *E. coli* | Cattle: observation of natural *E. coli* population | Higher concentration in grain-fed | Gilbert et al., 2005 |

# CHAPTER 2 - Effects of Dried Distiller's Grain on Cattle Fecal Prevalence and Growth of *Escherichia coli* O157 in Batch Culture Fermentations

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## Abstract

Distiller's grains (DG), a byproduct of ethanol production, are fed to cattle. Associations between *Escherichia coli* O157 prevalence and feeding DG were investigated in feedlot cattle (n = 379) allocated to 1 of 3 diets: steam-flaked corn (SFC) and 15% corn silage with 0 or 25% dried DG (DDG); or SFC with 5% corn silage and 25% DDG. Ten fecal samples were collected from each pen weekly for 12 weeks to isolate *E. coli* O157. Cattle fed 25% DDG with 5 or 15% silage had a higher ($P = 0.01$) prevalence of *E. coli* O157 than cattle fed 0% DDG. Batch culture ruminal or fecal microbial fermentations were conducted to evaluate the effect of DDG on *E. coli* O157 growth. The first study utilized microbial inocula from steers fed SFC or dry-rolled corn with 0 or 25% DDG, and included their diet as substrate. Ruminal microbial fermentations from steers fed DDG had higher *E. coli* O157 growth than no DDG ($P < 0.05$) when no substrate was included. Fecal fermentations showed no DDG effect on *E. coli* O157 growth. In the second study with DDG as a substrate, ruminal fermentations with 0.5 g had higher ($P < 0.01$) *E. coli* O157 concentrations at 24 h compared to 0, 1, or 2 g DDG. In fecal fermentations, 2 g DDG resulted in a higher concentration ($P < 0.05$) at 24 h compared to 0, 0.5, or 1 g DDG. Results indicate a positive association between DDG and *E. coli* O157 in cattle, and the findings will have important ramifications for food safety.

## Introduction

Cattle are major reservoirs for *Escherichia coli* O157, a significant foodborne pathogen (19, 24, 32). Asymptomatic colonization of *E. coli* O157 in cattle occurs in the lower gastrointestinal tract, specifically the mucosal surface of the rectum (31), and the organism is shed in feces (2, 6). There are multiple variables that influence the prevalence and shedding of *E. coli* O157 in ruminants (2, 34, 37). One of these factors is diet (3, 5, 6, 10, 14, 40), which may suggest that diet influences the physiological environment of the gut for survival and establishment of *E. coli* O157.

Different diet components have been evaluated to identify associations with *E. coli* O157 shedding. An epidemiologic study revealed a positive association between cattle receiving barley grain in the diet and *E. coli* O157 prevalence in feedlot cattle (8), which was confirmed by natural prevalence and challenge model studies (3, 5). Several studies have indicated that forage-fed cattle will shed *E. coli* O157 in the feces for a longer duration than grain-fed cattle (21, 40). Increased amounts of starch reaching the hindgut can increase volatile fatty acid (VFA) production and reduce pH in the hindgut, altering the environment for growth and survivability of the organism (14, 35).

The fermentation of cereal grains for ethanol production results in a co-product called distiller's grains (DG), which can be used as a livestock feed. The co-product is the 'spent' fraction that remains after distillation of ethanol. The solid fraction, called wet distiller's grain (WDG; approx. 30% dry matter {DM}), may be used as a feed or dehydrated to produce dried distiller's grains (DDG; approx. 90% DM). After enzymatic fermentation of the starch portion of grain, the remaining fraction is concentrated in other nutrient components, including protein, fiber, and lipid (38). Because of the properties in the remaining nutrients (energy or protein), DG is well suited for ruminant diets and has been shown to increase daily gain in finishing cattle (20). The absence of starch, and the presence of relatively high concentrations of ruminal escape protein and bran (fiber) components of DG are likely to impact the ecology of the hindgut. Previous work by Dewell et al. (2005), found that fecal samples from cattle fed brewer's grains, a fermentative product similar to DG, were more likely to be positive for *E. coli* O157 than from cattle not fed brewer's grains. In a previous study (25), we observed that feedlot cattle fed WDG had a higher prevalence of *E. coli* O157 on one of two collection days, however, the positive association was apparent on both days. The objectives of this study were to determine the fecal prevalence of *E. coli* O157 in feedlot cattle fed grain diets supplemented with DG, and also

determine if DG stimulated growth of *E. coli* O157 using *in vitro* fermentations with ruminal or fecal microbial inoculum.

## Materials and Methods

### *Study 1*

**Animals, treatments and sampling.** Yearling heifers (n = 379) were allocated randomly to one of three treatment groups. Diets consisted of a combination of steam-flaked corn (SFC), dried corn distiller's grains (DDG), and corn silage, and were formulated to meet National Research Council requirements (30). Pens were fed diets in bunks once daily so that only a trace amount of feed remained the following day. Treatments included SFC with 15% corn silage (DM basis), SFC with 25% DDG and 15% corn silage, and SFC with 25% DDG and 5% corn silage. Each treatment was replicated in eight pens with 15 to 16 heifers per pen. Cattle were housed in dirt-floor feedlot pens and fence-line water units were shared between adjacent pens. Ten freshly voided pen-floor fecal samples were collected from each pen once weekly for 12 weeks (August through October; n = 2,877), placed in sterile bags and immediately transported to the laboratory.

**Isolation of *E. coli* O157.** Fecal samples were cultured for *E. coli* O157 as described by Greenquist et al., (2005). Briefly, samples were kneaded for 30 sec and approximately 1 g of feces was placed in 9 ml Gram-negative (GN) broth with cefixime (0.5 mg/L), cefsoludin (10 mg/L), and vancomycin (8 mg/L). The GN tubes were enriched for 6 h at 37°C. After enrichment, immunomagnetic bead separation (IMS) was performed on 1 ml of GN broth, followed by plating 50 µL onto sorbitol-MacConkey agar with cefixime (0.5 mg/L) and potassium tellurite (2.5 mg/L; ct-SMAC). Plates were incubated overnight at 37°C, and up to six sorbitol negative colonies were picked and transferred to blood agar plates (BAP; Remel, Lenexa, KS). Following incubation at 37°C for 16 to18 h, colonies were tested for indole production and latex agglutination for the O157 antigen. *Escherichia coli* O157 isolates were further characterized by multiplex PCR identifying *stx*1, *stx*2, and *eae* genes (12). The multiplex PCR program used to amplify targets included: initial denaturation at 95ºC for 3 min, 30 cycles of 95ºC for 20 sec, 58ºC for 40 sec, and 72ºC for 90 sec, and a final elongation at 72ºC for 5 min.

## *Study 2*

**Animals and sample collection.** Twelve ruminally-cannulated Holstein steers (BW = 359 ± 54 kg), allotted randomly in a 2 × 2 factorial arrangement of dietary treatments, were used as sources of ruminal fluid and fecal inocula for this study. The dietary factors were grain type (SFC or dry-rolled corn {DRC}) and level of DDG (0 or 25%). Steers were housed in individual concrete-floored pens and fed approximately 3% of their body weight once daily. Steers were adapted to their treatment diets for at least 3 weeks prior to sample collection. Ruminal fluid was collected from each steer, immediately strained through four layers of cheesecloth, and transported to the laboratory in a flask sealed with a butyl rubber stopper. Fecal samples were collected at the same time from each steer via rectal grab, placed in sterile bags, and transported to the laboratory. An equal volume of Ringer's solution was added to the fecal samples to approximate the dry matter content to that of ruminal fluid, stomached for 30 sec, strained through four layers of cheesecloth (29), and placed in a flask with a butyl rubber stopper.

**Fermentation treatment and bacteriological procedures.** Batch culture fermentations were set up with or without substrate in 70-ml serum bottles sealed with butyl rubber stoppers fitted with Bunsen valves. The substrate was 0.5 g of the respective steer's whole diet that was ground. Each bottle contained 50 ml of a fermentation mixture composed of 33 ml of McDougall's buffer (28) and 17 ml of the ruminal fluid or fecal microbial inoculum (2:1 buffer to inoculum ratio). The buffer and the inoculum were added under flowing oxygen-free $CO_2$ gas (22) to create and maintain an anaerobic environment within the bottles. Fermentations were then inoculated anaerobically (under flowing $O_2$ –free $CO_2$) with 100 µl (approximately $10^3$ CFU/ml of fermentation) of a five-strain mixture of *E. coli* O157 (strains 01-2-1863, 01-2-7443, 01-2-10004, 1-2-10530, and 01-2-12329; 36) made resistant to 50 µg/ml naladixic acid ($Nal^R$). Fermentations were kept at 37°C in an orbital incubator (80 rpm) and sampled anaerobically (under flowing $O_2$ –free CO2) at 0, 6, 12, and 24 h to determine concentrations of $Nal^R$ *E. coli* O157. Samples were serially diluted in buffered peptone water (Sigma-Aldrich, St. Louis, MO) and 0.1 ml of appropriate dilution was spread, in triplicate, to ct-SMAC plates with naladixic acid (50 µg/ml; 15). Ruminal fluid and fecal microbial fermentations were set up in duplicates and repeated on a different day using new samples collected from the same animals.

### *Study 3*

Two ruminally-cannulated Holstein steers adapted to high grain diets served as donors to collect ruminal fluid and fecal samples. Steers, fed SFC with 6% alfalfa hay and supplemented with either 0 or 25% DDG, were adapted to diets for a minimum of two weeks prior to sample collection. Steers were housed by diet in dirt-floor feedlot pens and were bunk-fed *ad libitum* once daily. Fermentations with ruminal fluid or fecal microbial inoculum were set up as described above except DDG was added as substrate at 0, 0.5, 1, or 2 g per fermentation. Samples were removed at 0, 6, 12, and 24 h to determine concentrations of $Nal^R$ *E. coli* O157 as before. Fermentations were repeated on a different day using new samples collected from the same steers.

**Statistical analysis.** In study 1, prevalence of *E. coli* O157 was analyzed using logit models in PROC GENMOD of SAS (v. 9.1; Cary, NC) with the numerator being the number of positive samples per pen and the denominator being total samples per pen (1). Diet and week were investigated as explanatory effects and pen was included as a repeated effect. In studies two and three, analyses were conducted with the MIXED procedure of SAS to determine differences in the $\log_{10}$ concentration of $Nal^R$ *E. coli* O157. In study two, fixed effects included diet, substrate (ground feed included or excluded in fermentation), DDG (present or absent in the feed), and hour. Effects evaluated from study three included DDG substrate concentration (0, 0.5, 1 or 2 g), hour and presence of DDG in animal diet. When appropriate, linear contrast statements were included to account for the linear increase in substrate. Experiment repetition was included as a random effect for both studies two and three.

## Results

**Prevalence of *E. coli* O157 in feedlot cattle (Study 1).** The mean prevalence of *E. coli* O157 in pen-floor samples throughout the 12-week study was 7.4% (213 of 2,877). The mean weekly *E. coli* O157 prevalence for cattle fed steam-flaked corn based high-grain diets with 5 or 15% corn silage and supplemented with or without 25% DDG are shown in Fig. 2.1. Prevalence ranged from 2.9% (weeks 1 and 7) to 17.1% (week 10), but week was not significantly associated with *E. coli* O157 prevalence ($P > 0.05$). Analysis of the data showed that diet tended to be associated with *E. coli* O157 prevalence in cattle ($P = 0.06$); therefore, dietary effects were

further examined. The prevalence of *E. coli* O157 was higher ($P = 0.01$) in cattle fed SFC with 25% DDG and 15% corn silage when compared to cattle fed SFS with no DDG and 15% corn silage (Fig. 2.2). Likewise, cattle fed SFC with 25% DDG and 5% corn silage had a higher ($P = 0.01$) prevalence of *E. coli* O157 than cattle fed SFC with no DDG and 15% corn silage. However, no difference ($P > 0.5$) in the prevalence was observed between cattle fed DDG with either 5 or 15% corn silage (Fig. 2.2). Characterization of virulence genes from *E. coli* O157 isolates are shown in Table 2.1. As expected, all the isolates were positive for the *eae* gene and contained either *stx*1 or *stx*2 genes. Diet had no influence on the prevalence of *eae, stx*1, and *stx*2 genes in *E. coli* O157 isolates.

**Growth of *E. coli* O157 during *in vitro* fermentations with ground complete diets with or without distiller's grain as the substrate (Study 2).** Ruminal fluid fermentations from cattle fed SFC had a higher concentration of $Nal^R$ *E. coli* O157 than fermentations from cattle fed DRC, but the difference was significant only at 24 h ($P < 0.01$; Fig. 2.3). The ruminal microbial fermentations from cattle fed DDG had higher $Nal^R$ *E. coli* O157 concentrations at 24 h compared to cattle fed no DDG ($P < 0.05$) when substrate was not included in the fermentation (Table 2.2). However, if substrate was included in ruminal fluid microbial fermentations, there were no differences in $Nal^R$ *E. coli* O157 concentrations between fermentations containing DDG or no DDG ($P > 0.4$). In fecal microbial fermentations, DDG had no effect on the concentration of $Nal^R$ *E. coli* O157, regardless of substrate inclusion (Table 2.2). There were, however, differences in $Nal^R$ *E. coli* O157 concentrations between cattle fed DRC and SFC. This effect was only statistically significant when substrate was excluded from the fermentation. Cattle fed SFC had higher concentrations of $Nal^R$ *E. coli* O157 in fecal fermentations than those fed DRC ($P < 0.01$) at 12 and 24 h sampling intervals.

**Growth of *E. coli* O157 during *in vitro* fermentation with DDG substrate (Study 3).** *In vitro* fermentations with DDG as a substrate revealed several statistical differences in the concentration of $Nal^R$ *E. coli* O157 in both ruminal fluid and fecal microbial fermentations. In the 24 h sample of ruminal fluid fermentations, the concentration of $Nal^R$ *E. coli* O157 was significantly higher when 0.5 g of DDG substrate was included compared to 0 ($P < 0.01$), 1 ($P < 0.001$), or 2 g ($P < 0.001$) DDG (Table 2.3). When no DDG substrate was included in the

ruminal fluid fermentations, a higher concentration of *Nal*$^R$ *E. coli* O157 was observed at 24 h than when 1 ($P < 0.01$) or 2 g ($P < 0.001$) of DDG substrate was included. The fecal microbial fermentations revealed a different response in *Nal*$^R$ *E. coli* O157 concentration when different concentrations of DDG substrate were included (Table 2.3). Inclusion of 2 g of DDG substrate in fecal fermentations resulted in a higher *Nal*$^R$ *E. coli* O157 concentration at 24 h compared to 0 ($P < 0.05$), 0.5 ($P < 0.05$), and 1 g ($P < 0.01$) DDG substrate. No other differences in *Nal*$^R$ *E. coli* O157 concentration were significant in 24 h fecal microbial fermentations (Table 2.3). No difference in *Nal*$^R$ *E. coli* O157 concentration was found between the donor steers supplemented with 0% DDG and 25% DDG for either ruminal fluid or fecal microbial inoculum ($P > 0.4$).

## Discussion

The need to examine a potential association between feeding cattle distiller's grains or other fermentation byproducts and *E. coli* O157 prevalence has been suggested (32). Recently, a multi-state epidemiologic study conducted in feedlots observed that the odds of an *E. coli* O157 positive fecal sample were six times higher if cattle were fed brewers grains, a fermentative product similar to DG (9). These authors suggested that the increased odds of an *E. coli* O157 positive sample may be due to components of the brewer's grain or management factors associated with brewer's grain feeding. The results from the present study are in agreement with a previous study that showed a higher prevalence of *E. coli* O157 in cattle fed DG compared to cattle fed diets lacking DG (25). For that study, we sampled approximately 370 yearling heifers and concluded that the prevalence of *E. coli* O157 in fecal samples collected twice during a 150-day feeding period (days 122 and 136) from cattle fed wet DG (WDG) was higher compared to cattle not fed WDG. In contrast to the previous study, the present study allowed us to follow the prevalence of *E. coli* O157 in cattle throughout the 12-week finishing period. Interestingly, in the present study, cattle fed DDG with 5% corn silage had *E. coli* O157 prevalence between the two other diets (both containing 15% corn silage with or without DDG) at most sampling weeks. Distiller's grain replaced a portion of the corn in these diets. Because the starch in DG has been utilized during fermentation, addition of the byproduct to corn-based diets decreases the total starch content and increases fiber (bran) content of the feed (38). Previous work has shown that feeding forage-based diets increased the duration of shedding and/or concentration of *E. coli* O157 in the feces compared to animals fed a grain-based diet (21, 26, 40). Possibly, feeding DG

altered the hindgut environment favorably, either by decreasing starch (14) or by increasing the fiber to cause higher prevalence of *E. coli* O157. There was considerable week to week variation in the prevalence of *E. coli* O157 in this study. Such variation is not surprising because a number of factors influence prevalence and such variation has been previously reported (37). Also, there are a number of non-O157 serotypes that are becoming increasingly important as food-borne pathogens, and cattle have been shown to harbor many of these serotypes (4). It is important to determine whether DG has influence on non-O157 serotypes, however, these analyses were not included in the current study.

Other rationale for the distiller's grain association with prevalence seen in this study is that DG contains some component(s) stimulatory for *E. coli* O157 growth. To determine if DG can stimulate the growth of *E. coli* O157, *in vitro* fermentations were conducted. Both ruminal fluid and fecal microbial fermentations were used to examine this relationship. *In vitro* fermentations with ruminal fluid or fecal microbial inoculum have been used to assess gut microbial activity and interaction, and digestibility of substrates (11, 29, 41). Chaucheyras-Durand et al. (2006) have used *in vitro* fermentations with ruminal fluid or fecal microbial suspensions to evaluate gut biotic and abiotic factors on growth and survival of *E. coli* O157. They observed that growth of *E. coli* O157 in ruminal fluid fermentation was inhibited by the resident microbial flora. The site of *E. coli* O157 colonization in cattle is the hindgut and not the rumen (17, 27, 33, 40). Although the reasons for preference to hindgut are not known, it is logical to surmise that the ecosystem of the hindgut is relatively more hospitable to *E. coli* O157 than the rumen. In the intestine of ruminants, microbial counts in colon and rectum were higher than at any other location (39). Fecal microbial suspensions used during *in vitro* fermentation studies in sheep have been shown to contain microbial counts representative of the colon and rectum (7). In addition, fermentation processes and microbial populations differ between the rumen and hindgut (29), so fecal microbial fermentations may better represent hindgut fermentation compared to ruminal fermentations.

Feeding DG may alter the microbial populations in the rumen (16), and/or prevent depression in ruminal pH that could normally occur in corn diets without distiller's grain, possibly because of decreased starch content (13), both of which may enhance the viability of *E. coli* O157 in the rumen. In ruminal fluid microbial fermentations, when substrate was excluded, there was a higher concentration of $Nal^R$ *E. coli* O157 at 24 h from cattle fed DDG than cattle fed

no DDG. This increase may be because of higher pH in fermentations containing DG than without DG. Alternatively, there may have been a stimulatory component(s) present in the DG diet allowing for *E. coli* O157 growth. Possibly, a masking effect occurred when substrate was included in the fermentations (increased fermentation products and consequent reduction in pH), resulting in no differences between the two diets from these fermentations. Surprisingly, there was no DDG effect in the fecal microbial fermentations, whether substrate was included or not. This may possibly be because the stimulatory component(s) of the DG did not reach the hindgut in cattle fed DDG, and thus was not present in the fecal microbial inoculum.

In addition to a DDG effect in ruminal fluid fermentations from study 2, the type of grain processing in the animal diet (steam-flaked or dry-rolled) was associated with the concentration of *Nal*$^R$ *E. coli* O157 in both ruminal fluid and fecal microbial fermentations. Fermentations with inocula from cattle fed steam-flaked corn had higher *Nal*$^R$ *E. coli* O157 concentrations than fermentations with inocula from cattle fed DRC. Previous work has shown that grain processing, specifically steam-flaked versus dry-rolled grains fed to cattle, was related to the fecal shedding of *E. coli* O157 (14). Two grain types, wheat and sorghum, were used in that study, and steam-flaking compared to dry-rolling of both grains resulted in a higher prevalence of *E. coli* O157. The *in vitro* fermentation results from our study are in agreement with that observation. Grain processing (heat, moisture, etc.) increases the digestibility of the grain within the rumen (23), thereby reducing the amount of starch reaching the hindgut and not allowing for increased VFA and decreased pH in that location (35).

Study 3 evaluated DDG added in increasing amounts to ruminal fluid or fecal microbial fermentations. In both instances, adding DDG was associated with higher concentrations of *Nal*$^R$ *E. coli* O157. In ruminal fluid fermentations, the concentration of 24 h *Nal*$^R$ *E. coli* O157 increased over the initial (0 h) concentrations, suggesting DG stimulated growth of the organism. In the ruminal fluid fermentation, adding 0.5 g of DDG substrate stimulated the growth of *Nal*$^R$ *E. coli* O157; however, when DDG was included at concentrations above 0.5 g there was no apparent change. This may possibly be a result of decreased pH, associated with fermentations of DG, inhibiting *E. coli* O157. Unfortunately, the pH values were not measured in this study. Perhaps not surprisingly, the results were quite different in fecal microbial fermentations. These fermentations appeared to have a more "dose-dependent" response to increases in DDG substrate on *Nal*$^R$ *E. coli* O157 concentrations. There was no stimulation of growth of *E. coli* O157 as

observed in the ruminal fluid fermentations; however, providing 2 g of DDG did result in higher concentrations at 24 h compared to other DDG doses, likely because of some protective or stimulatory component in DG.

In conclusion, our study found a positive association between DG feeding and the fecal prevalence of *E. coli* O157 in cattle, which confirms our preliminary observation. Our observation has potentially serious ramifications. Distiller's grains are likely to become more available and a popular feed supplement because of the projected growth of ethanol as a biofuel. Feeding programs that elicit increases in the prevalence of *E. coli* O157 are likely to be met with strong opposition by beef producers, and especially by beef processors. The utilization of distiller's byproducts as a component of feedlot diets may depend, in part, on our ability to devise feeding strategies that do not compromise the perceived safety of beef products. Therefore, it is important to determine the mechanism of the association because such knowledge could lead to potential intervention strategies. *In vitro* fermentation studies provided some evidence that DG may actually stimulate the growth of *E. coli* O157. We hypothesize that there may be two possible mechanisms for *E. coli* O157 association with DG; a decreased starch concentration (DG replaced 25% of grain in this study) reaching the hindgut and impacting the ecology to favor *E. coli* O157, or DG has stimulatory component(s) that enhances growth of the organism. Further research is needed to test our hypotheses.

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**Figure 2.1 Prevalence of *E. coli* O157 in pen floor fecal samples from cattle fed SFC-based high-grain diets with 5 or 15% corn silage and supplemented or not supplemented with 25% DDG.**

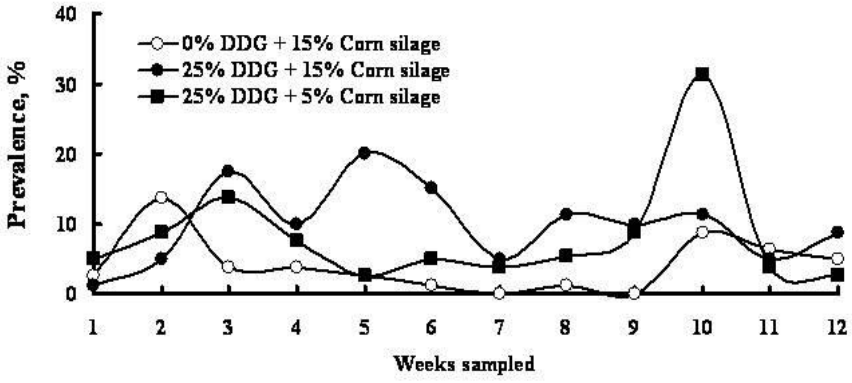
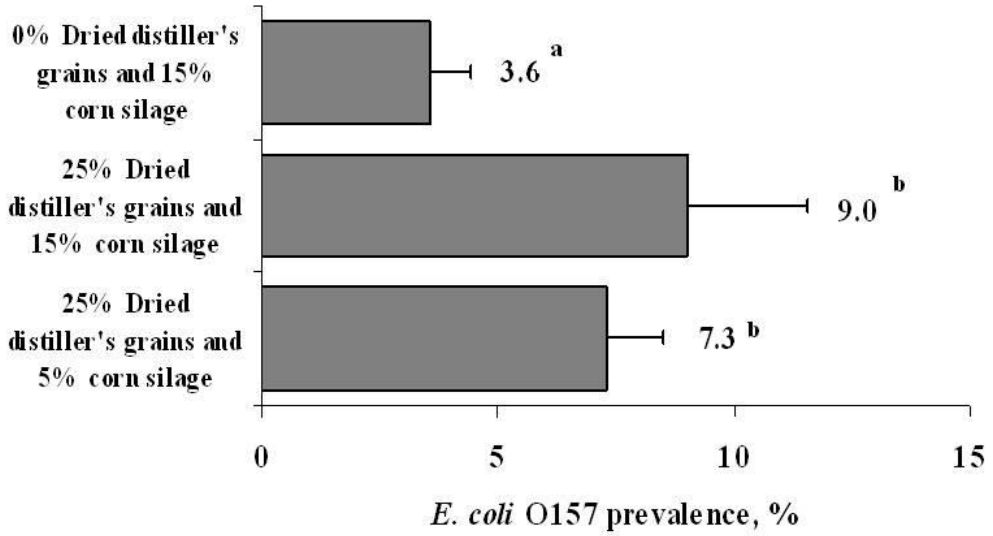
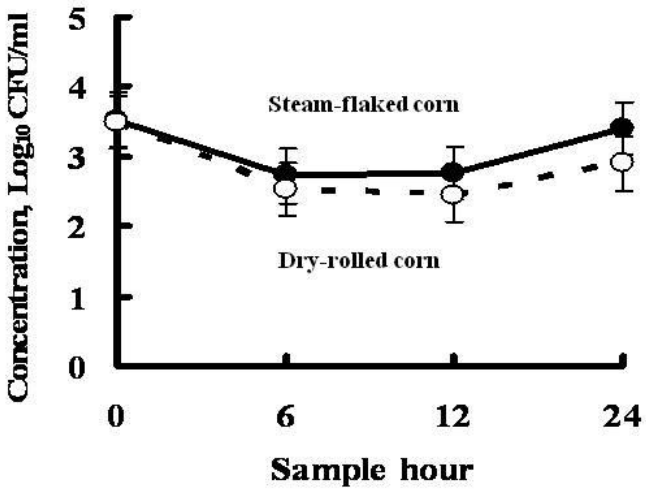


**Figure 2.2 Cumulative prevalence of *E. coli* O157 in pen floor fecal samples from cattle fed SFC-based high-grain diets with 5 or 15% corn silage and supplemented or not supplemented with 25% DDG.**




**The error bars indicate standard errors of the means. Bars with different superscripts are significantly different ($P < 0.05$).**

**Figure 2.3 Concentrations of Nal$^R$ *E. coli* O157 cultured from *in vitro* ruminal microbial fermentations (study 2).**




**Ruminal fluid inocula were obtained from donor steers fed diets containing either DRC (○) or SFC (●). The difference in the Nal$^R$ *E. coli* O157 concentration between the DRC and SFC diets was significant at 24 h ($P < 0.01$).**

**Table 2.1 Number and percentages of *E. coli* O157 isolates positive for the *eae* gene and Shiga toxin-producing genes (*stx*1 and *stx*2).**

| Virulence gene(s) | No. (%) of *E. coli* O157 isolates positive with the following diet[a]: No DDG +15% corn silage (n = 39) | 25% DDG + 15% corn silage (n = 96) | 25% DDG + 5% corn silage (n = 78) |
|---|---|---|---|
| *eae* | 39 (100.0) | 96 (100.0) | 78 (100.0) |
| $stx_1$ only | - | 3 (3.1) | 2 (2.6) |
| $stx_2$ *only* | 27 (69.2) | 71 (74.0) | 58 (74.4) |
| $stx_1$ and $stx_2$ | 12 (30.8) | 22 (22.9) | 18 (23.1) |

**[a] Isolates were obtained from a prevalence study which allocated feedlot cattle to treatments with or without 25% dried distiller's grains (DDG) and 5 or 15% corn silage**

**Table 2.2 Mean concentrations of Nal$^R$ *E. coli* O157 and standard error in ruminal fluid or fecal microbial fermentations at 24 h (study 2)**

| Microbial inoculum | Substrate included in fermentations[a] | DDG in diet[b] | Concn of *Nal*$^R$ *E. coli* O157 ($log_{10}$ CFU/ml) | SEM ($log_{10}$ CFU/ml) | *P* value |
|---|---|---|---|---|---|
| Ruminal fluid | Yes | Yes | 2.57 | | |
| | | No | 2.79 | 0.24 | 0.43 |
| | No | Yes | 3.91 | | |
| | | No | 3.33 | 0.24 | 0.04 |
| Feces | Yes | Yes | 2.83 | | |
| | | No | 2.83 | 0.13 | 0.99 |
| | No | Yes | 2.76 | | |
| | | No | 2.72 | 0.13 | 0.78 |

**[a] The substrate included in fermentations was the ground whole diet of steers that served as donors of ruminal fluid or feces for *in vitro* fermentations.**

**[b] DDG was included in steer's diet at a concentration of 25%.**

**Table 2.3. Mean concentrations of Nal$^R$ *E. coli* O157 and standard errors of the means in ruminal fluid or fecal microbial fermentations at 12 and 24 h (study 3)**

| DDG concn in fermentation (g) | Concn ($log_{10}$ CFU/ml) of *Nal*$^R$ *E. coli* O157 | | | | | |
|---|---|---|---|---|---|---|
| | Ruminal fluid fermentation | | | Fecal microbial fermentation | | |
| | 12 h | 24 h | SEM | 12 h | 24 h | SEM |
| 0 | 2.37 [a] | 3.45 [a] | 0.25 | 2.39 [a] | 2.24 [a] | 0.25 |
| 0.5 | 2.56 [a] | 4.07 [b] | 0.26 | 2.59 [a,b] | 2.27 [a] | 0.25 |
| 1.0 | 2.14 [a] | 2.66 [c] | 0.25 | 2.67 [a,b] | 2.17 [a] | 0.25 |
| 2.0 | 2.15 [a] | 2.38 [c] | 0.25 | 2.90 [b] | 2.76 [b] | 0.25 |

**[a, b, c] Different superscripts within a column indicate statistical significance ($P < 0.05$).**

# CHAPTER 3 - Feeding Supplemental Dried Distiller's Grains Increases Fecal Shedding of *Escherichia coli* O157 in Experimentally Inoculated Calves

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## Abstract

*Escherichia coli* O157 is an important foodborne pathogen and asymptomatic cattle serve as major reservoirs for human infection. We have shown a positive association between feeding distiller's grains and *E. coli* O157 prevalence in feedlot cattle. The objective of this study was to determine the effect of feeding dried distiller's grain (DDG) on fecal shedding of *E. coli* O157 in calves experimentally inoculated with *E. coli* O157. Holstein calves (5 per treatment group), fed steam-flaked corn-based high-grain diets supplemented with 0% (control) or 25% DDG, were orally inoculated with a five-strain mixture ($6 \times 10^9$ CFU/calf) of nalidixic acid-resistant ($Nal^R$) *E. coli* O157. Fecal samples were taken three times per week for six weeks to determine the prevalence and concentration of $Nal^R$ *E. coli* O157. At the end of the study (day 43), calves were euthanized and necropsied. Ruminal, cecum, colon, and rectal contents, and rectoanal mucosal swab (RAMS) samples were collected at necropsy to determine $Nal^R$ *E. coli* O157 concentration. There was a trend for an interaction between treatment and fecal sampling day. The

concentration of *Nal*$^R$ *E. coli* O157 in the feces was significantly higher in fecal samples from calves fed DDG compared to control calves on days 35, 37, 39, and 42. At necropsy, the concentration of *Nal*$^R$ *E. coli* O157 was higher in the cecum ($P = 0.01$), colon ($P = 0.03$), and rectum ($P = 0.01$) from calves fed DDG compared to control animals. The number of sites at necropsy positive for *Nal*$^R$ *E. coli* O157 was higher in calves fed DDG compared to calves in the control treatment ($P < 0.001$). Our results indicate that *E. coli* O157 gut persistence and fecal prevalence increased in calves fed DDG, which potentially have important implications for food safety.

## Introduction

Shiga-toxin producing *Escherichia coli* O157 is a major foodborne pathogen, and ruminants, particularly cattle, are primary reservoirs (Bach et al., 2002; Gyles, 2007; Hussein, 2007). The organism colonizes hindgut and is shed in the feces, which serves as a major source of contamination of food products (Callaway et al., 2003). In a review of *E. coli* O157 outbreaks between 1982 and 2002, Rangel et al. (2005) reported that the pathogen caused 73,000 ilnesses in the US annually. Transmission route for the outbreaks included foodborne (52%), person to person (14%), waterborne (9%), animal contact (3%), laboratory-related (0.3%) and unknown (21%). Among the foodborne outbreaks, 38% was attributed to ground beef and 21% to produce (Rangel et al., 2005). Diet has been implicated as a factor in the prevalence of *E. coli* O157 in cattle (Van Baale et al., 2004; Dewell et al., 2005; Fox et al., 2007). Cattle orally inoculated with *E. coli* O157 have been used to assess dietary impacts on fecal shedding. Van Baale et al. (2004) reported that *E. coli* O157 was shed for a longer duration in orally challenged calves fed a forage-based diet, compared to a grain-based diet. Another challenge study reported that a higher number of steers were *E. coli* O157 positive when fed barley grain compared to those fed corn grain (Buchko et al., 2000).

Distiller's grains (DG), a co-product of ethanol production by yeast fermentation of enzymatically hydrolyzed grain starch, is an effective feed supplement in finishing cattle diets (Firkins et al., 1985). The co-product can be fed as dry (DDG) or wet (WDG), with or without dehydration to remove the liquid fraction, respectively. Distiller's grains constitute the components of cereal grains that remain after starch fermentation, effectively concentrating the non-starch nutrients approximately three-fold (Spiehs et al., 2002). The use of DG has been

shown to improve daily gain in beef cattle (Ham et al., 1994; Al-Suwaiegh et al., 2002) and increase milk yield and feed efficiency in dairy cattle (Kleinschmit et al., 2006). Distiller's grain usage in cattle is likely to increase with further expansion of the ethanol industry.

Previously, natural prevalence studies have indicated a positive association between *E. coli* O157 prevalence and feeding brewers grain or distiller's grains (both WDG and DDG) in feedlot cattle (Dewell et al., 2005, Jacob et al., 2008a, b). Therefore, an experimental inoculation study was deemed necessary to confirm the positive association between *E. coli* O157 prevalence and feeding supplemental DG. The objective of this study was to compare the fecal shedding patterns and concentration of *E. coli* O157 in orally inoculated calves fed diets with or without DDG from corn grain. We hypothesized that feeding DDG would increase fecal prevalence and concentration of *E. coli* O157.

## Materials and Methods

**Animals and treatment groups.** Ten Holstein bull calves (BW = 90.7 ± 22.7 kg) were randomly assigned to one of two dietary treatments (five calves per treatment group): steam-flaked corn (SFC) based high grain diets with 0% (control) or 25% DDG. Calves were fed a series of four step-up diets (stepped–up approximately every 5 days) with their respective diets for approximately three weeks, during which time they were housed in two outdoor pens with a common fenceline. Once calves were adjusted to the final diet {0 or 25% DDG, steam-flaked corn (84 or 61%), alfalfa hay (6%), corn steep liquor (6%), and a vitamin and mineral supplement (4 or 2%)} they were moved to individual pens (5' × 10') within a biosafety level-2 facility and allowed to acclimate for one week prior to oral inoculation with *E. coli* O157. Calves in each group were housed in five consecutive pens per isle (one treatment per isle) and each pen contained walls tall enough to prevent physical contact between adjacent animals. Diets were fed once daily and all calves had access to fresh water. Pens were scrapped and cleaned once daily.

***Escherichia coli* O157 inoculation.** Calves were orally dosed with a five-strain mixture of Shiga-toxin producing *E. coli* O157 isolated from beef feedlots (Sargeant et al., 2003) and made resistant to 50 µg/ml nalidixic acid ($Nal^R$ *E. coli* O157). Isolates were plated on blood agar (BAP; Remel, Lenexa, KS) and grown overnight at 37˚C. A single colony was selected,

inoculated into 10 ml of tryptic soy broth (TSB; Difco; BD, Sparks, MD) and incubated overnight at 37˚C. One ml of TSB was inoculated into 100 ml of TSB and incubated for 7 h at 37˚C. All strains were pooled together after incubation and 1 ml of pooled culture was serially diluted ten-fold in peptone water (Remel) and spread plated (4 plates per dilution) onto sorbitol-MacConkey agar (Difco) plates with cefixime (0.5 mg/L), potassium tellurite (2.5 mg/L), and nalidixic acid (50 µg/ml; ctn-SMAC) to determine the concentration of *Nal*$^R$ *E. coli* O157. Each calf was dosed orally, via gastric tube, with 5 ml of pooled culture mixed with 200 ml of 1% sterile skim milk (Oxoid, Basingstoke Hampshire, UK). The final inoculum for each calf contained $6.0 \times 10^9$ CFU of *Nal*$^R$ *E. coli* O157.

**Fecal sampling and detection or enumeration of *Nal*$^R$ *E. coli* O157.** Fecal samples were collected from each animal before inoculation (day 0) to determine the prevalence of wild-type *E. coli* O157, as described previously (Greenquist et al, 2005). Briefly, one gram of fecal sample was enriched in 9 ml of Gram Negative broth (Difco) with cefixime (0.5 mg/L), cefsoludin (10 mg/L), and vancomycin (8 mg/L; GNccv). Immunomagnetic bead separation (Dynal, Inc., New Hyde Park, NY) was performed and samples were plated onto sorbitol-MacConkey agar (Difco) plates with cefixime (0.5 mg/L), potassium tellurite (2.5 mg/L). Six sorbitol-negative colonies were picked and colonies were tested for indole production and O157 antigen latex agglutination (Oxoid).

After oral challenge with *Nal*$^R$ *E. coli* O157 (day 0), fecal samples were collected from each calf three times a week (Monday, Wednesday, and Friday). At each sampling, control calves were sampled before DDG-supplemented calves and personnel changed suits (Tyvek, DuPont; Wilmington, DE) between treatment groups. Foot baths with bleach were used and gloves were changed between each pen and isle. Fecal samples were collected by rectal palpation, placed in sterile bags, and transported to the laboratory immediately. A tube containing 9 ml GNccv was weighed, approximately 1 g of fecal sample was added, and the tube was re-weighed to determine the weight of the sample. One ml of fecal suspension was serially diluted ten-fold in buffered peptone water. One hundred microliters of appropriate dilutions were plated, in triplicate, on ctn-SMAC to determine the concentration of *Nal*$^R$ *E. coli* O157. Plates were incubated overnight at 37˚C and the sorbitol-negative colonies were counted. The remaining GNccv broth was enriched for 6 h at 37 ºC, after which, 1 ml was removed and

inoculated into 9 ml of fresh GNccv broth (secondary enrichment) and incubated for 16 to 18 h at 37ºC. If colonies were not recovered by direct plating, 100 µl of secondary enrichment was plated onto ctn-SMAC. A colony from each direct or enrichment plate was picked, plated on BAP and tested for indole production and O157 antigen latex agglutination.

At the termination of the study (day 43), all calves were euthanized and necropsied. Contents from the rumen, cecum, colon, and rectum were collected and the concentration of *Nal*$^R$ *E. coli* O157 was determined as above. Also, the rectum was cut open, the mucosa was rinsed with water to remove visible fecal material and the area, 3 to 5 cm proximal to the rectoanal junction, was swabbed with a foam-tipped applicator (RAMS; VWR International, Buffalo Grove, IL, catalog #10812-022; Fox et al., 2008). Swabs were immediately placed in 3 ml of GNccv broth, transported to the laboratory, vortexed and processed for detection of *Nal*$^R$ *E. coli* O157 by direct plating or enrichment as above.

**Statistical analysis.** Fecal concentrations of *Nal*$^R$ *E. coli* O157 were $\log_{10}$ transformed before data analyses. If *Nal*$^R$ *E. coli* O157 was not detected by direct plating, but enrichment was positive, the lowest detectable concentration was assigned, accounting for sample weight ($10^1$ CFU/g). The concentration in RAMS samples was not determined because the size of the inoculum was unknown; therefore, swabs were considered positive (direct plating or enrichment) or negative. For live animal fecal collections (day 2 through 42), differences in *Nal*$^R$ *E. coli* O157 concentrations between treatment groups (control or DDG), sampling day and treatment × sampling day effects were analyzed using the MIXED procedure in SAS (Version 9.1, SAS Institute, Cary, NC). Sampling day and the treatment × sampling day effects were included anticipating *a priori* that fecal concentrations would be high the first few days after inoculation, and then gradually decreasing with any treatment effects occurring only after 7-10 days post-inoculation. The MIXED procedure also was used to detect differences in *Nal*$^R$ *E. coli* O157 concentration in gut contents collected at necropsy with treatment, site (rumen, cecum, colon, and rectum), and treatment × site effects included in the final model. For both live animal and necropsy sample analyses, animal was included as a repeated effect. Two way comparisons were conducted using least squares means estimates.

The prevalence (positive by direct plating or by enrichment) of *Nal*$^R$ *E. coli* O157 in live animals was analyzed using logistic regression in GENMOD of SAS. This analysis compared

the cumulative prevalence for each animal between day 21 (the first study day in which at least one animal was negative for *Nal*$^R$ *E. coli* O157) and the termination of the study (day 42). Treatment was included as the explanatory variable. Initially, a repeated measures analysis was used to assess the probability of an animal being fecal-positive on each sample day from days 21 to 42, but this model did not converge. Prevalence of *Nal*$^R$ *E. coli* O157 in necropsy samples also was analyzed using logistic regression in GENMOD of SAS. The cumulative prevalence of *Nal*$^R$ *E. coli* O157 at the five necropsy sites for each calf was the evaluated response and treatment was included as an explanatory variable.

## Results

**Fecal shedding of *E. coli* O157.** Fecal samples from all calves were negative for *E. coli* O157 prior to oral inoculation. One calf in the control diet was treated with Banamine® (Schering Plough Animal Health Corp., Summit, NJ) over a one-week period (on days 5 through 8 and day 11 after inoculation) because of high body temperature and loss of appetite. Normal body temperature and appetite were restored after treatment and the calf was kept in the study.

Two days after oral inoculation with *Nal*$^R$ *E. coli* O157, all calves were positive by direct plating. All calves in both treatments were positive for *Nal*$^R$ *E. coli* O157 through sampling day 18, after which the cumulative prevalence of *Nal*$^R$ *E. coli* O157 (between days 21 and 42) was higher in DDG-fed calves compared to calves in the control group ($P < 0.0001$; Fig. 3.1). Sampling day had a significant effect on the concentration of *Nal*$^R$ *E. coli* O157 in fecal samples ($P < 0.001$). The treatment × day interaction ($P = 0.07$) was sufficient to warrant further investigation, therefore, the differences between treatments were examined on each collection day (Fig. 3.2). On day two, the *Nal*$^R$ *E. coli* O157 concentration was higher in fecal samples from calves fed no DDG than from calves fed DDG ($P < 0.01$). However, there were no differences in the concentrations of *Nal*$^R$ *E. coli* O157 between treatment groups after day two until day 35 of the study. The *Nal*$^R$ *E. coli* O157 concentrations in fecal samples were higher in calves fed DDG compared to control calves (Fig. 3.2) on days 35 ($P = 0.02$), 37 ($P = 0.03$), 39 ($P = 0.02$), and 42 ($P < 0.01$).

***E. coli* O157 in gut contents and on rectoanal mucosa.** All calves in the DDG group were positive for *Nal*$^R$ *E. coli* O157 in at least one site in the gut (rumen, cecum, colon, rectum, or

RAMS) at necropsy compared to only three of five control calves (Table 3.1). The number of sites at necropsy positive for *Nal*$^R$ *E. coli* O157 was higher in calves fed DDG compared to calves in the control treatment ($P < 0.001$). Of the five calves in the DDG group, four were positive in all samples collected at necropsy and the fifth calf was positive only in cecal contents. In two of three control calves, *Nal*$^R$ *E. coli* O157 was only isolated from the rectal contents or on the rectoanal mucosa (Table 3.1). For ruminal contents, four of five calves fed DDG were positive for *Nal*$^R$ *E. coli* O157, whereas none of the control calves were positive. In addition, four of five DDG calves were culture positive for *Nal*$^R$ *E. coli* O157 in RAMS compared to only one calf in the control group (Table 3.1).

Feeding DDG increased ($P < 0.01$) *Nal*$^R$ *E. coli* O157 concentrations in gut contents collected at necropsy (Fig. 3.3). The *Nal*$^R$ *E. coli* O157 concentrations in DDG-fed calves were higher than control calves in cecal ($P = 0.01$), colonic ($P = 0.03$), and rectal ($P = 0.01$) contents. As all ruminal content samples were positive only after enrichment (approx. $10^1$ CFU/g), low concentrations were calculated and no statistical differences were evident between treatment groups.

## Discussion

Supplementation of calves fed high-grain diets with 25% DDG significantly increased the cumulative prevalence and concentration of *E. coli* O157 in feces following oral inoculation with *Nal*$^R$ *E. coli* O157. This may imply that the organism can persist and colonize the gut more readily in calves fed DDG compared to no DDG control calves. Necropsy data further supports *E. coli* O157 persistency in the gut of DDG-supplemented calves. Because of the preferential colonization of *E. coli* O157 on the recto-anal mucosa (Naylor et al., 2003), the RAMS technique was developed to swab the rectoanal junction and was shown to be more sensitive than fecal samples from cattle in detecting *E. coli* O157 (Rice et al., 2003; Greenquist et al., 2005). The RAMS technique was used in our study only at necropsy and four of five calves supplemented with DDG were positive, while only one control calf was RAMS positive. There is evidence to suggest that mucosal carriage of *E. coli* O157 at the rectoanal junction is associated with long-duration fecal shedding and high concentration of fecal excretion (Cobbold et al., 2007; Davis et al., 2006; Lim et al., 2007; Low et al., 2005).

This study confirms our previous observations from two natural prevalence studies that have shown a positive association between DG supplementation and *E. coli* O157 prevalence (Jacob et al., 2008a, b). In the first study, we observed that the prevalence of *E. coli* O157 in fecal samples was higher from cattle fed 25% WDG compared to cattle fed diets without WDG on one of two collection days during the 150-day feeding period (Jacob et al., 2008a). In the second feedlot study, cattle ($n$ = 379) were fed SFC-based diets supplemented with 0 or 25% DDG (similar to the present study), and the cumulative prevalence of *E. coli* O157 from pen floor fecal samples was higher ($P$ = 0.01) in DDG-fed cattle compared to control cattle throughout the 12-week finishing period (Jacob et al., 2008b). These two studies suggested that positive associations between DG supplementation and prevalence of *E. coli* O157 occurred with dry or wet DG. Synge et al. (2003) in their investigation of management factors influencing the shedding of *E. coli* O157 by beef cows in Scotland observed that the housed cows supplemented with DG exhibited a higher shedding rate than cows not given DG. Additionally, Dewell et al. (2005) reported, in a multi-state epidemiological feedlot study. that the odds of an *E. coli* O157 positive fecal sample, were six times higher in cattle fed brewer's grains, a fermentative co-product similar to DG, than in cattle without brewer's grains.

The mechanism to explain the relationship between *E. coli* O157 and supplementation of distiller's grains is not known. It appears that DG may contain components that directly or indirectly stimulate growth of *E. coli* O157. Indirect stimulation is probably mediated via the impact of DG on ruminal or hindgut microbial populations. We have shown that addition of DDG to *in vitro* fermentations with ruminal or fecal (representing hindgut microbial fermentation) microbial inoculum resulted in a higher concentration of inoculated $Nal^R$ *E. coli* O157 after 24 h incubation compared to fermentations without added DDG (Jacob et al., 2008b). Growth stimulation observed within a short *in vitro* incubation (24 h) suggests that the more likely reason for an association is the direct effect of DG on *E. coli* O157. The DG supplement includes the non-starch fractions of the corn grain, as well as yeast cells and their metabolic intermediates and end products from ethanol production. Therefore, the factor(s) in DG responsible for stimulation of *E. coli* O157 may be associated with the yeast (cells or products) or unfermented fractions of the grain. The soluble fraction obtained after separation of solids, generally by centrifugation, is often condensed by heating and added to the DG as condensed distiller's solubles (Rausch and Belyea, 2005). The soluble fraction is more likely to contain

yeast fermentation intermediates and products, other than volatile compounds, such as ethanol, which are removed during distillation or dehydration (Fron et al., 1996). Analyses of distiller's soluble fractions from several sources have identified lactate, fumarate, malate, and succinate as components (Fron et al., 1996). Although the effect of these organic acids on *E. coli* O157 is not known, it is well known that they stimulate the growth of certain ruminal bacteria, particularly the lactate fermenting bacteria, such as *Selenomonas ruminantium* and *Megasphaera elsdenii* (Martin, 1998). Bach et al. (2003) have previously reported that the yeast *Saccharomyces cerevisiae* subsp. *boulardii* inhibited *E. coli* O157 strains in batch culture fermentations; however, inhibition did not occur in continuous culture.

Much of the attention DG has received is focused on three major macronutrients, protein, fiber, and lipids, because of their usefulness as protein or energy sources for livestock. We cannot rule out the possibility of unfermented macro or micronutrients of DG providing direct stimulation of *E. coli* O157. More likely, the effect of macronutrients is mediated via alteration of the ruminal or hindgut microbial ecosystem. Because DG replaces a portion of cereal grain of the diet, the reduction in dietary starch content also contributes to changes in ruminal or hindgut fermentation. Fox et al. (2007) showed that grain processing (dry-rolling verse steam-flaking) impacted the prevalence of *E. coli* O157, possibly because of differences in the ability of starch to reach the hindgut. Grain processing methods, such as steam-flaking, increase ruminal digestibility of the grain and reduce the amount of starch reaching the hindgut (Huntington, 1997). An altered hindgut ecology impacting the growth of competing microorganisms, an increased hindgut pH, or other mechanisms associated with decreased starch flow to the hindgut may be a means for the increased *E. coli* O157 concentration or prevalence (Fox et al., 2007). Also, the impact of DG or condensed distiller's products on ruminal microbial population and their fermentation products could influence *E. coli* O157. The altered ruminal microbial fermentation is evidenced by increased molar proportion of propionic acid in DG supplemented cattle (Ham et al., 1994) or increased cultural counts of lactate-utilizing bacteria with a two-fold increase in the *in vitro* lactic acid fermentation in steers supplemented with condensed distiller's solubles (Fron et al., 1996). An altered rumen ecosystem promoting growth and persistence of *E. coli* O157 is supported by our observation of the presence of $Nal^R$ *E. coli* O157 in ruminal contents from four out of five calves fed DDG in this study.

The primary colonization of *E. coli* O157 in ruminants is the hindgut (Grauke et al., 2002; Van Baale et al., 2004), specifically the rectoanal mucosal junction (Naylor et al., 2003). Although reasons for persistence or colonization in the hindgut are undetermined, suggested rationale includes a more hospitable environment for *E. coli* colonization and proliferation compared to the rumen (Van Baale et al., 2004; Fox et al., 2007). In our experiment, all DDG calves were culture positive in one or more regions of the hindgut (cecum, colon, rectum or rectoanal mucosa) and the three calves in the control group that were positive for *E. coli* O157 were negative in the rumen but positive in the hindgut. In DG-supplemented cattle, more nutrients, particularly bran and protein, could possibly flow into the hindgut and alter the microbial ecosystem in favor of growth and persistence of *E. coli* O157. Previous studies have shown that forage feeding is positively associated with *E. coli* O157 in ruminants (Kudva et al., 1997; Van Baale et al., 2004), possibly because of altered hindgut ecology. Van Baale et al. (2004) reported that forage-fed cattle inoculated with *E. coli* O157:H7 shed the organism in the feces for a longer duration than grain-fed calves. Increased protein degradation in the hindgut because of increased flow of protein could possibly elevate pH (increased ammonia production) thus favoring growth and persistence of *E. coli* O157.

In conclusion, this study confirmed previous observations that feeding cattle DG was positively associated with *E. coli* O157 shedding. The mechanism by which this occurs is unknown; however, we hypothesize that altered hindgut ecology or a component in DG that stimulates *E. coli* O157 proliferation and colonization may be responsible for this positive association. Further research is needed to delineate the mechanism responsible for this association. Given the growing trend for use of DG in cattle finishing diets, our finding of a positive association with prevalence of *E. coli* O157 is significant and has potentially important ramifications for food safety.

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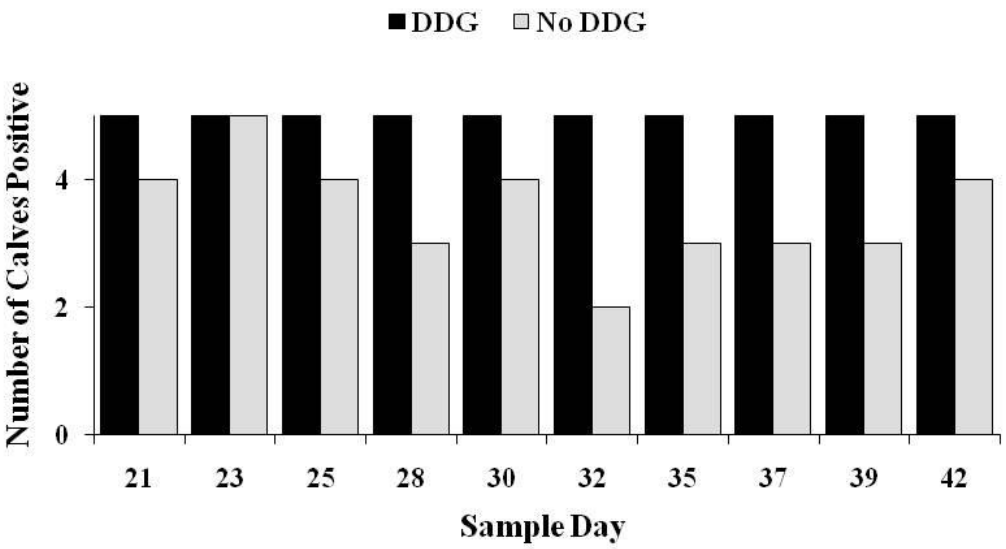
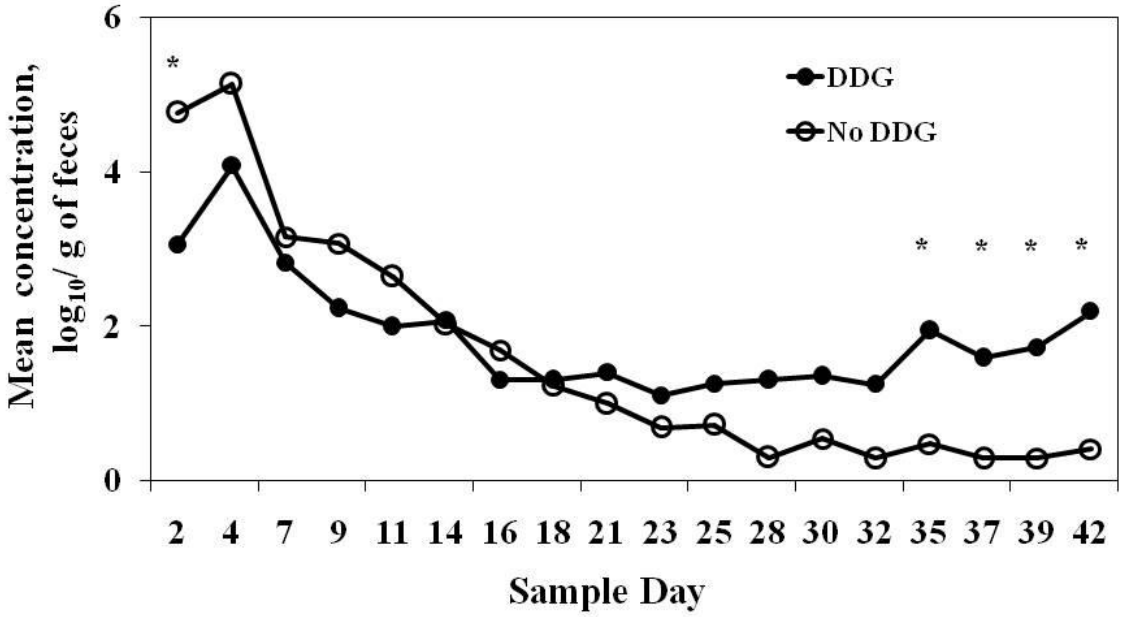
**Figure 3.1 The number of fecal samples positive for nalidixic acid-resistant *Escherichia coli* O157 from calves fed high-grain diets with (n = 5) or without (n = 5) 25% supplemental dried distiller's grains (DDG) between study days 21 and 42.**



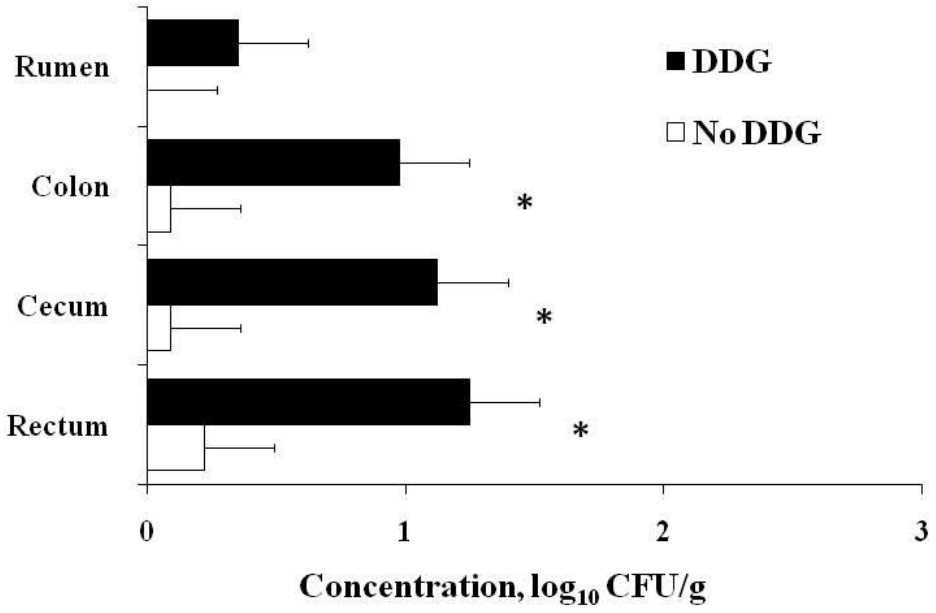

**The cumulative prevalence between days 21 and 42 was higher ($P < 0.001$) in calves fed DDG compared with control calves.**

**Figure 3.2 The mean $log_{10}$ concentration of nalidixic acid-resistant *Escherichia coli* O157 in fecal sample of calves (n = 5 per treatment) fed high-grain diets with or without 25% supplemental dried distiller's grains (DDG) across sample days.**




**The concentration was significantly different (*) between treatments on days 2, 35, 37, 39, and 42 ($P < 0.05$).**

**Figure 3.3 The mean $\log_{10}$ concentration and SE of nalidixic acid-resistant *Escherichia coli* O157 in gut contents at necropsy from calves fed high grain diets with or without 25% supplemental dried distiller's grains (DDG; five calves per treatment).**




*** Indicates statistical difference between calves fed DDG and those fed the control diet ($P < 0.05$).**

**Table 3.1 Prevalence of nalidixic acid-resistant *Escherichia coli* O157 in gut contents and on the rectoanal mucosa at necropsy in calves fed high-grain diets with 0 or 25% dried distiller's grains (DDG).**

| Calf no. | DDG, % of diet | Gut contents | | | | Rectoanal mucosa | Total number of gut locations positive (%) |
|---|---|---|---|---|---|---|---|
| | | Rumen | Cecum | Colon | Rectum | | |
| 1 | 0 | - | + | + | + | - | 5 of 25 (20) |
| 2 | 0 | - | - | - | - | + | |
| 3 | 0 | - | - | - | - | - | |
| 4 | 0 | - | - | - | - | - | |
| 5 | 0 | - | - | - | + | - | |
| 6 | 25 | + | + | + | + | + | 21 of 25 (84) |
| 7 | 25 | + | + | + | + | + | |
| 8 | 25 | + | + | + | + | + | |
| 9 | 25 | + | + | + | + | + | |
| 10 | 25 | - | + | - | - | - | |

**+: Positive for nalidixic acid-resistant *E. coli* O157 by direct plating or by enrichment;**

**-: negative for nalidixic acid-resistant *E. coli* O157 by enrichment.**

# CHAPTER 4 - Feeding Dried or Wet Distiller's Grains at Various Inclusion Levels to Feedlot Cattle Affects Fecal Shedding of *Escherichia coli* O157:H7

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## Abstract

Our objectives were to evaluate the prevalence of *Escherichia coli* O157:H7 in cattle fed 0, 20 or 40% dried (DDG) or wet distiller's grains (WDG), and to assess whether removing DG from diets prior to slaughter affected fecal shedding of *E. coli* O157:H7. Eight hundred forty steers were allocated to seventy pens (12 steers/pen) in a research feedlot. Treatments were no DG (control), 20% DDG or WDG, and 40% DDG or WDG, and each was replicated in 14 pens. In phase one, eight fecal samples were collected from each pen every two weeks for 12 weeks for isolation of *E. coli* O157:H7. In phase two, half of pens with DG were transitioned to the no DG, control diet and eight fecal samples were collected from all pens weekly for four weeks and tested for *E. coli* O157:H7. Each sample was also evaluated by direct plating to identify high shedders. During phase one, the prevalence of *E. coli* O157:H7 was 20.8% for overall shedding and 3.2% for high shedders. The inclusion level but not form of DG significantly affected *E. coli* O157:H7 shedding. Prevalence of overall *E. coli* O157:H7 or high shedders were not different between cattle fed diets with 0 or 20% DG; however, cattle fed 40% DG had a higher prevalence of overall *E. coli* O157:H7 and high shedders than those fed either 0 or 20% DG ($P \leq 0.05$). During the second phase, overall and high shedder prevalence estimates were 3.3% and < 0.1% respectively, and there were no differences between DG form, inclusion level, or removing DG

from diets. Results from this study indicate that the DG form had no impact on *E. coli* O157:H7; however, fecal shedding was associated with the inclusion level of DG in the diet.

## Introduction

Cattle are asymptomatic reservoirs for *Escherichia coli* O157:H7, a foodborne pathogen associated with gastrointestinal disease in thousands of Americans each year. The organism colonizes the hindgut of cattle (20, 30) and is shed in cattle feces. Once shed, *E. coli* O157:H7 can contaminate food and water, creating a food safety risk (22). The contamination of beef products occurs during the slaughter process and is associated with the prevalence of *E. coli* O157:H7 in the feces and on the hides of cattle at harvest (6, 10, 14).

The prevalence of *E. coli* O157:H7 in cattle has been associated with many factors including season, geographic location and diet. Previous work has shown that cattle fed diets containing distiller's grains (DG), an ethanol fermentation co-product, have a higher prevalence of *E. coli* O157:H7 than cattle fed diets without DG (12, 31). Distiller's grains are a valuable feed commodity for cattle producers, and the use of these co-products has increased with the expansion of the ethanol industry (16, 19). Distiller's grains for use in cattle diets are available in wet (WDG) or dry (DDG) forms. The association between feeding DG and *E. coli* O157:H7 prevalence appears to be independent of the DG form (12, 31), but no study has directly compared the two forms. The level of supplementation of DG in cattle diets generally ranges from 10 to 50% depending on whether it is used as a protein or energy source; as a protein supplement, it is included at 10 to 15% and as an energy source the level is generally dictated by the availability of the co-product and the price of grain (16). There is evidence that the *E. coli* O157:H7 prevalence is different for cattle fed different levels of DG (21). However, no study has specifically evaluated the relationship between *E. coli* O157:H7 prevalence and DG inclusion level. Evaluation of these two factors (form and inclusion level) is important for our understanding of the association between DG and *E. coli* O157:H7 in cattle.

We also were interested in determining whether removing the DG component of the diet would lower the fecal prevalence of *E. coli* O157:H7. Such a strategy may lead to potential mitigation options, and would provide further evidence of a positive association between feeding DG and *E. coli* O157:H7 prevalence in cattle. In this two-phase study, our objectives were to 1) concurrently evaluate the effect of DG inclusion level and form on *E. coli* O157:H7 prevalence

in feedlot cattle, and 2) determine if removing DG from cattle diets subsequently reduces the fecal prevalence of *E. coli* O157:H7.

## Materials and Methods

**Phase One: Animals, Treatments and Sampling.** The study was conducted during the summer and fall of 2009. Eight hundred forty steers (BW = 414 ± 131 kg) were randomly allocated to one of 70 pens (12 steers/pen) in a commercial research feedlot in Nebraska. Cattle were fed high-grain, corn-based finishing diets (Table 4.1), with one of five treatments randomly assigned once within a block of five pens. The treatments were 0% DG (control), 20% DDG, 20% WDG, 40% DDG, and 40% WDG. Each treatment was replicated in 14 pens. Diets were formulated to meet nutritional requirements and were designed to be as similar as possible, differing only in the inclusion of DG. Cattle began final finishing diets after a one month transition and were fed once per day with continual access to water. After cattle began finishing diets, eight fresh pen floor fecal samples were collected from each pen once every two weeks for 12 weeks. Care was taken to avoid contamination of samples from other feces or from pen floors and all efforts were made to collect each sample from a different animal within that pen. Samples were collected with plastic spoons, placed in whirl pack bags (Nasco, Fort Atkinson, WI) and transported on ice to the laboratory.

**Phase Two: Animals, Treatments and Sampling.** After 12 weeks on phase one finishing diets, DG was removed from half (7 of 14 pens) of each the DG-fed treatments to initiate phase two of the study. Two consecutive blocks of five pens (see phase one above) were used to create one block for phase 2. Within the new block of 10 pens, one pen receiving 20% DDG, 20% WDG, 40% DDG, and 40% WDG was randomly selected to transition to the control (no DG) diet. First, half of the pens in the 40% DDG or WDG group were changed to 20% DDG or WDG with a concurrent increase in the cracked corn grain. After approximately one week, the 20% DG was replaced with corn grain and at the same time half of the pens in the 20% DDG or WDG group were changed to 0% DG. Therefore, phase 2 had nine treatments: 0% DG (control), 20% DDG, 20% WDG, 40% DDG, 40% WDG, 20% DDG-removed (REM), 20% WDG-REM, 40% DDG-REM, 40% WDG-REM. After cattle had been acclimated to new diets (2 weeks), 8 pen floor fecal grab samples were collected from each pen once weekly for four weeks.

**Detection of *E. coli* O157:H7.** Approximately one gram of fecal sample was placed into 9 ml of Gram Negative (GN) broth (Difco; BD, Sparks, MD, USA) supplemented with cefixime (0.5 mg/L), cefsulodin (10 mg/L), and vancomycin (8 mg/L; $GN_{ccv}$). Samples were vortexed and incubated for 5.5 h at 37°C, after which immunomagnetic bead separation (IMS) was performed on 1 ml of enrichment. After IMS, samples were plated onto sorbitol-MacConkey agar supplemented with cefixime (0.5 mg/L) and potassium tellurite (2.5 mg/L; CT-SMAC). Plates were incubated overnight at 37°C, and up to six sorbitol negative colonies were streaked onto blood agar plates (Remel, Lenexa, KS). After an overnight incubation at 37°C, colonies were tested for indole production and latex agglutination for the O157 antigen (Oxoid, Remel) and positive isolates were further characterized by multiplex PCR identifying *rfbE* (O157), *eae* (intimin), *stx1* (Shiga toxin 1), *stx2* (Shiga toxin 2), *hly*A (hemolysin), and *fliC* (flagella) genes (2, 3, 7).

In addition to detection by enrichment and selective plating, fecal samples were categorized into low and high shedders of *E. coli* O157:H7 by a semi-quantitative, direct plating method (10, 24). Briefly, a swab of fecal suspension in $GN_{ccv}$ broth (prior to enrichment) was spread onto a top quadrant of a CT-SMAC plate. A loop was used to streak from the quadrant for isolation and plates were incubated for approximately 20 h at 37°C. Up to six sorbitol negative colonies were picked from CT-SMAC plates, transferred to blood agar plates, incubated at 37°C overnight, evaluated for indole production and latex agglutination for the O157 antigen, and confirmed by multiplex PCR as described above. Following both the semi-quantitative and IMS methods, samples were categorized into high-shedder positive (positive isolate obtained from a direct-streak plate) and enrichment-positive (positive isolate obtained only with the IMS method).

**Statistical Analysis.** The pen-level fecal prevalence and the prevalence of cattle shedding *E. coli* O157:H7 at high concentrations (high shedders) were the outcomes of interest in the study. Descriptive statistics on prevalence outcomes were assessed prior to multivariable analysis. Data were then analyzed with generalized linear models for count data using Poisson and negative binomial distributions with log links in PROC GENMOD of SAS (v. 9.1, Cary, NC). A negative binomial model was only used when there was evidence of overdispersion in the Poisson model.

Pen was the experimental unit and the count of positive samples (or count of high shedders) was the outcome, which was offset by the natural log of the number of samples processed for each pen. A repeated (random) effect was included in all models to account for the lack of independence among samples within pens over time. Score statistics for generalized estimating equations were used to assess statistical significance of effects for DG form, inclusion level, collection day, and first-order interactions. Phase two data were analyzed in different, but similarly specified models. In addition to variables evaluated in phase one models, phase two models included dichotomous variable indicating whether or not DG was removed from diets. When analyzing phase two data, *E. coli* O157:H7 outcomes from cattle fed only the control diet were not included since the objective was to evaluate the effect of removing DG on *E. coli* O157:H7 prevalence estimates. Results were considered statistically significant at $P < 0.05$.

## Results

**Phase 1.** The overall fecal prevalence of *E. coli* O157:H7 during phase one (6 collections over 12 weeks) was 20.8% (695 of 3,350). The model-adjusted estimates of cumulative prevalence were 15.5, 19.2, 18.6, 25.6, and 24.7% for pens of cattle fed control, 20% DDG, 20% WDG, 40% DDG, and 40% WDG diets, respectively (Fig. 4.1). Collection day was associated with *E. coli* O157:H7 fecal prevalence ($P < 0.01$). In addition, the level at which DG was included in cattle diets was associated with fecal prevalence of *E. coli* O157:H7 ($P = 0.05$), while DG form was not associated with prevalence ($P = 0.8$). Pens of cattle fed 20% DG (wet or dried) did not have a statistically different *E. coli* O157:H7 prevalence than cattle fed the control diet ($P = 0.4$); however, prevalence in cattle fed 40% DG (wet or dried) differed from prevalence for cattle fed control ($P = 0.02$) or 20% DG ($P = 0.05$) diets. The interaction of DG form and inclusion level was not statistically significant ($P = 0.9$).

The overall sample prevalence of cattle shedding *E. coli* O157:H7 at high concentrations during phase one was 3.2% (107 of 3,353) and model-adjusted prevalence estimates were 2.1, 2.4, 2.4, 4.8, and 4.3% for cattle fed control, 20% DDG, 20% WDG, 40% DDG, and 40% WDG diets, respectively (Fig 4.2). Similar to overall shedding results, collection day was associated with high shedder prevalence ($P < 0.01$). In addition, the level of DG inclusion was associated with *E. coli* O157:H7 high shedder prevalence ($P < 0.01$), but DG form ($P = 0.7$) and the interaction ($P = 0.7$) were not significant. The prevalence of high shedders was higher in cattle

fed diets with 40% DG compared to those fed no DG ($P < 0.01$) or 20% DG ($P < 0.01$); prevalence of high shedders was not different between cattle fed control diets and diets with 20% DG ($P = 0.6$).

**Phase 2.** Fecal prevalence of *E. coli* O157:H7 during phase two was 3.3% (60 of 1,792) overall and < 0.1% (1 of 1,792) of pen floor samples were considered high shedders. Prevalence of *E. coli* O157:H7 varied by collection day during the second phase of this study ($P < 0.01$). The model-adjusted estimates of cumulative prevalence were 0.7, 2.6, 3.7, and 3.7 for cattle fed 20 % DDG, 20% WDG, 40% DDG, and 40% WDG, respectively, and were 4.8, 1.9, 1.9, and 3.0% for cattle fed 20% DDG- REM, 20% WDG-REM, 40% DDG-REM, and 40% WDG-REM diets, respectively. The inclusion level and form of DG were not associated with *E. coli* O157:H7 prevalence during this phase. In addition, DG removal from treatments was not associated with prevalence ($P = 0.7$).

## Discussion

Our study confirms the previous observation that cattle fed wet or dried DG diets had an increased fecal prevalence of *E. coli* O157:H7. In addition, our study shows that not only overall prevalence, but also high shedder prevalence was dependent on the level at which the co-product was included. Previous studies documenting the relationship between *E. coli* O157:H7 prevalence and feeding DG differed in the inclusion level (10 to 50%) and form of DG (wet or dried) fed to cattle; further, the magnitude of the reported association has not been consistent (12, 13, 31). Although both dried and wet DG have been studied, the two have not been directly compared in terms of their impact of *E. coli* O157:H7 prevalence. The current study was designed to evaluate DG inclusion level and DG form (dried or wet), concurrently, in order to understand how previous inconsistencies in prevalence may be related to different diet composition. There was no difference between the two forms of DG and there was no interaction between inclusion level and DG form; thus, we can conclude that the impact of wet versus dried products does not differ.

The amount of DG included in cattle diets is variable, with most studies using 40 to 50% DG on a dry matter basis as the upper limit (8, 11, 28). The optimum inclusion level for animal performance ranges between 15 and 30% and is related to additional factors including primary

grain type, source, and grain processing (4, 16). Price of grain, availability, and location of DG plants are all factors influencing the level of DG fed to cattle (29). A previous study evaluating the efficacy of an *E. coli* O157:H7 vaccine included DG at different levels of cattle diets (0, 10, 20, 30, 40 and 50%) and observed that inclusion level may be associated with *E. coli* O157:H7 prevalence (21). We also observed that DG inclusion level was associated with *E. coli* O157:H7 prevalence. Our study suggests that at 20% DG inclusion, there was no significant increase in *E. coli* O157:H7 prevalence compared to cattle not fed DG; however, when DG was included at 40% of the diet, the fecal prevalence was significantly higher.

Different inclusion levels of DG, as well as nutrient component variability's between DG sources (15, 26) may explain the inconsistencies among previous studies regarding the magnitude of association between *E. coli* O157:H7 and DG in the diet. Because *E. coli* O157:H7 is primarily found in the hindgut of cattle (20, 30), it is likely affected by changes in the hindgut ecology, such as pH, volatile fatty acids (VFA) concentrations and nutrients availability. Distiller's grains are low in starch content, so the amount of starch reaching the hindgut is less than in a grain-fed animal. On the other hand, more protein is likely to reach the hindgut because DG protein is less fermentable in the rumen (16). Fecal pH and VFA concentrations have been used as indicators of hindgut fermentation (18). Previous work has shown that total VFA concentration in the feces decreases linearly with increasing levels of DG fed to cattle (27). Wells et al. (31) evaluated *E. coli* O157:H7 shedding in cattle fed 40% DG diets and observed no relationship with fecal pH; however, the concentrations of $_{L}$-lactate and total VFA were higher in *E. coli* O157:H7 positive compared to negative fecal samples. In that study, differences in fecal pH and VFA concentrations were not examined between cattle fed 0 and 40% WDG, which may have provided additional information on the differences between diets. We did not measure fecal pH, $_{L}$-lactate, or VFA concentrations in our study; however, it is likely that cattle fed increasing amounts of DG have different physiological conditions and microbial populations in the hindgut.

In addition to fecal prevalence of *E. coli* O157:H7 being positively associated with feeding 40% DG to cattle, we also observed that a greater number of cattle fed these diets shed the organism at high concentrations. This observation is in agreement with the study reported by Wells et al. (31) who showed that the number of samples that were quantifiable (shedding higher concentrations) for *E. coli* O157:H7 was higher in cattle fed 40% WDG compared to cattle fed

control diets. Although the significance of cattle shedding high concentrations of *E. coli* O157:H7 is not fully understood, there is evidence that at harvest the prevalence of the high shedders is positively associated with hide and carcass contamination (10, 14). It is also proposed that high shedders are responsible for increased transmission of *E. coli* O157:H7 within a cohort (1, 17); possibly by increasing the concentration of the organism in the environment (27). Although the reason why cattle shed high concentrations of *E. coli* O157:H7 is not known, we can surmise that more cattle shedding higher concentrations of the organisms increases the food safety risk. It is not known why DG feeding increases high shedders. It has been suggested that DG may stimulate growth of *E. coli* O157 directly (12); however, our results seem to suggest that the mechanism may be similar to that promoting *E. coli* O157:H7 at normal concentrations since the trends between inclusion levels and both fecal prevalence and high shedder prevalence were similar.

The difference between wet and dried DG is a dehydrating process, which makes transportation of the product to cattle producers easier (16). Despite potential differences in nutrient content between WDG and DDG, the prevalence and concentration of *E. coli* O157:H7 in cattle fed similar amounts seemed unaffected; perhaps indicating that the observed association with DG may be more a function of starch displacement than added nutrients. This hypothesis has been proposed by others evaluating the effect on *E. coli* O157:H7 prevalence due to diet regimens that alter the availability of starch in the hindgut (13). Fox et al. (9) reported a higher prevalence of *E. coli* O157:H7 in cattle fed steam-flaked corn compared to dry-rolled corn; the authors hypothesized that the difference may be related to the amount of starch reaching the hindgut which would be higher in dry-rolled than steam-flaked corn diets. The role of starch on *E. coli* O157:H7 in the hindgut of cattle remains unknown, yet differences in starch content seem common in diets which alter *E. coli* O157:H7 shedding.

The prevalence of *E. coli* O157:H7 in cattle during the second phase of our trial was quite low (3.3%), which limited our ability to effectively evaluate whether removal of DG had any effect on fecal prevalence of *E. coli* O157:H7. We had anticipated that the prevalence and concentration of *E. coli* O157:H7 in cattle removed from DG would decrease to control values seen in the first phase of the study, providing further evidence for the positive association between DG and *E. coli* O157 prevalence. Instead, prevalence in all treatments dropped significantly, as did the number of high shedders. Although there was no statistical difference in

prevalence between cattle fed DG diets and those fed diets where the DG was removed, these results should be interpreted with caution. The samples for the second phase were collected in late fall; the fact that we had low prevalence during this time may not be surprising considering the seasonal prevalence of *E. coli* O157:H7, which typically peaks during summer months (5, 23, 25). In this study, we transitioned cattle from diets containing DG to control finishing diets over two weeks to prevent disruption in intake and maintain rumen health. Even if we had we observed a reduction of *E. coli* O157:H7 shedding, it would be challenging to develop widespread adoption of such an intervention strategy, particularly with the risk of reduced of animal performance.

In conclusion, this study confirmed that feeding cattle distiller's grains was associated with increased fecal shedding of *E. coli* O157:H7 and an increased number of cattle shedding the organism at high concentrations. Additionally, we have shown that the form of DG, wet or dry, had no impact on the association; however, the fecal shedding of *E. coli* O157:H7 was dependent on the level at which DG was included.

## Acknowledgements

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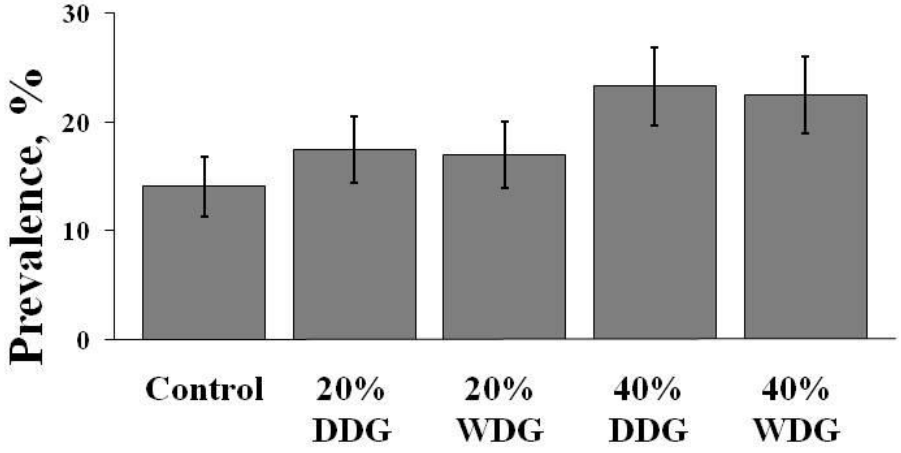
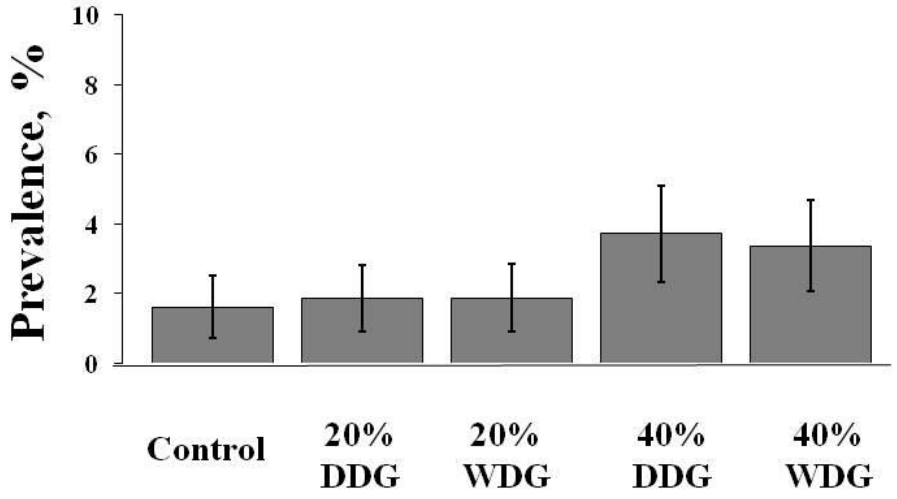
**Figure 4.1 Model-adjusted cumulative prevalence estimates (and 95% confidence intervals) of fecal *Escherichia coli* O157:H7 shedding in pens of cattle fed diets with 0, 20, or 40% wet or dried distiller's grains (WDG; DDG) for twelve weeks.**




**Prevalence estimates for cattle fed WDG and DDG were not statistically different; however, cattle fed 40% WDG or DDG had a higher fecal prevalence of E. coli O157:H7 than cattle fed either 0 or 20% ($P \leq 0.05$).**

**Figure 4.2 Model-adjusted cumulative prevalence estimates (and 95% confidence intervals) of *Escherichia coli* O157:H7 high shedders in pens of cattle fed diets with 0, 20, or 40% wet or dried distiller's grains (WDG; DDG) for twelve weeks.**




**After analysis, WDG and DDG types were not statistically different; however, cattle fed 40% WDG or DDG had a higher high shedder prevalence than cattle fed either 0 or 20% DG ($P < 0.01$).**

**Table 4.1 Dietary Treatment Composition.**

| Item | Control | 20 % Dried DG | 40 % Dried DG | 20% Wet DG | 40% Wet DG |
|---|---|---|---|---|---|
| *Ingredient* | | | *%, As-fed basis* | | |
| Cracked Corn | 82.9 | 68.2 | 48.9 | 51.8 | 29.7 |
| Soybean meal | 2.03 | 0.0 | 0.0 | 0.0 | 0.0 |
| Ground alfalfa | 7.63 | 7.79 | 7.93 | 5.91 | 4.82 |
| Dried distillers grains | 0.0 | 18.4 | 37.5 | 0.0 | 0.0 |
| Wet distillers grains | 0.0 | 0.0 | 0.0 | 38.1 | 62.0 |
| Low protein liquid supplement | 0.0 | 5.60 | 5.70 | 4.25 | 3.46 |
| High protein liquid supplement | 7.49 | 0.0 | 0.0 | 0.0 | 0.0 |
| *Chemical Composition* | | | *%, Dry matter basis* | | |
| Dry matter | 80.7 | 82.1 | 83.2 | 65.2 | 55.3 |
| Crude protein | 10.1 | 15.9 | 20.9 | 19.8 | 25.3 |
| Soluble protein | 5.87 | 4.38 | 5.72 | 4.91 | 6.06 |
| Acid detergent fiber | 5.08 | 7.63 | 10.5 | 10.6 | 14.2 |
| Neutral detergent fiber | 10.4 | 15.0 | 20.0 | 18.4 | 23.5 |
| Crude fat | 4.69 | 6.17 | 7.57 | 8.21 | 10.4 |
| Sulfur | 0.16 | 0.27 | 0.42 | 0.34 | 0.47 |
| ME, Mcal/kg | 3.16 | 3.22 | 3.21 | 3.35 | 3.43 |

# CHAPTER 5 - Evaluation of Feeding Dried Distiller's Grains with Solubles and Dry-Rolled Corn on the Fecal Prevalence of *Escherichia coli* O157:H7 and *Salmonella* spp. in Cattle

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## Abstract

*Escherichia coli* O157:H7 and *Salmonella* are foodborne pathogens that reside in the gut of cattle and are shed in the feces. Previous work indicated a positive association between feeding cattle distiller's grains (DG) and an increase in *E. coli* O157:H7 prevalence. Feeding processed grains also has been shown to affect fecal prevalence of *E. coli* O157:H7. The objective of this study was to evaluate the effect of feeding DG and dry-rolled corn (DRC), alone or in combination, on fecal prevalence of *E. coli* O157:H7 and *Salmonella* in finishing cattle. Cattle were allotted to pens (n = 28) and fed dietary treatments (n = 150 days) structured in a 2×2 factorial arrangement; the factors were 0 or 25% dried corn DG with solubles (DDGS) and 0 or 25% DRC in steam-flaked corn-based high-grain diets. Fecal samples were collected from each pen floor before initiating dietary treatments and at least once every 2 weeks after final diets began. Overall prevalence of *E. coli* O157:H7 in fecal samples was 5.1%. There were no significant effects of DDGS, DRC, or sampling time on *E. coli* O157:H7 prevalence ($P > 0.20$). Overall prevalence of *Salmonella* in pen floor fecal samples was 23.7%, and sampling week affected prevalence ($P < 0.01$), ranging from < 1% (week 1) to 77.5% (week 17). *Salmonella* prevalence was not affected by cattle diet and no work had previously reported an association between either DG or DRC and *Salmonella* prevalence. Lack of an association between *E. coli* O157:H7 prevalence and feeding DG or DRC is contrary to previous observations. Further

research is needed to understand inconsistencies between studies of *E. coli* O157:H7 prevalence and potential associations with DG and grain processing methods.

## Introduction

*Escherichia coli* O157:H7 and *Salmonella* are foodborne pathogens harbored within the gastrointestinal tract of food animals (Callaway et al., 2003; Gyles et al., 2007). These organisms, which are shed in the feces of cattle, can contaminate food products and result in human illness (Mead et al., 1999; Rangel et al., 2005). Factors that contribute to the prevalence and fecal shedding of foodborne pathogens in cattle require further evaluation to facilitate development of strategies to mitigate the human health effects of these pathogens.

Distiller's grains (DG), which are byproducts of ethanol production that are available in a wet or dry form, are valuable as feed for livestock. Use of these products in cattle production systems has increased considerably as a consequence of rapid expansion of the fuel ethanol industry (National Agricultural Statistics Service, 2007). Also, reduced availability and higher price of corn make DG an attractive energy source for cattle (Klopfenstein et al., 2008). Previously, we reported a positive association between fecal shedding of *E. coli* O157:H7 and feeding cattle wet or dried DG (Jacob et al., 2008a, b, c). Positive associations between *E. coli* O157:H7 prevalence and byproduct feeding also were observed in a study of beef cows fed DG in Scotland (Synge et al., 2003) and in an epidemiologic study of U.S. feedlot cattle fed brewer's grains, a fermentation byproduct of the brewery industry (Dewell et al., 2005). It has been suggested that DG contains components that directly or indirectly stimulate growth of *E. coli* O157:H7 (Jacob et al., 2008a, b). Indirect stimulation may be mediated via the effect of DG on the hindgut microbial ecosystem, the major site of *E. coli* O157:H7 persistence or colonization (Grauke et al., 2002; Van Baale et al., 2004). Because DG replaces a portion of cereal grain in the diet, the reduction in dietary starch and flow of non-starch fractions, particularly bran, lipid and protein, into the hindgut could alter the microbial ecosystem to favor of growth and persistence of *E. coli* O157:H7. Therefore, one potential strategy to mitigate the DG effects on *E. coli* O157:H7 shedding in cattle is to feed diets that create environment in the hindgut inhospitable for the organism.

A previous study showed that feeding grain processed by dry-rolling rather than steam-flaking, reduced fecal shedding of *E. coli* O157 (Fox et al., 2007). Possibly, dry-rolling allowed

more starch to reach the hindgut where it was fermented to acids, thus making the hindgut inhospitable to the survival of *E. coli* O157 (Fox et al., 2007). Therefore, our objectives in this study were to compare fecal shedding of *E. coli* O157:H7 in cattle fed steam-flaked corn (SFC)-based high-grain finishing diets with 0 or 25% DG supplementation and 0 or 25% dry-rolled corn (DRC). The effect of feeding DG on fecal shedding of *Salmonella* is not known. Because the intestinal tract of cattle also can contain *Salmonella* (Callaway et al., 2008), which can be shed in the feces (Sanchez et al., 2002; Kunze et al., 2008), our study also determined the effect of DG supplementation with or without DRC on fecal shedding of *Salmonella.*

## Materials and Methods

**Animals, Treatments, and Sampling Schedule.** Seven hundred crossbred yearling heifers were blocked by body weight and randomly allotted to one of 28 feedlot pens; each pen contained approximately 25 animals. Pens provided approximately 17$m^2$ of surface area and 40 cm linear bunk space per animal. Pens were divided by pipe fences and a common watering unit was shared between adjacent pens. Cattle in all pens were fed SFC-based high grain finishing diets with approximately 6% alfalfa hay (Table 5.1). Cattle were transitioned through a series of four step-up diets (specific for each treatment) before beginning their respective finishing diets. Four dietary treatments were used, and each treatment was replicated in seven pens. Treatments were structured in a 2 × 2 factorial arrangement; factor one was the level of supplementation with dried corn distiller's grains with solubles (DDGS; 0 or 25% of diet dry matter) and factor two was the level of supplementation with DRC (0 or 25% of diet dry matter). Each treatment was randomly assigned to one of four adjacent pens.

Fecal samples were collected from the pen floor to determine prevalence of *E. coli* O157:H7 and *Salmonella* spp. The first sample collection occurred prior to the first step-up diet. Once cattle were transitioned to the final fishing diet, fecal samples were collected once every 1 to 2 weeks for the remaining 19 weeks of the experiment. Because of low fecal prevalence of *E. coli* O157:H7, sample collection after week 12 occurred weekly for the isolation of *E. coli* O157:H7. For isolation of *Salmonella*, samples were processed once every 2 weeks throughout the study. Sampling of cattle on finishing diets occurred from June through October 2007. At each collection, 10 fresh fecal pats were identified and sampled from the pen floor, taking care to avoid ground contamination. Approximately 150 g of fecal material were obtained per sample,

placed into a pre-labeled whirl pack bag (Nasco, Fort Atkinson, WI), and transported in a cooler to the laboratory. Samples were refrigerated overnight at 4˚C before processing.

**Isolation of *E. coli* O157:H7 and *Salmonella* spp.** The procedure used for the isolation of *E. coli* O157:H7 has been described previously (Greenquist et al., 2005). Approximately 1 g of feces was enriched in 9 mL of Gram Negative (GN) broth (Difco; BD, Sparks, MD, USA) with cefixime (0.5 mg/L), cefsulodin (10 mg/L), and vancomycin (8 mg/L) at 37˚C for 6 h. One mL of the enriched sample was subjected to immunomagnetic bead separation (IMS; Invitrogen; Carlsbad, CA, USA) followed by plating onto sorbitol-MacConkey agar (Difco) with cefixime (0.5 mg/L) and potassium tellurite (2.5 mg/L). After plates were incubated overnight (37˚C), sorbitol negative colonies (up to six) were selected and plated on blood agar plates (Remel, Lenexa, KS). Following an overnight incubation (37˚C), colonies were tested for indole production and agglutination with the O157 antigen (Oxoid, Remel). Further confirmation of isolates as *E. coli* O157:H7 was done using PCR determination for the virulence genes *eae, fliC, hly*A, *stx*1, and *stx*2. Primers used in the multiplex PCR for *eae, hly*A, *stx*1, and *stx*2 were designed by Fagan et al. (1999) and PCR conditions were: initial denaturation at 95˚C for 3 min, 30 cycles of 95˚C for 20 sec, 58˚C for 40 sec, and 72˚C for 90 sec, and a final elongation at 72˚C for 5 min. The gene for H7 flagella, *fliC*, was detected using primers designed by Gannon et al. (1997) with the same running conditions as previously described. Amplified DNA fragments were electrophoresed using a 2% (w/v) agarose gel (Fisher Scientific; Houston, TX, USA). Gels were stained with ethidium bromide (0.5 µg/mL), visualized with UV light and imaged with a Gel-Doc 2000 fluorescent imager (Bio-Rad, Hercules, CA).

*Salmonella* isolates were obtained using a procedure adapted from that described by Barkocy-Gallagher et al. (2002). Approximately 10 g of feces were enriched in 90 mL of tryptic soy broth (TSB; Difco) for 2 h at 25˚C, 6 h at 42˚C, and overnight at 4˚C. After enrichment, 10 mL of TSB were inoculated in 90 mL of tetrathionate broth (TTB; Difco) and incubated for 24 h at 37˚C. One mL of enriched TTB was subjected to IMS, using anti-*Salmonella* beads, followed by enrichment in 10 mL of Rappaport-Vassiliadis (RV; Difco) broth at 42˚C for 16 to 18 h. Finally, 50 µL of RV broth were plated onto Hektoen enteric agar (Difco) supplemented with novobiocin (15 mg/L). Hektoen plates were incubated overnight at 37˚C, after which up to three typical black colonies, suggestive of *Salmonella*, were picked and plated onto blood agar plates.

Agglutination with polyvalent O (A-I and Vi) antisera (Difco) were used to confirm *Salmonella* isolates, and isolates were subsequently serogrouped based on group-specific agglutination.

**Statistical Analyses.** Fecal prevalence of *E. coli* O157:H7 and *Salmonella* within pens were the primary outcomes of interest in this study. Prevalence estimates were the total number of culture-positive (*E. coli* O157:H7 or *Salmonella*) fecal samples per pen at each collection divided by the total number of samples per pen obtained at each collection. Data were analyzed using logit models in PROC GENMOD of SAS (v. 9.1, Cary, NC). Pen was the experimental unit and a repeated measure was included in the models because all pens were sampled on multiple weeks. The final model for *E. coli* O157:H7 included main effects of DDGS, DRC, and sampling week as well as DDGS × DRC and week × DRC interactions. The three-way interaction and DDGS × week interaction were not included because the model would not converge. The final model for *Salmonella* included the main effects (DDGS, DRC and sampling week) and the DDGS × DRC interaction, again because the model would not converge with all interactions. A chi-square analysis using PROC FREQ of SAS was used to evaluate a potential association between the fecal presence of *E. coli* O157:H7 and *Salmonella* within each fecal sample, regardless of the dietary treatments cattle received.

## Results

The average daily feed intakes (kg dry matter/day) were 8.4, 8.6, 8.7, and 8.7 for SFC, SFC+DDGS, SFC+DRC, and SFC+DRC+DDGS treatment groups, respectively. There were no significant effects of grain processing, DDGS addition, or their interactions on daily feed intake. Initial prevalence of *E. coli* O157:H7 in fecal samples prior to feeding cattle transition diets (week 0) was 15.7% (44 of 280 samples). *Salmonella* baseline prevalence was 28.6% (80 of 280 samples). Of the 80 *Salmonella* isolates obtained when cattle initially arrived, 71.2% (57 of 80 isolates) were serogroup B, 25% (20 of 80 isolates) were serogroup C1, and 3.8% (3 of 80 isolates) were serogroup E. There were no differences in the prevalence of either *E. coli* O157:H7 or *Salmonella* between treatment groups prior to beginning dietary treatment ($P > 0.05$).

Overall cumulative prevalence of *E. coli* O157:H7 in pen floor fecal samples after cattle began final finishing diets (weeks 1 to 19) was 5.1% (183 of 3,560 samples). Figure 5.1 shows

the cumulative prevalence from pen floor fecal samples for each treatment group. Prevalence of *E. coli* O157:H7 was numerically higher ($P = 0.25$) in pens of cattle fed DDGS (5.7%) or DDGS with DRC (5.0%) compared with pens fed SFC only (2.7%) or SFC with DRC (4.0%). In the final logit model, there were no effects on *E. coli* O157:H7 prevalence associated with feeding 25% DDGS ($P = 0.25$), 25% DRC ($P = 0.55$), or both 25% DDGS and 25% DRC ($P = 0.42$). In addition, prevalence did not differ by week ($P > 0.2$). The prevalence of *E. coli* O157:H7 was generally quite low throughout the finishing period, displaying only occasional minor spikes (Fig. 5.3). Mean fecal prevalence (arithmetic means) of *E. coli* O157:H7 across pens for each diet at all sampling times are shown in Table 5.2.

Overall cumulative prevalence of *Salmonella* in pen floor fecal samples collected after cattle began final diets was 23.7% (606 of 2,557 samples). The serogroup distribution for *Salmonella* isolates was: 88% C1 (529 of 603 isolates), 11% B (68 of 603 isolates), 1% E (6 of 603 isolates), and 0.5% (3 of 603 isolates) that did not agglutinate with antisera from any serogroup evaluated. Figure 5.2 shows the cumulative prevalence of *Salmonella* collected from each treatment group for the entire study. Feeding DDGS, DRC, or the combination had no effect on *Salmonella* prevalence ($P = 0.9$, $P = 0.7$, and $P = 0.3$, respectively). *Salmonella* prevalence differed by week ($P < 0.01$), and least square mean estimates ranged from < 1% on the first sampling week after final diets began to 78% on week 17 (Fig.5.3). Mean fecal prevalence (arithmetic means) of *Salmonella* across pens for each diet at all sampling times are shown in Table 5.3. There was no association between the presence of *E. coli* O157:H7 and *Salmonella* ($P > 0.4$) within individual fecal samples.

## Discussion

There were no significant effects of including 25% DDGS in the diet on prevalence of *E. coli* O157:H7 in cattle. However, cumulative *E. coli* O157:H7 prevalence estimates across pens in this study from cattle fed DDGS were about two-fold higher than in cattle fed no DDGS. Overall prevalence of *E. coli* O157:H7 was low (< 6%), which might have affected our ability to find statistical differences. It is also possible that variation in nutrient compositions of distillery co-products is a factor affecting the fecal shedding of *E. coli* O157:H7. Distiller's grains are concentrated in protein, fiber, and lipid components, and the concentrations of nutrients in the co-product can be variable (Spiehs et al., 2002). Some of this variation results from differences

in nutrient concentrations of cereal grains used for fermentation. Also, differences in types of yeasts, fermentation, distillation, centrifugation efficiencies, drying processes, and the amount of solubles blended into the feed co-product could influence nutrient composition. Relative amounts of DG and distiller's solubles mixed together varies depending on the ethanol plant and the production of the wet or dry form.

Previous work has indicated a positive association between feeding cattle fermented grain co-products (distiller's or brewer's grains) and an increased fecal shedding of *E. coli* O157 (Synge et al., 2003; Dewell et al., 2005; Jacob et al., 2008a, b, c). In an investigation of management factors influencing the shedding of *E. coli* O157 by beef cows in Scotland, Synge et al. (2003) reported that feeding DG was associated with increased *E. coli* O157 shedding in beef cows that were housed; however they found no difference in grazing animals supplemented with DG. An epidemiologic assessment of factors contributing to *E. coli* O157 prevalence in U. S. feedlot cattle reported fecal samples were more likely to be positive in cattle fed brewer's grains compared to those not fed brewer's grains (Dewell et al., 2005). In previous natural prevalence and experimental inoculation studies, we have observed an increased prevalence of *E. coli* O157:H7 in cattle fed 25% DDGS compared to control cattle fed no DDGS (Jacob et al., 2008a, b, c). The mechanism for the association between DG and *E. coli* O157:H7 shedding is not known but was hypothesized to be either direct stimulation of *E. coli* O157:H7 by a component of DG or mediated via an indirect effect of DG on gut microbial populations (Jacob et al., 2008a). The high concentrations of protein, fiber, and lipid components and decreased starch concentration (the component removed during ethanol production) likely affect the microbial ecology of the gastrointestinal tract in animals supplemented with DG. In the rumen, changes in fermentation products and microbial shifts including a trend for more amylolytic and lactate-utilizing bacteria were observed in cattle fed DG (Fron et al., 1996). However, the effect of DG supplementation on the hindgut ecosystem has not been determined. *Escherichia coli* O157:H7 commonly is found in the hindgut of cattle (Grauke et al., 2002); therefore, changes in the environment of this region may affect fecal shedding of the organism.

Our data indicated no significant relationship between DDGS and *Salmonella* shedding. Although prevalence of *Salmonella* varied by week, we did not detect any DDGS by week interaction. Few studies have evaluated the influence of diet or hindgut ecology on *Salmonella* shedding in feedlot cattle. Berry et al. (2006) assessed forage versus corn silage-based diets on

the prevalence of several foodborne pathogens including *Salmonella*; because *Salmonella* recovery was low, no conclusion was drawn. Experimental inoculation studies with cattle manure revealed that *Salmonella* Typhimurium declined faster in manure from cattle fed straw, which had a higher pH and fiber content, compared with grass silage plus corn silage (Franz et al., 2005). Additionally, feeding tallow, whole cottonseed, or cottonseed hulls was associated with increased *Salmonella* prevalence (Losinger et al., 1997). The variation of *Salmonella* prevalence by week in this study was substantial, although there was generally an increasing trend. Although the reason for this variation is unknown, *Salmonella* prevalence in dairy and feedlot cattle has previously been shown to vary by factors including geographic region and season (Fedorka-Cray et al., 1998; Dargatz et al., 2003; Edrington et al., 2004). It is possible that environmental factors including temperature, pen conditions, and bird migration, among others influenced the *Salmonella* prevalence in this study, however, those factors were not evaluated and we cannot speculate to such a large variation.

Grain processing method, particularly steam-flaking compared with dry-rolling, has been associated with decreased feed intake and increased feed efficiency and fecal pH in cattle (Barajas and Zinn, 1998; Fox et al., 2007; Depenbusch et al., 2008). Steam-flaking of grains enhances ruminal digestion of the starch component within the rumen (Huntington, 1997). Steam-flaking also may alter the hindgut ecosystem in cattle to promote *E. coli* O157:H7 or *Salmonella* shedding. Fox et al. (2007) observed that cattle fed wheat or sorghum diets processed by steam-flaking resulted in higher shedding rates compared with shedding rates of cattle fed the same grains processed via dry-rolling, suggesting that increased hindgut fermentation may impede *E. coli* O157 survival. In another study, prevalence of *E. coli* O157 also tended to decrease between cattle fed diets containing dry-rolled corn and steam-flaked corn (Depenbusch et al., 2008). Intervention strategies incorporated prior to cattle being harvested may be an effective means to control the spread of foodborne pathogens. One advantage to manipulating feed ingredients as a means of preharvest intervention is that the change may be easily implemented (Loneragan and Brashears, 2005). Prevalence of *E. coli* O157:H7 or *Salmonella* was not affected by inclusion of 25% DRC into finishing diets in this study. Additionally, we observed no interaction between DG and DRC. It is possible that supplementation with only 25% DRC was insufficient to impact hindgut ecology to inhibit *E. coli* O157:H7. Previous work evaluating differences in *E. coli* O157 between grain processing

methods used diets composed primarily of either dry-rolled or steam-flaked grains (Fox et al., 2007; Depenbusch et al., 2008). In this study, particularly for control diets with DRC, the major component of the diet was SFC.

We also were interested in potential associations between fecal shedding of the two organisms. Smith et al. (2005) reported that pens of cattle tested using ropes and positive for *Salmonella* were 0.58 times as likely to be positive for *E. coli* O157, indicating a potential inverse relationship. In our study, we found no relationship (positive or negative) between *E. coli* O157:H7 and *Salmonella* within pen floor fecal samples.

In conclusion, we observed no significant effects from inclusion of DDGS on *E. coli* O157:H7 prevalence, which is inconsistent with previous data. Whether lack of a statistically significant difference is reflective of a low overall prevalence of *E. coli* O157:H7, differences in nutrient composition of the byproduct, or lack of a true biological effect needs further study. There also was no indication that byproduct feeding affected *Salmonella* shedding in finishing cattle. Supplementing SFC diets with 25% DRC also did not affect prevalence of either *E. coli* O157:H7 or *Salmonella*. Further research is needed to better understand inconsistencies between studies of *E. coli* O157:H7 prevalence and potential associations with DG and grain processing methods if dietary interventions are to become an effective means of reducing potential food safety risks.

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**Figure 5.1 Cumulative prevalence (and 95% confidence intervals) of *Escherichica coli* O157:H7 in pen floor fecal samples across pens of cattle fed a steam-flaked corn (SFC)-based high grain diet, supplemented with 25% dried corn distiller's grains with solubles (SFC + DDGS), supplemented with 25% dry-rolled corn (SFC + DRC), or supplemented with 25% DDGS and 25% DRC (SFC + DDGS + DRC).**

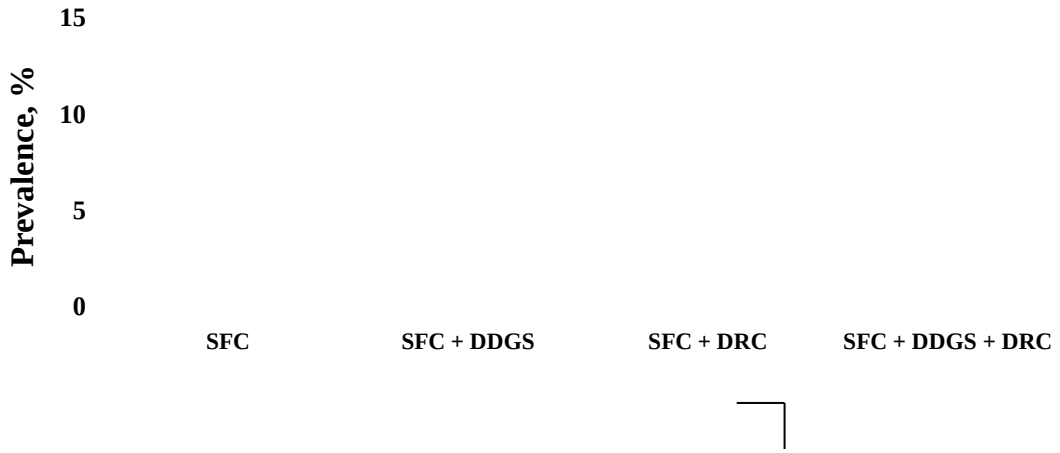

**Figure 5.2 Cumulative prevalence (and 95% confidence intervals) of *Salmonella* O157:H7 in pen floor fecal samples across pens of cattle fed a steam-flaked corn (SFC)-based high grain diet, supplemented with 25% dried corn distiller's grains with solubles (SFC + DDGS), supplemented with 25% dry-rolled corn (SFC + DRC), or supplemented with 25% DDGS and 25% DRC (SFC + DDGS + DRC).**

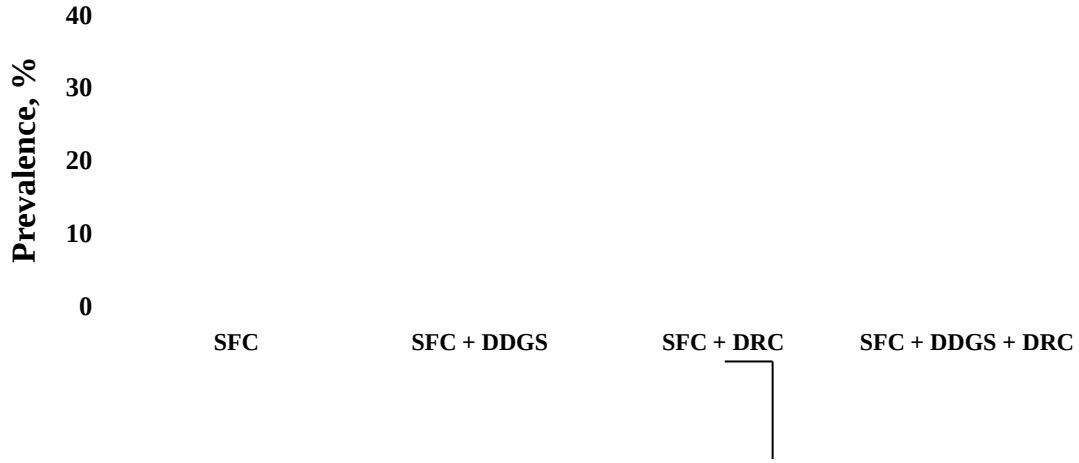

**Figure 5.3 Weekly least squares mean prevalence estimates of *Escherichia coli* O157:H7 and *Salmonella* species from pen floor fecal samples across pens of cattle fed steam-flaked corn diets with or without dried distiller's grains with solubles and dry-rolled corn.**

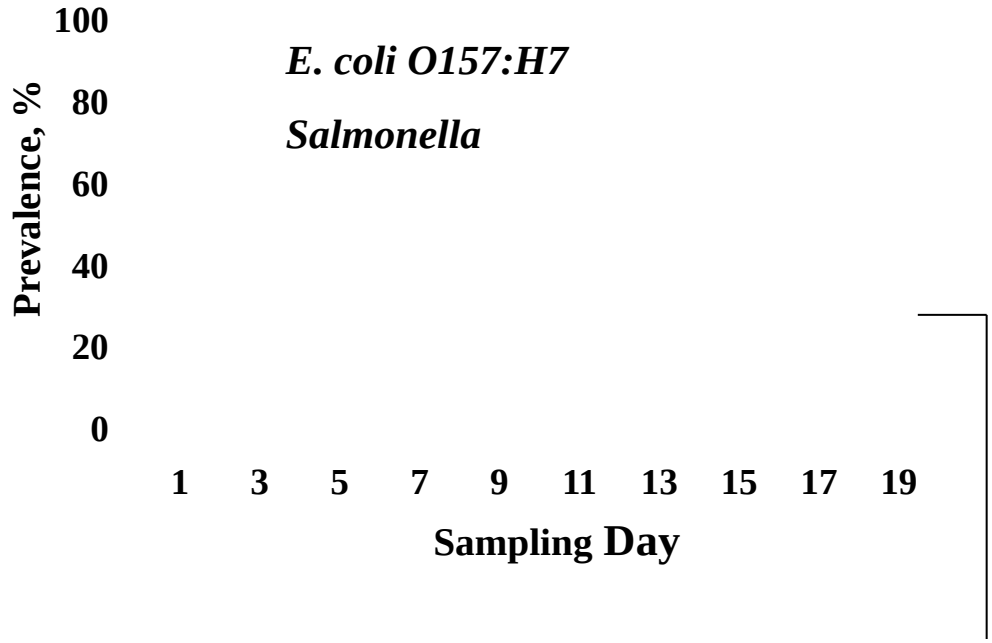

**Table 5.1 Ingredient and nutrient compositions of the final steam-flaked corn (SFC) grain-based diets with or without supplemental dried corn distiller's grains with solubles (DDGS) and dry-rolled corn (DRC).**

| *Ingredient or Nutrients* | *SFC diet* | *SFC diet with DDGS* | *SFC diet with DRC* | *SFC diet with DDGS and DRC* |
|---|---|---|---|---|
| **Ingredients, % dry matter** | | | | |
| SFC | 82.1 | 58.2 | 56.8 | 33.1 |
| DDGS | - | 25.4 | - | 25.3 |
| DRC | - | - | 25.5 | 25.3 |
| Alfalfa hay | 5.9 | 5.9 | 5.9 | 5.8 |
| Corn steep liquor | 6.5 | 6.4 | 6.4 | 6.4 |
| Supplement[1] | 5.5 | 4.2 | 5.5 | 4.1 |
| **Nutrients, %** | | | | |
| Crude protein (CP) | 14.0 | 16.0 | 14.0 | 16.0 |
| Non-protein nitrogen (as CP equivalent) | 3.5 | - | 3.5 | - |
| Neutral detergent fiber | 10.6 | 15.8 | 10.6 | 15.8 |
| Ether extract | 3.8 | 5.7 | 3.8 | 5.7 |
| Calcium | 0.7 | 0.7 | 0.7 | 0.7 |
| Phosphorus | 0.4 | 0.5 | 0.4 | 0.5 |

**[1]Formulated to meet or exceed nutritional requirements and contained 0.055 mg/kg melengestrol acetate, 33 mg/kg monensin, and 9.9 mg/kg tylosin.**

**Table 5.2 Mean prevalence (%) and standard deviation across pens of *Escherichia coli* O157:H7 from cattle fed a steam-flaked corn-based high-grain diet (SFC), SFC supplemented with 25% dried corn distiller's grains with solubles (SFC + DDGS), SFC supplemented with 25% dry-rolled corn (SFC + DRC), or SFC supplemented with 25% DDGS and 25% DRC (SFC + DDGS + DRC).**

| *Week* | *SFC* | *SFC + DDGS* | *SFC + DRC* | *SFC + DDGS + DRC* |
|---|---|---|---|---|
| Pre-Treatment | 12.9 ± 16.0 | 12.9 ± 7.6 | 21.4 ± 14.6 | 15.7 ± 17.2 |
| 1 | 4.3 ± 5.3 | 11.4 ± 16.8 | 5.7 ± 7.9 | 8.6 ± 12.1 |
| 3 | 0 ± 0 | 2.9 ± 4.9 | 2.9 ± 4.9 | 1.4 ± 3.8 |
| 5 | 1.4 ± 3.8 | 17.1 ± 23.6 | 4.3 ± 7.9 | 10.0 ± 10.0 |
| 7 | 10.0 ± 12.9 | 7.1 ± 18.9 | 0 ± 0 | 5.7 ± 9.8 |
| 9 | 2.9 ± 7.6 | 2.9 ± 7.6 | 1.4 ± 3.8 | 1.4 ± 3.8 |
| 11 | 2.9 ± 4.9 | 5.7 ± 11.3 | 1.4 ± 3.8 | 2.9 ± 4.9 |
| 12 | 0 ± 0 | 2.9 ± 4.9 | 0 ± 0 | 4.3 ± 11.3 |
| 13 | 2.9 ± 4.9 | 1.4 ± 3.8 | 1.4 ± 3.8 | 4.3 ± 7.9 |
| 14 | 5.7 ± 7.9 | 8.6 ± 14.6 | 10.0 ± 15.3 | 17.1 ± 25.6 |
| 15 | 1.4 ± 3.8 | 2.9 ± 7.6 | 4.3 ± 5.3 | 5.7 ± 11.3 |
| 16 | 0 ± 0 | 5.7 ± 11.3 | 8.6 ± 14.6 | 2.9 ± 7.6 |
| 17 | 0 ± 0 | 2.5 ± 5.0 | 10.0 ± 14.1 | 0 ± 0 |
| 18 | 5.0 ± 5.8 | 10.0 ± 14.1 | 10.0 ± 11.5 | 2.5 ± 5.0 |
| 19 | 12.5 ± 25.0 | 12.5 ± 18.9 | 22.5 ± 28.7 | 12.5 ± 18.9 |

**Table 5.3 Mean prevalence (%) and standard deviation across pens of *Salmonella* species from cattle fed a steam-flaked corn-based high-grain diet (SFC), SFC supplemented with 25% dried corn distiller's grains with solubles (SFC + DDGS), SFC supplemented with 25% dry-rolled corn (SFC + DRC), or SFC supplemented with 25% DDGS and 25% DRC (SFC + DDGS + DRC).**

| *Week* | *SFC* | *SFC + DDGS* | *SFC + DRC* | *SFC + DDGS + DRC* |
|---|---|---|---|---|
| Pre-Treatment | 40.0 ± 22.4 | 25.7 ± 21.5 | 27.1 ± 18.0 | 21.4 ± 18.6 |
| 1 | 0 ± 0 | 0 ± 0 | 1.4 ± 3.8 | 0 ± 0 |
| 3 | 4.3 ± 7.9 | 4.3 ± 7.9 | 1.4 ± 3.8 | 1.4 ± 3.8 |
| 5 | 14.3 ± 12.7 | 10.0 ± 15.3 | 7.1 ± 12.5 | 7.1 ± 4.9 |
| 7 | 27.1 ± 18.0 | 10.0 ± 19.1 | 11.4 ± 19.5 | 12.9 ± 17.0 |
| 9 | 32.9 ± 40.3 | 28.6 ± 18.6 | 22.9 ± 21.4 | 41.4 ± 26.1 |
| 11 | 22.9 ± 33.0 | 10.0 ± 15.3 | 10.0 ± 22.4 | 24.3 ± 29.9 |
| 13 | 25.7 ± 29.4 | 35.7 ± 35.5 | 32.9 ± 38.2 | 38.6 ± 38.9 |
| 15 | 44.3 ± 33.6 | 35.7 ± 33.6 | 48.6 ± 21.9 | 51.4 ± 40.6 |
| 17 | 70.0 ± 46.9 | 82.5 ± 28.7 | 75.0 ± 37.9 | 82.5 ± 28.7 |
| 19 | 40.0 ± 29.4 | 5.0 ± 10.0 | 35.0 ± 47.3 | 42.5 ± 34.0 |

# CHAPTER 6 - Animal- and Truckload-Level Associations between *E. coli* O157:H7 in Feces and on Hides at Harvest and Contamination of Pre-evisceration Beef Carcasses

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## Abstract

Cattle feces and hides contribute to carcass contamination with *Escherichia coli* O157:H7, ultimately impacting beef safety. Primary objectives of our cross-sectional study were to evaluate associations among fecal, hide and pre-evisceration carcass prevalence of *E. coli* O157:H7 and assess factors affecting carcass contamination. Fecal, hide and pre-evisceration carcass samples were collected from up to 32 cattle on each of 45 truckloads presented to a Midwest abattoir. Enrichment and selective culture were used to assess fecal, hide, and carcass prevalence, and direct plating was used to identify cattle shedding high concentrations of *E. coli* O157:H7 in feces. Fecal, hide and carcass prevalence of *E. coli* O157:H7 within truckload were significantly ($P < 0.05$) correlated with each other. Enriched fecal sample prevalence was 13.8%, while high shedder prevalence was 3.3%. We found 38.5% of hides and 10.5% of carcasses positive for *E. coli* O157:H7. We used logistic regression to assess animal- and truckload-level factors affecting the probability of carcasses testing positive for *E. coli* O157:H7. All truckload-level predictors significantly affected the probability of an *E. coli* O157:H7-positive carcass, including presence of a high shedder within the truckload (OR=4.0; CI, 1.6 to 10.1), high (> 25%) within-truckload fecal prevalence (OR=19.3; CI, 4.7 to 79.0), and high (> 50%) within-truckload hide prevalence (OR=7.7; CI, 3.1 to 19.6); the only significant animal-

level predictor was having a positive hide (OR=1.6; CI, 1.0 to 2.6). Our study suggests that pre-harvest targets for reducing *E. coli* O157:H7 contamination of carcasses should focus on truckload (cohort)-level and hide mitigation strategies.

## Introduction

For more than two decades, the importance *Escherichia coli* O157:H7 as a foodborne pathogen has been well recognized. Cattle, an asymptomatic reservoir for *E. coli* O157:H7 shed the organism in their feces which can subsequently contaminate food products (*27*). Because of the threat to human health, *E. coli* O157:H7 is considered an adulterant, resulting in millions of pounds of beef recalls every year (*30*). Although intervention strategies targeted at processing reduce the prevalence of the pathogen in beef products (*34*), the number of recalls implies that further interventions are necessary.

Contamination of beef carcasses with *E. coli* O157:H7 occurs during harvest and is associated with both fecal and hide prevalence (*14, 19*). Cattle typically shed *E. coli* O157:H7 at concentrations less than $10^2$ CFU/g feces, however, some cattle, generally referred to as high shedders, can shed the organism at concentrations greater than $10^4$ CFU/g feces (*8, 16, 20*). Modeling studies have predicted that these cattle may be responsible for increased on-farm transmission the organism (*22*). Similarly, cattle shedding high concentrations of *E. coli* O157:H7 in feces also may be responsible for increased transmission during transportation and lairage, resulting in an increased risk for carcass contamination at harvest. Previously, Fox et al. (*16*) reported that the presence of both low and high fecal shedders within a truckload of cattle were significantly associated with carcass prevalence of *E. coli* O157:H7; however, loads containing one or more high shedders had the highest risk of carcass contamination. One limitation to this previous work was the lack of hide prevalence information; hides are believed to be a primary source of bacterial contamination of carcasses, probably due to proximity to carcasses at harvest (*19, 23*). Further defining the importance of hides as a transmission vehicle, at the animal and truckload levels, as well as the contribution of low and high shedders to hide contamination, may help to more accurately target pre-harvest intervention strategies for *E. coli* O157:H7.

Feeding distiller's grains has been recently linked to increased fecal shedding of *E. coli* O157:H7 in finishing cattle (*17, 18, 33*). Previous studies evaluating distiller's grains were

conducted using pre-harvest pen floor fecal samples or experimental challenge studies (*17, 18, 33*). It is unknown whether the relationship remains after cattle are transported and held in lairage, two events that may impact *E. coli* O157:H7 prevalence (*1*). Our objectives for this cross-sectional study were three-fold: 1) further define and characterize the relationships among cattle shedding *E. coli* O157:H7 in their feces (at low and high concentrations) and hide and carcass contamination at harvest 2) assess animal and truckload-level factors affecting carcass contamination with *E. coli* O157:H7 and 3) evaluate the potential relationship between feeding cattle distiller's grains and *E. coli* O157:H7 presence and magnitude in cattle feces, and presence on hides and carcasses at harvest.

## Materials and Methods

**Cattle selection and sample collection.** Fecal, hide, and pre-evisceration carcass samples were collected from truckloads (a cohort of cattle transported and lairaged together) of finished cattle at a Midwest commercial abattoir on ten sampling days during the summer months (June, July and August) of 2008. Matched fecal, hide, and carcass samples from a minimum of 20 and maximum of 32 animals were collected from 45 truckloads of cattle. In truckloads with more than 32 cattle, the first 32 animals were sampled, and in truckloads with less than 32, a minimum of 20 cattle or all animals were sampled. This sample size was derived from an *a priori* calculation designed to detect an *E. coli* O157:H7 positive truckload if it had a minimum 5% carcass prevalence within truckloads. Information on the cattle source (feedlot) was provided by abattoir personnel and a maximum of two loads of cattle from each source were used for collection on any given sampling day. After samples were processed for *E. coli* O157:H7, feedlots were contacted by telephone to obtain ingredient composition of diets fed during the finishing phase of sampled cattle. These data were used to evaluate potential associations between fecal, hide and carcass presence of *E. coli* O157:H7 and the feeding of distiller's grains to cattle during the final finishing phase.

Hide and carcass samples were collected as previously described (*14, 29*). Briefly, hide samples were collected by swabbing a 500 $cm^2$ area of the perineum using 2 × 2-inch sterile gauze pads moistened with 5 ml of sterile water. Gauze pads were placed directly into sterile bags. Carcasses were swabbed using a Speci-Sponge (Nasco, Fort Atkinson, WI) soaked with 15 ml of buffered peptone water (BPW) and held with a nitrile glove. Each carcass was swabbed

immediately after hide removal, but prior to evisceration or any post-harvest intervention. The area (approximately 1,000 $cm^2$) on the right side of each carcass, from the tail head over the rump and part of the round, was swabbed with the sponge. The sponge was then placed back into the Speci-Sponge bag containing BPW. Feces were collected post-evisceration from intact rectums (after cutting rectums open) with a plastic spoon. The spoon with feces was placed into a Whirl-pack bag. Care was taken to avoid contamination by changing gloves between animals, and placing different sampling personnel at the hide, carcass and fecal sample collection locations. All samples were placed in a cooler with ice packs and transported to the laboratory for processing within 24 hours.

**Isolation and semi-quantification of *E. coli* O157:H7 from feces.** Approximately one gram of fecal sample was placed into a tube containing 9 ml of Gram Negative (GN) broth (Difco; BD, Sparks, MD, USA) with cefixime (0.5 mg/L), cefsulodin (10 mg/L), and vancomycin (8 mg/L; $GN_{ccv}$), vortexed and incubated for 5.5 h at 37°C. After incubation, immunomagnetic bead separation (IMS) was performed on 1 ml of enrichment, followed by plating onto sorbitol-MacConkey agar with cefixime (0.5 mg/L) and potassium tellurite (2.5 mg/L; CT-SMAC). Plates were incubated overnight at 37°C, and up to six sorbitol negative colonies were streaked on blood agar plates (Remel, Lenexa, KS). After overnight incubation at 37°C, colonies were tested for indole production and latex agglutination for the O157 antigen (Oxoid, Remel), and further characterized by multiplex PCR identifying *rfbE* (O157), *eae* (intimin), *stx1* (Shiga toxin 1), *stx2* (Shiga toxin 2), *hly*A (hemolysin), and *fliC* (flagella) genes (*4*, *5*, *15*).

A semi-quantitative method was used to categorize fecal culture positive cattle into low and high shedders (*16*, *28*). Briefly, a swab of 1:10 diluted fecal suspension in $GN_{ccv}$ broth was spread onto the top quadrant of a CT-SMAC plate prior to enrichment. A loop was used to streak for isolation and plates were incubated for approximately 20 h at 37°C. From direct streaked CT-SMAC plates, up to six sorbitol negative colonies were transferred to blood agar plates, evaluated for indole production and latex agglutination for the O157 antigen, and confirmed by multiplex PCR as described above. Following both the semi-quantitative method and IMS method, samples were categorized into high-shedder (HS) positive (positive isolate obtained from a direct-streak plate), enrichment-positive (positive isolate obtained only with the IMS method), and fecal-positive (isolate obtained from either method).

**Isolation of *E. coli* O157:H7 from hide and carcass samples.** Hide and carcass sponge samples were cultured for *E. coli* O157:H7 using previously described procedures (*14*). Briefly, 20 ml brilliant green bile (BGB) broth was added to hide samples, which were enriched for 6 h at 37°C. Carcass sponge samples were enriched in 90 ml BGB for 10 h at 37°C. After enrichment, IMS separation was performed on 1 ml of BGB broth, followed by plating onto CT-SMAC. Further isolation, identification, and characterization were identical to the procedures described for fecal samples.

**Statistical Analyses.** Descriptive statistics for high-shedder, enrichment-positive, fecal-positive, hide, carcass, and animal (any sample positive) prevalence were calculated in Microsoft Office Excel 2007 (Redmond, WA). Either enrichment-positive fecal samples or fecal-positive samples (positive by either enrichment or direct streak) were used in analyses; these variables were never included together because the categories were highly correlated (data not shown). Spearman rank-order correlation coefficients (with Fisher's Z-transformations), level of significance, and 95% exact confidence intervals (CI) were determined for truckload-level hide, carcass, high-shedder and fecal enrichment-positive prevalence using PROC CORR of SAS (v. 9.1, SAS Institute, Inc. Cary, N.C.). Logistic regression, modeled using generalized estimating equations, was used to determine associations among independent variables and prevalence estimates (PROC GENMOD of SAS). Unconditional associations were evaluated between an *E. coli* O157:H7 positive sample from a high-shedder, fecal enrichment, hide, or carcass and sampling day or a final finishing diet with or without distiller's grains. Samples from truckloads where the final finishing diet information was not available were not included in analyses of dietary effects. An individual's high-shedder or enrichment fecal results were considered as explanatory variables when evaluating the probability of an animal's hide to test positive for *E. coli* O157. Hide, high-shedder, or enrichment fecal results were assessed as explanatory variables while evaluating the probability of an individual carcass to test positive for *E. coli* O157:H7. A covariance structure (compound symmetry) was used to account for the lack of independence within truckload for all animal-level analyses. Wald's Chi-square tests and a *P*-value of 0.05 were used to assess statistical significance.

In addition, logistic regression (as above) was used to evaluate truckload-level effects on the probability of an individual carcass testing positive for *E. coli* O157:H7. A combination of both biological and statistical considerations were used to collapse hierarchically coded truckload-level variables into like-groups by hide, enrichment-positive and high shedder sample prevalence (*32*). For hide prevalence, truckloads of cattle were categorized into two groups, 0-49% and 50-100% *E. coli* O157:H7 prevalence. High shedding truckloads were also categorized into two groups, no high-shedders in the truckload (0% prevalence), or at least one high-shedder in the truckload (> 0% prevalence). Enrichment-positive fecal prevalence for truckloads was categorized into one of three groups: no *E. coli* O157:H7 positive fecal samples (0%), 1-25%, or >25% prevalence.

A final logistic model, specified as described above, was developed to evaluate significant animal- and truckload-level factors affecting the probability of detecting a positive carcass. All unconditional associations and first order interactions were screened for significance ($P < 0.15$) prior to being included in the final model. Backwards elimination ($P < 0.10$) was used to fit the final model (*13*). Odds ratios with 95% confidence intervals were calculated from model coefficients and robust standard errors.

## Results

Samples were collected from 1,379 animals and 45 truckloads of cattle in this study. Because of logistical constraints, not all sample types (hide, carcass, and fecal) were collected from each animal. In total, all four sample outcomes (high-shedder, enrichment-fecal, hide and carcass) were available for 1,330 animals. Of cattle with all four sample outcomes, 45.6% (607 of 1,330) had at least one sample test positive for *E. coli* O157:H7.

Ingredient composition or components of final finishing diets were known for only 33 of the 45 truckloads of cattle. There were 18 truckloads of cattle fed diets with DG and 15 truckloads of cattle that were fed diets containing no DG. Data obtained regarding the inclusion of DG were diverse; feedlots varied in the form of DG fed to cattle (truckloads were fed wet DG, n = 12; dried DG, n = 2; both wet and dried DG, n = 3; unknown form, n = 1), the inclusion level (range = 3 to 40%), and the basis of inclusion level (dry matter vs. as-fed). Truckloads of cattle fed diets either with or without DG also were not represented equally within sampling days.

**Prevalence of *E. coli* O157:H7 in feces.** The overall prevalence of *E. coli* O157:H7 in fecal samples in this study was 14.4% (194 of 1,351 samples). The percentage of fecal samples classified as high shedders was 3.3% (34 of 1,351), and enrichment-positive fecal prevalence was 13.8% (186 of 1,351 samples). There were no differences in any model results when using enrichment-positive versus fecal-positive data, so all model results are reported for enrichment-positive fecal samples. Sampling day prevalence of *E. coli* O157:H7 in enrichment fecal samples ranged from 0 to 26.4% (mean, 11.9%; median, 13.4%), while the prevalence of high-shedders across collection days ranged from 0 to 6.3% (mean, 3.0%; median, 3.0%). The prevalence of enrichment-positive fecal samples across truckloads ranged from 0 to 62.5% (mean, 14.1%; median, 6.5%). The prevalence of high shedders across truckloads of cattle ranged from 0 to 21.9% (mean, 3.4%; median, 0%). In this study, feeding DG was not associated with *E. coli* O157:H7 high-shedder ($P = 0.95$) or fecal enrichment-positive ($P = 0.60$) prevalence. However, none of the models assessing the effects of DG feeding would converge when a variable for sampling day was included.

**Prevalence of *E. coli* O157:H7 on hides.** Hide prevalence of *E. coli* O157:H7 was 38.5% (528 of 1,370 samples) and ranged from 0 to 88.2% (mean, 41.1%; median, 34.7%) across collection days. Across truckloads, prevalence ranged from 0 to 100% (mean 39.9%; median, 34.4%). Diets containing DG were not associated with hide *E. coli* O157:H7 prevalence ($P = 0.46$) based on unconditional comparisons. Hide prevalence within truckloads was significantly correlated with both truckload high-shedder ($R^2 = 0.55$; $P < 0.01$) and enrichment-positive ($R^2 = 0.55$; $P < 0.01$) prevalence (Fig 6.1).

An individual animal's hide being positive for *E. coli* O157:H7 was unconditionally associated with both the animal's high shedder and enrichment-positive fecal status. Given an animal was a high shedder, the odds of that animal's hide being positive were 2.8 (CI, 1.2 to 6.8) times higher than if the animal was not a high shedder. In addition, an animal with an enrichment-positive fecal sample was 2.7 (CI, 1.5 to 4.8) times more likely to have a positive hide sample than if the animal was enrichment-negative.

**Prevalence of *E. coli* O157:H7 on carcasses.** Pre-evisceration carcass prevalence of *E. coli* O157:H7 was 10.5% (143 of 1,365 samples) overall. Across collection days, prevalence ranged

between 0 and 33.3% (mean, 9.9%; median, 6.2%). Between truckloads, the prevalence ranged from 0 to 65.6% (mean, 10.6%; median, 3.1%). Diets containing DG were not associated with carcass prevalence ($P = 0.22$) based on unconditional comparisons. At the truckload-level, carcass prevalence was correlated to hide ($R^2 = 0.70$; $P < 0.01$), high shedder ($R^2 = 0.41$; $P < 0.01$), and enrichment-positive ($R^2 = 0.50$; $P < 0.01$) prevalence (Fig 6.2).

The probability of an animal's carcass testing positive for *E. coli* O157:H7 was unconditionally associated with that animal's enrichment-fecal and hide status; however, the animal's high-shedding status did not significantly impact carcass contamination results ($P = 0.11$). Fecal enrichment-positive animals were 2.9 (CI, 1.6 to 5.6) times more likely to have a positive carcass than animals with negative fecal samples after enrichment. An animal with a positive hide was 5.5 (CI, 3.1 to 9.7) times more likely to have a positive carcass than an animal with a negative hide.

The final multivariable model evaluating factors affecting carcass contamination with *E. coli* O157:H7 included both animal- and truckload-level factors. All animal-level variables (high-shedder, fecal enrichment, and hide) and truckload-level variables (low or high hide prevalence, the presence of a high shedder, and 0, low or high prevalence of fecal enrichment *E. coli* O157:H7) were associated with the outcome on unconditional analyses ($P < 0.15$) and thus included in the initial multivariable model. A categorical variable representing sampling day was also included in the initial multivariable model. Four variables, individual hide status, high-shedder group, enrichment fecal sample group and hide group, remained in the final multivariable model (Table 6.1); no first order interactions were statistically significant. After controlling for the truckload-level effects (low or high hide prevalence, the presence of a high shedder, and 0, low or high prevalence of fecal enrichment *E. coli* O157:H7), we found that a carcass was 1.6 (CI, 1.0 to 2.6) times more likely to be positive if that animal had a positive versus a negative hide sample, which tended ($P = 0.07$) to be significant. All other variables were significant at $P < 0.05$. If a carcass came from a truckload with a hide prevalence > 50%, it was 7.7 (CI, 3.1 to 19.6) times more likely to be positive than if the truckload had a prevalence of 0-49%, after controlling for the other effects in the final model. Similarly, an *E. coli* O157:H7-positive carcass was 4.0 (CI, 1.6 to 10.1) times more likely if the truckload had at least one high shedder compared to truckloads that lacked a high shedder. Finally, if the truckload had 1-25% fecal enrichment prevalence or > 25% enrichment prevalence, the odds of a positive

carcass sample were 3.9 (CI, 1.3 to 12.0) and 19.3 (CI, .4.7 to 79.0) higher, respectively, than if the truckload was negative for *E. coli* O157:H7 based on enriched fecal samples.

The predicted probabilities for detecting a positive *E. coli* O157:H7 carcass presented in Fig 6.3 were determined under various scenarios using the animal and truckload-level factors identified in the final logistic regression model (Table 6.1). Under a best case scenario where an animal's hide was negative and the animal came from a truckload with no truckload level risk factors (truckload hide prevalence ≤ 50%, fecal enrichment prevalence ≤ 1%, and no high fecal shedder present), the probability that the carcass would test positive for *E. coli* O157:H7 was 0.009 (CI, 0.003 to 0.29). This is in contrast to a worst case scenario where the animal's hide tested positive for *E. coli* O157:H7, and the animal came from a truckload with all truckload-level risk factors (truckload hide prevalence > 50%, fecal enrichment prevalence > 25%, at least one high shedder) where the probability of detecting a positive carcass was 0.54 (CI, 0.34 to 0.86). The probability of detecting a positive carcass when the animal's hide was positive, and there were no truckload level risk factors was 0.01 (CI, 0.004 to 0.05). In contrast, when the animal's hide was negative and the animal came from a high risk truckload, the predicted probability of an *E. coli* O157:H7 positive carcass was 0.33 (CI, 0.18 to 0.60).

## Discussion

Our study was unique in the simultaneous quantification of effects of both individual animal and truckload-level factors in finished cattle that contribute to pre-evisceration carcass contamination with *E. coli* O157:H7. Our study provides a logical extension to the work of Fox et al. *(16)*, by providing animal and truckload-level hide contamination data, which we found significantly impacted carcass contamination. We did not track or compare *E. coli* O157:H7 isolates based on microbial characteristics as other have done previously (*2,12*), but rather focused on presence and prevalence of *E. coli* O157:H7 within defined cohorts (truckloads) of animals and their carcasses. Interestingly, all three truckload-level factors (presence of at least one animal shedding high concentrations of *E. coli* O157:H7 in the feces, within truckload fecal and hide prevalence of > 50%) significantly contributed to the probability that a carcass was positive. In contrast, only an animal's positive hide, and not its fecal *E. coli* O157:H7 status, tended to affect the probability that the carcass would test positive. We acknowledge that carcass samples in this study were taken prior to evisceration, a step which may be more likely to

facilitate contact between an animal's fecal material and carcass. Still, this work, along with others (*16*), demonstrates that truckload- or cohort-level prevalence estimates of *E. coli* O157:H7 (fecal and hide) are more important predictors of carcass contamination than that individual animal's *E. coli* O157:H7 status at harvest.

Prevalence estimates for *E. coli* O157:H7 in this study were similar to those previously reported for feedlot cattle at harvest. Pre-evisceration carcass contamination was our primary outcome of interest, and we observed 10.5% of carcasses were positive. This prevalence estimate is well within the 3 to 47% range previously reported (*2, 6, 7, 16, 34*). Previous work has shown that *E. coli* O157 isolates obtained from carcasses are frequently found in feces from animals within the same truckload (*12*). In our study, fecal *E. coli* O157:H7 prevalence, obtained after enrichment, was 13.8%, and 71% of loads contained at least one enrichment-positive animal. These estimates of prevalence are similar to other observations (*16, 20, 25*). Within truckload fecal prevalence (as determined by enrichment) was correlated with hide and carcass prevalence within a truckload of cattle. This was in agreement with other studies measuring fecal prevalence (at the feedlot or slaughter facility) and subsequent carcass prevalence (*14, 16, 34*). However, the presence of *E. coli* O157:H7 in feces of an individual animal was not significantly associated with their carcass testing positive based on our final multivariable model.

To further define the role of feces with regard to carcass contamination, we used a semi-quantification procedure to identify cattle shedding *E. coli* O157:H7 in their feces at high concentrations. The prevalence of high shedders in this study was 3.3%; other studies using direct plating methods have shown high shedder prevalence at harvest of 3.7% (*16, 20*). There is some inconsistency in the literature regarding the fecal concentration (e.g. $10^3$ or $10^4$ CFU/g feces) chosen to classify cattle as high shedders for *E. coli* O157:H7 (*16, 20, 25, 28*). Because of the nature of the direct plating procedure we used, there is a possibility for misclassification of fecal samples, potentially an overestimation of high shedders. However, despite potential misclassification, our study and others have shown that these different fecal shedding designations are associated with important epidemiologic parameters (*3, 16*). Although the specific concentration at which cattle become high shedders is not known, cattle shedding more *E. coli* O157:H7 are believed to increase transmission within a given cohort (*3, 10, 22*). Data from our study indicate that these high shedding cattle also may be responsible for increased

transmission within a truckload of cattle during shipment or at lairage because the prevalence of high shedding animals was correlated to hide prevalence ($R^2 = 0.55$). However, the directionality of the association between hide and high shedding prevalence cannot be determined in our study. There also was a relationship between the presence of at least one high shedding animal and an individual's carcass testing positive for *E. coli* O157:H7 (OR = 4.0). Fox et al. (*16*) similarly reported this association, at an even larger magnitude (OR = 16.2) than we report here: however, we were able to control for hide effects, which was not accounted for in the previous study. Interestingly, we found that a particular animal's high shedder status was not significantly associated with contamination of its carcass. Together with the enrichment results, these data imply that although fecal and high shedder prevalence are associated with carcass prevalence within a truckload, they may not be useful in predicting an individual's pre-evisceration carcass status. Therefore, the direct source of *E. coli* O157:H7 on an individual's carcass may be something other than that animal's feces.

Hide *E. coli* O157:H7 prevalence for this study was 38.5%, also within the range (11 to 71%) previously reported (*2, 6, 7, 14*). We saw tremendous variability in hide prevalence among truckloads of cattle and sampling days. Previously, transportation and lairage of cattle prior to harvest have been shown to impact *E. coli* O157:H7 prevalence on hides, and ultimately carcasses, by serving as the direct source of bacteria or an environment facilitating the spread of the organism (*1, 2, 11*). Transportation and lairage environments may enhance hide contamination because of the close contact of animals. Previous work has shown these areas can account for a substantial proportion of clonal isolates found on hides and subsequently carcasses at processing, more so then attributed to the feedlot where cattle originated (*2, 9, 21*). The hypothesis that hides are an important source of carcass contamination was confirmed in our study where cattle with *E. coli* O157:H7 positive carcasses were 1.6 times more likely to come from an animal with a positive hide sample. In addition, a carcass from a truckload with substantial hide prevalence (> 50%) was 7.7 times more likely to test positive for *E. coli* O157:H7 than if the truckload had a hide prevalence of < 50%. Thus, the within-truckload hide prevalence had a larger magnitude of effect on carcass contamination than the presence of *E. coli* O157:H7 on an individual's hide.

The hindgut is the primary location of *E. coli* O157:H7 colonization in cattle, where it is then shed in the feces (*24, 31*). Thus, for hide contamination to occur, the organism first must be

transmitted from feces to the hide prior to, during, or post-transport to the processing plant. Even after controlling for the effects of hide contamination, we still observed that fecal prevalence and concentration can impact carcass contamination with *E. coli* O157:H7. Although high shedders were not directly associated with carcass contamination at the animal level in our multivariable model, both high shedder and fecal enrichment prevalence were correlated to hide prevalence within load (Fig 6.1), which ultimately can result in carcass contamination (Table 6.1). In other words, even though hides may be the direct source of carcass contamination (*19*), hide prevalence is strongly correlated with fecal prevalence and concentration of *E. coli* O157 within a cohort of cattle.

Our study also evaluated the previous observation that cattle fed finishing diets containing distiller's grains had a higher fecal prevalence of *E. coli* O157:H7 compared to cattle fed no distiller's grains (*17, 18, 33*). However, no differences were seen in the high-shedder, enrichment-fecal, hide, or carcass prevalence of *E. coli* O157:H7 between the two diet designations evaluated in this study. Unfortunately, uncontrolled potential confounding variables such as sampling day may have limited our ability to accurately describe the relationship. Prevalence estimates did vary by sampling day and distiller's fed cattle were not presented equally among days. Statistical models estimating the probability of a high-shedder, enrichment-fecal, hide, or carcass sample to be positive for *E. coli* O157:H7, given finishing diet (with or without DG), would not converge if sampling day was included; thus, the unconditional results we obtained may not truly reflect the distiller's grains association and should be interpreted with caution. We also had tremendous variability in the type (wet versus dry) and inclusion level of distiller's grains, factors that may affect *E. coli* O157:H7 shedding (*26*). Because truckloads of cattle fed diets with and without DG were available on only 5 of 10 collection days and there was variability in the level and type of distiller's grains fed, our power to demonstrate a difference may have been limited. Finally, we evaluated samples at one beef processing plant during this study and because *E. coli* O157:H7 prevalence can differ between plants, our results may be somewhat limited in scope.

In conclusion, our results indicate important animal and truckload-level risk factors for carcass contamination with *E. coli* O157:H7. Although we demonstrated truckload-level correlations among fecal (both high and low shedders), hide, and carcass prevalence that have been previously observed, our use of multivariable models to simultaneously quantify effects of

multiple factors affecting carcass contamination provides a unique assessment of these factors. Interestingly, all truckload level factors (at least one high shedder, and high fecal and hide *E. coli* O157:H7 prevalence within the cohort) significantly contributed to carcass contamination, whereas only one animal-level factor (hide contamination) was associated with carcass contamination. These data point to truckload or cohort level mitigation strategies, particularly those reducing hide contamination, being most effective in reducing *E. coli* O157:H7 contamination of beef carcasses.

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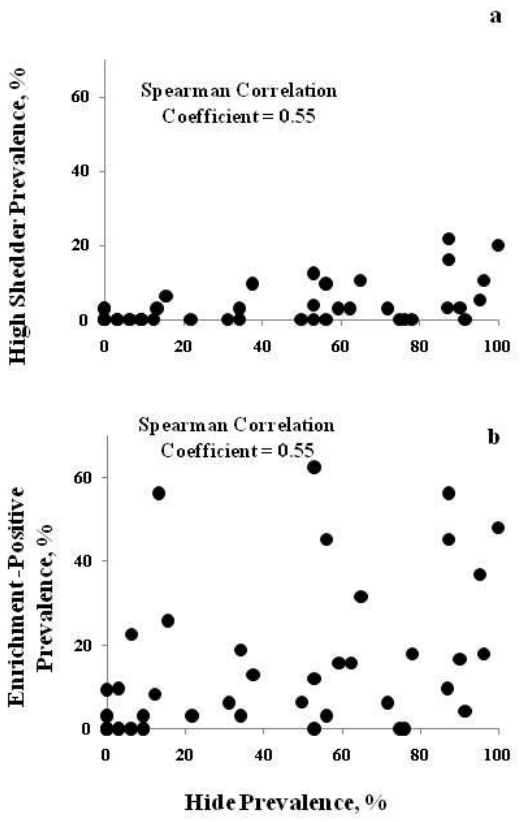
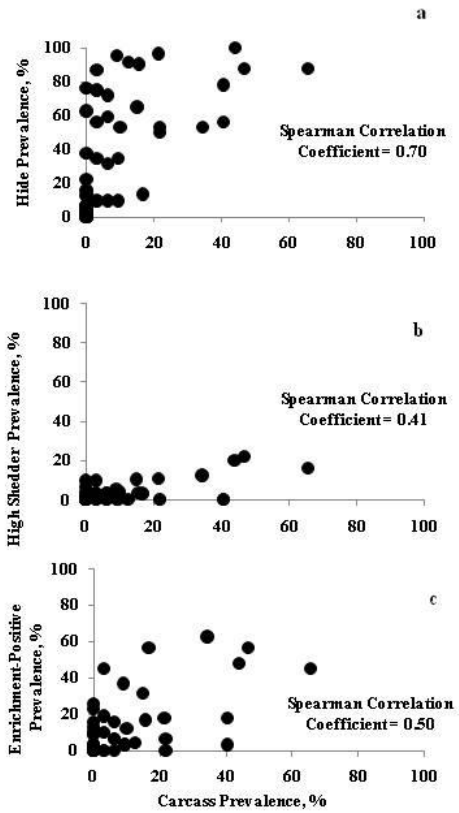
**Figure 6.1 Correlations between truckload-level hide prevalence and high-shedder[a] (a) or enrichment-positive[b] (b) fecal prevalence of *Escherichia coli* O157:H7.**




*E. coli* O157:H7 present in fecal samples at a high concentration as determined by direct plating (semi-quantification). [b]Fecal sample determined to be positive for *E. coli* O157:H7 by immunomagnetic separation following enrichment

**Data are from 45 truckloads of cattle harvested at an abattoir during summer months of 2008.**

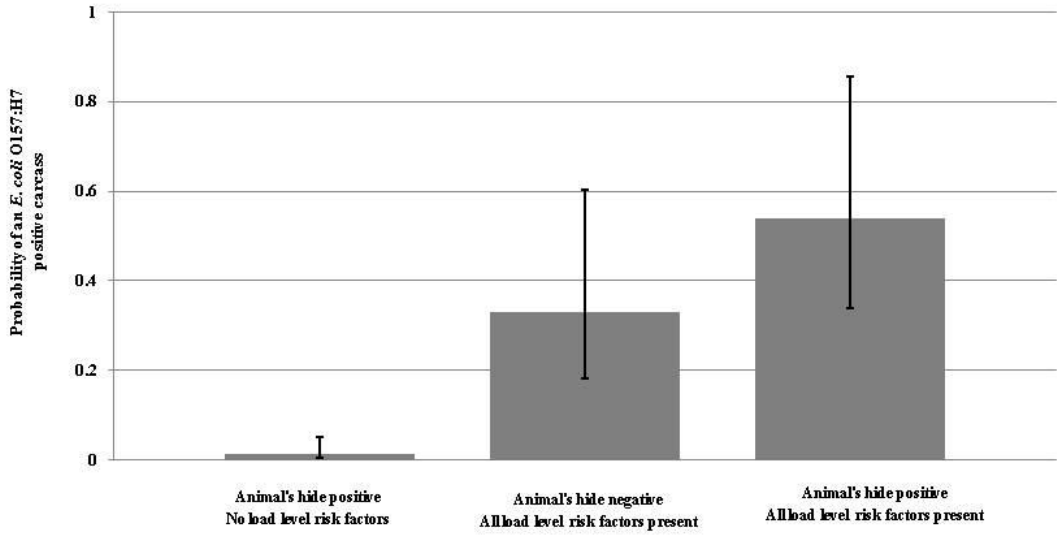
**Figure 6.2 Correlations between truckload-level carcass prevalence and hide (a), high-shedder[a] (b) or enrichment-positive[b] (c) fecal prevalence of *Escherichia coli* O157:H7.**




[a]*E. coli* O157:H7 present in fecal samples at a high concentration as determined by direct plating (semi-quantification). [b]Fecal sample determined to be positive for *E. coli* O157:H7 by immunomagnetic separation following enrichment

**Data are from 45 truckloads of cattle harvested at an abattoir during summer months of 2008.**

**Figure 6.3 Probability of an *Escherichia coli* O157:H7 positive carcass given animal and truckload level risk factors identified by a logistic regression model[a] of data on 45 truckloads of cattle harvested at a Midwest U.S. abattoir during summer months of 2008.**




[a]Model was estimated using generalized estimating equations (PROC GENMOD; SAS v. 9.1) with a correlation structure to account for lack of independence within truckloads of cattle.

**Truckload level risk factors include: within truckload hide prevalence of > 50%, at least one fecal high shedder (based on a direct culture method for feces) within the truckload, and within truckload fecal prevalence of > 25% (based on an enrichment and selective culture procedure). Error bars are 95% confidence intervals based on robust standard errors.**

**Table 6. 1. Results from a multivariable logistic regression model[a] evaluating factors associated with probability of detecting *Escherichia coli* O157:H7 on a pre-evisceration beef carcass.**

| Effect | *P* - value | *B* | Robust standard error | Odds ratio | 95% CI Odds ratio | |
|---|---|---|---|---|---|---|
| Intercept | | -4.72 | 0.61 | | | |
| Animal's hide positive for *E. coli* O157:H7 | 0.07 | 0.49 | 0.24 | 1.6 | 1.0 | 2.6 |
| Within truckload hide prevalence > 50% | < 0.01 | 2.05 | 0.47 | 7.7 | 3.1 | 9.6 |
| High shedder present within truckload[b] | 0.03 | 1.40 | 0.47 | 4.0 | 1.6 | 0.1 |
| Levels of within truckload fecal prevalence[c] | | | | | | |
| 0% | | Reference | - | - | - | |
| 1 to 25% | | 1.37 | 0.57 | 3.9 | 1.3 | 2.0 |
| | < 0.01 | | | | | |
| > 25% | | 2.96 | 0.72 | 19.3 | 4.7 | 9.0 |

**[a]Model was estimated using generalized estimating equations (PROC GENMOD; SAS v. 9.1) with a correlation structure to account for lack of independence within truckloads of cattle.**

**[b]*E. coli* O157:H7 present in at least one fecal sample at a high concentration as determined by direct plating (semi-quantification).**

**[c]Determined to be positive for *E. coli* O157:H7 by immunomagnetic separation following enrichment.**

# Appendix A - Effect of a Probiotic Product (PrimaLac® 454 F/G) on Fecal Shedding of *Escherichia coli* O157:H7 in Cattle

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## Abstract

Probiotics have been proposed as a pre-harvest intervention strategy to reduce the prevalence of *E. coli* O157:H7 in cattle. PrimaLac® 454 F/G is used as a feed additive for feedlot cattle, however, its efficacy for *E. coli* O157:H7 reduction has not been evaluated. The objective of this study was to determine the efficacy of PrimaLac® 454 F/G probiotic on the fecal shedding of *E. coli* O157:H7 in cattle experimentally inoculated with the organism. Holstein calves were randomly assigned to one of two treatments: control (n = 8) and probiotic (PrimaLac® 454 F/G ; n= 7). Each calf was administered a standard, distiller's grains-containing, high-grain commercial diet and was experimentally inoculated with five strains of nalidixic acid-resistant *E. coli* O157:H7. Fecal samples were collected from each calf three times per week for six weeks to determine the presence and concentration of *E. coli* O157:H7. At the termination of the study, calves were euthanized and necropsied and gut contents were collected from the rumen, cecum, colon, and rectum, and recto-anal mucosal swab samples were collected. Feeding calves PrimaLac® 454 F/G probiotic was associated with a lowered concentration of $Nal^R$ *E. coli* O157:H7 initially (days 5, 7, and 9), however, throughout the course of the study the two treatment groups behaved similarly. At necropsy more calves had more sites positive for $Nal^R$ *E. coli* O157:H7 in the PrimaLac® 454 F/G treatment group compared to the control calves,

although the concentration was only significantly different at the rectum. Further work is needed to determine the efficacy of PrimaLac® 454 F/G on *E. coli* O157:H7 reduction in cattle.

## Introduction

*Escherichia coli* O157:H7, a foodborne pathogen, continues to be a major public health concern in the United States. Ruminants are a major reservoir for this organism, in which the organism colonizes the hindgut and is shed in the feces. Cattle feces are a major source of contamination of food products and initially, adulterated ground beef was identified as the culprit for transmission to humans. However, *E. coli* O157:H7 outbreaks have also been associated with consumption of contaminated water and produce and by direct contact with animals (Rangel et al., 2005). Intervention strategies are used successfully on beef hides and carcasses at slaughter to reduce the risk of the pathogen entering the food chain (Koohmaraie et al., 2005). However, interventions at abattoirs do not preclude fecal contamination of water and fresh produce. Therefore, efforts to reduce shedding of *E. coli* O157:H7 in beef cattle prior to harvest are important (Callaway et al., 2002; Callaway et al., 2003; LeJeune and Wetzel, 2007; Loneragan and Brashears, 2005).

The use of probiotics, also called direct-fed microbials, as a pre-harvest intervention strategy appears to be a promising approach to reduce *E. coli* O157:H7 in cattle (Diez-Gonzales and Shamberger, 2004). Several research efforts focused on the potential of lactic acid bacteria (*Lactobacillus acidophilus* or other species) to reduce prevalence of *E. coli* O157 in feedlot cattle (Brashears et al., 2003; Elam et al., 2003; Lema et al. 2001; Younts-Dahl et al., 2005; Peterson et al., 2007). Probiotics have either an indirect effect by altering microbial populations and products of fermentation in the gastrointestinal tract or a direct inhibitory or competitive effect on *E. coli* O157, thus reducing the ability of *E. coli* O157 to survive in the gut and shed in feces (Ohya et al., 2000, Diez-Gonzales and Shamberger, 2004; Krehbiel et al., 2003; LeJeune and Wetzel, 2007; Loneragan and Brashears, 2005).

The product PrimaLac® 454 F/G is used as a feed additive for feedlot cattle. It is a mixture of four bacterial species and consists of *Lactobacillus acidophilus* fermentation product dehydrated, *Lactobacillus casei* fermentation product dehydrated, *Bifidobacterium thermophilum* fermentation product dehydrated, and *Enterococcus faecium* fermentation product dehydrated, in

rice hulls, calcium carbonate, and vegetable oil carrier. The effect of this product on fecal shedding of *E. coli* O157:H7 has not been determined.

## Methods and Materials

**Study treatments and animals.** Fifteen Holstein calves, approximately 4 months of age were used in the study. Calves were adapted to a corn-based high-grain diet with dried distiller's grains. Dried distiller's grains were included because previous research suggests that the fermentation co-product increases the concentration of *E. coli* O157:H7 shed in experimentally inoculated calves (Jacob et al., 2008). For an adaptation period, calves were group fed for forty-six days, and went through two step-up diets prior to beginning the final diet. After adaptation, calves were transported to a Biosafety Level (BL)-2 animal facility (two adjacent barns) at the Kansas State University CVM Animal Resource Facility. Each barn had 8 pens and housed 4 calves for each treatment. Each calf was housed individually and barriers prevented contact between calves housed adjacently. Calves were fed diets once a day (Table A.1). Each calf was fed at approximately 2% of body weight. Each animal was randomly assigned to one of two treatments: control (n = 8) or probiotic-fed (n = 7), and each treatment group was housed in four (or three) continuous pens.

Calves in the probiotic group were given 6 g/head/day of PrimaLac® 454 F/G (Star Labs; Clarksdale, MO) product as a top dress beginning on the day they entered the BL-2 facility (a week before experimental inoculation). Calves in the control group were given 6 g/head/day ground soy hulls as a placebo treatment, administered similarly to the probiotic. Both groups of calves were orally inoculated, via a stomach tube, with $3.5 \times 10^9$ CFU of a five-strain mixture (strain FRIK920, FRIK1123, FRIK2000, 01-2-12329, 01-2-08970) of nalidixic acid-adapted *E. coli* O157:H7 (50 µg; $Nal^R$) after a week of acclimation. The strains were previously isolated from feces of feedlot cattle (Sargeant et al., 2003). Immediately prior to challenge, fecal samples were obtained from each animal to test for resident *E. coli* O157:H7. After oral inoculation, fecal samples were collected from each animal three times per week for six weeks. Samples were tested for the presence and concentration of $Nal^R$ *E. coli* O157:H7 at each collection. After the study, calves were euthanized and gastrointestinal contents (rumen, cecum, colon, and rectum) were collected and analyzed for the presence and concentration of $Nal^R$ *E. coli* O157:H7. Also, the rectoanal mucosa was swabbed and tested for the presence of $Nal^R$ *E. coli* O157:H7.

**Pre-challenge fecal samples**. All animals were tested for resident *E. coli* O157:H7 prior to challenge. Fecal samples, obtained from each animal by rectal grab, were kneaded and approximately 1 g of fecal material was placed in 9 mL of Gram Negative (GN) broth supplemented with cefixime (0.05mg/L), cefsulodin (10.0 mg/L), and vancomycin (8.0 mg/L; GNccv). Samples were vortexed for 1 min and incubated for 5.5 h at 37°C. Immunomagnetic separation (IMS; Dynal, Inc.) was performed following enrichment, and 50 µL of product was plated onto sorbitol MacConkey agar supplemented with cefixime (50 ng/mL) and potassium tellurite (2.5µg/mL; CT-SMAC). Plates were incubated overnight at 37°C and up to six sorbitol negative colonies from each sample were picked and streaked onto blood agar plates. Blood agar plates were incubated overnight at 37°C and colonies were tested for indole production, the presence of the O157 antigen using latex agglutination, and confirmation of species with PCR analysis of *eae*, *fliC*, *stx*1, *stx*2, *hylA*, and *rfb*E virulence genes (Bai et al., 2008; Bertrand et al., 2007; Fagan et al., 1999).

**Post-challenge fecal or necropsy samples**. Fresh fecal or gut content samples obtained from each animal by rectal grab, were kneaded and placed in a pre-weighed 9 mL tube containing GNccv. Each tube was re-weighed after the sample was added to determine the amount of sample. Tubes were vortexed for one minute, and 0.1 mL of each sample was transferred to the first well of a 96-well Microtitre plate containing 0.9 mL of peptone water. Ten-fold serial dilutions were made and 100 µL of each dilution was spread in triplicate onto CT-SMAC with nalidixic acid (50µg/ml; CT-SMAC-N). The plates were incubated overnight at 37°C, after which time sorbitol negative colonies were counted. Up to 3 colonies from the appropriate dilution were picked and confirmed to be $Nal^R$ *E. coli* O157:H7 testing for indole production and latex agglutination with the O157 antigen.

In addition to the above procedure, which has a detection limit of $> 10^2$ per g of feces, an enrichment was completed for every fecal sample in GNccv for 6 h at 37°C. After incubation, 1 mL was removed and incubated in another 9 mL GNccv broth for 18 h at 37°C. Following incubation, 100 µL was spread onto CT-SMAC-N and incubated overnight at 37°C. A maximum of 3 colonies per sample were picked, streaked onto blood agar and confirmed as above. This procedure allows for detection of the organism below the detection limit of direct

plating, and the minimum detectable concentration was calculated for each positive sample. The recto-anal mucosal swab samples were enriched and processed as described above using 3 mL of GNccv.

**Statistical Analysis.** The concentration of fecal *Nal*$^R$ *E. coli* O157:H7 for live calf data, as well as necropsy data, was compared between the two treatment groups. Concentrations were $\log_{10}$ transformed prior to analysis. Data were analyzed using the Mixed Procedure of SAS (v. 9.1), with a repeated measure statement for calf within each collection day for when needed. Treatment, barn, and sampling day were fixed effects for the live calf data. Treatment, barn, and gastrointestinal location (site) were included for fixed effects in the necropsy model. All interactions were included in both models. Least-squares means were used to determine differences of dietary effects on significant associations.

## Summary of Results

**Pre-Challenge Fecal Samples.** Prior to *Nal*$^R$ *E. coli* O157:H7 challenge, fecal grab samples were collected from each animal to test for resident *E. coli* O157:H7. One of the fifteen calves (PrimaLac® treatment group) was positive for *E. coli* O157:H7 at the time of challenge. Whether this strain was different from those used to challenge the animals was not determined, however, it was resistant to nalidixic acid.

**Post-Challenge Fecal Samples.** The average concentration of *Nal*$^R$ *E. coli* O157:H7 in fecal samples of calves in both the PrimaLac® probiotic and control treatment groups throughout the course of the study is shown in Fig. A.1. Overall, there was no treatment effect for this study ($P = 0.41$), however, there was a tendency for a treatment by day interaction ($P = 0.08$). When this interaction was investigated, fecal concentrations of *Nal*$^R$ *E. coli* O157:H7 on days 5, 7, and 9 of the study were significantly different between treatments ($P < 0.05$). On each of these days, the concentration of *Nal*$^R$ *E. coli* O157:H7 was higher in the control group compared to the PrimaLac® probiotic administered group. This indicates that initially, the concentration of *Nal*$^R$ *E. coli* O157:H7 dropped faster in calves administered the probiotic treatment, however, after the 9$^{th}$ day both treatments behaved similarly.

Figure A.2 shows the percent of calves that were positive for *Nal*$^R$ *E. coli* O157:H7 (by either direct plating or enrichment) on each day of collection. Although the concentration of *Nal*$^R$ *E. coli* O157:H7 was lower in calves fed PrimaLac® on days 5, 7, and 9, all calves in both treatments remained positive for the organism until day 11. After day 11, calves began to have undetectable *Nal*$^R$ *E. coli* O157:H7 concentrations, however, once negative the animal did not necessarily remain so throughout the remainder of the study. Additionally, the calves in the west barn of the facility had a higher concentration of *Nal*$^R$ *E. coli* O157:H7 than calves in the east barn ($P < 0.05$), however, there was not a treatment × barn ($P > 0.3$) or day × barn ($P > 0.4$) interaction. It is unknown why there was a difference between the two barns, however, high variability between calves is not unexpected.

**Necropsy Samples.** The results for the presence of *Nal*$^R$ *E. coli* O157:H7 at each gastrointestinal location (direct plating or enrichment) of calves at necropsy are shown in Table A.2. One calf in the probiotic treatment group tested positive for *Nal*$^R$ *E. coli* O157:H7 in every gut location. This calf also tested positive for the organism the previous two collections before necropsy. Typically, *E. coli* O157:H7 colonizes cattle at the recto-anal mucosal junction and is shed in the feces. Locations located above this region may indicate transient organisms passing through the GI tract. Even though the number of sites positive for *Nal*$^R$ *E. coli* O157:H7 differ substantially between the two treatment groups (5 v. 34%), the concentration of *Nal*$^R$ *E. coli* O157:H7 was only significantly different at the rectum ($P < 0.001$; Fig. A.3). There was no effect of the barn the calves were housed in, or any barn interactions observed at necropsy ($P > 0.1$).

In conclusion probiotics have been proposed as a pre-harevest intervention strategy for reducing *E. coli* O157:H7 in cattle prior to harvest. In this study PrimaLac® 454 F/G lowered the concentration of *Nal*$^R$ *E. coli* O157:H7 initially (days 5, 7, and 9), however, throughout the rest of the study the two treatment groups behaved similarly. The mechanism responsible for the reduction in *E. coli* O157:H7 concentration on days 5, 7, and 9 is not known. At necropsy more calves had more sites positive for *Nal*$^R$ *E. coli* O157:H7 in the PrimaLac® 454 F/G treatment group compared to the control calves, although the concentration was only significantly different at the rectum. Further work is needed to determine the anti-*E. coli* O157:H7 properties of

PrimaLac® 454 F/G in cattle, although this study does not indicate it effectively reduces *E. coli* O157:H7 for a long duration, even with continual feeding of the product.

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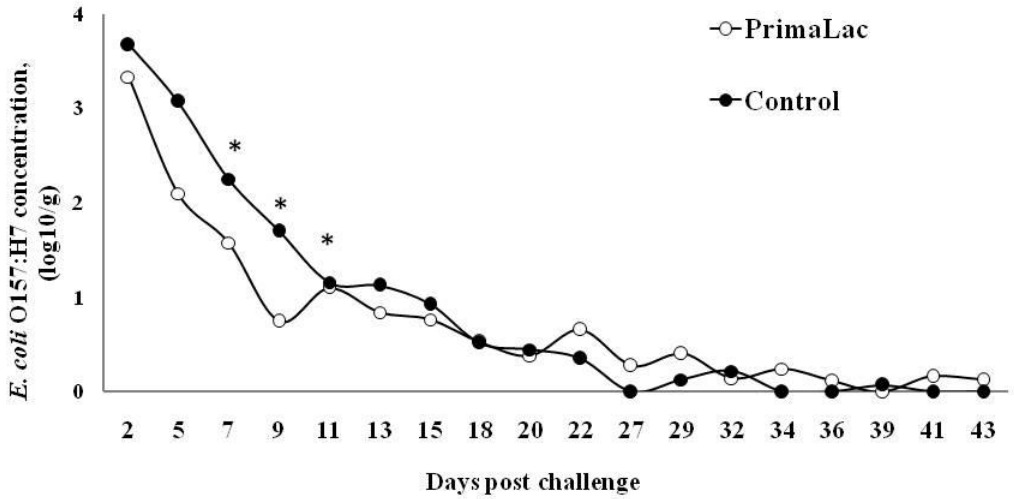
**Figure A.1. The mean fecal concentration of *E. coli* O157:H7 in experimentally inoculated calves administered either control (n = 8) or PrimaLac® probiotic (n = 7) treatments.**




*** Indicates statistical significance ($P < 0.05$) between PrimaLac and control treatment groups.**

**Figure A.2 The percent of *E. coli* O157:H7 experimentally inoculated calves positive for the organism when administered control (n = 8) or PrimaLac® probiotic (n = 7) treatments.**

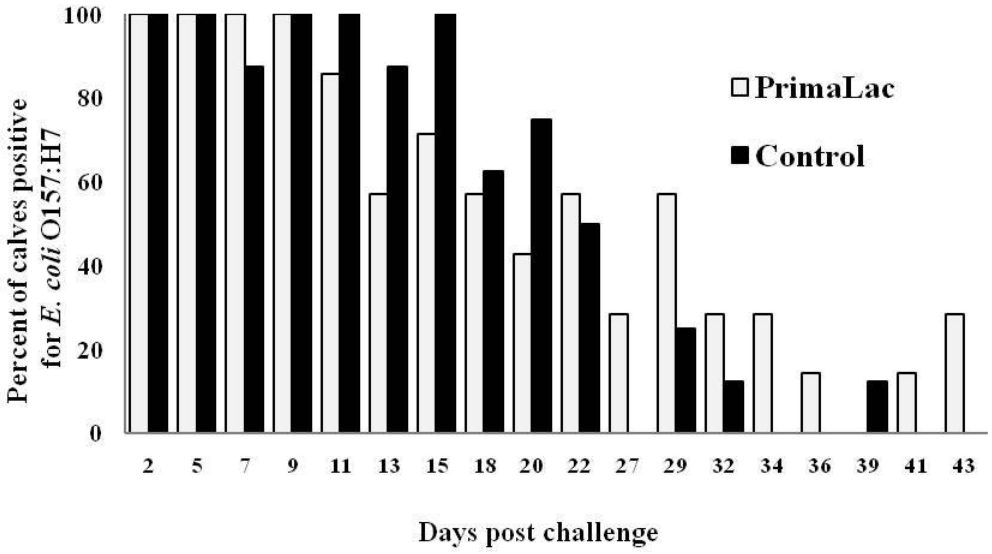
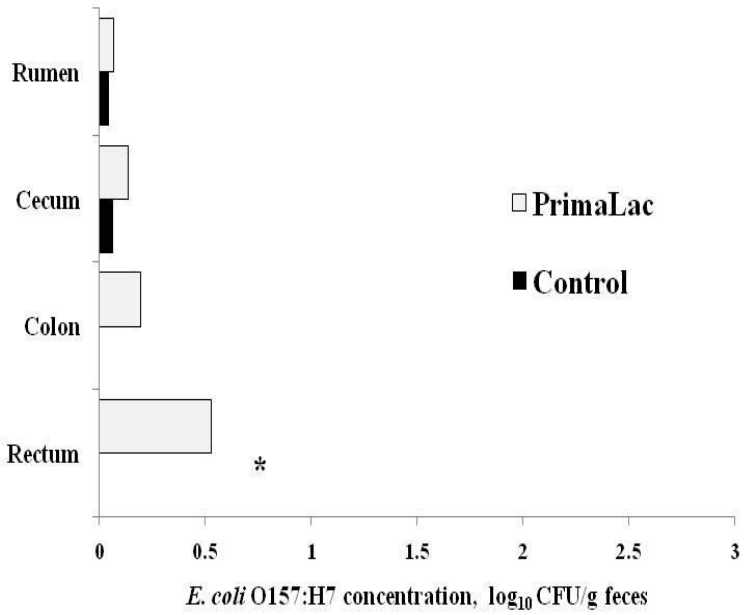


**Figure A.3 The mean concentration of Nal$^{R}$ *E. coli* O157:H7 in the gut contents of calves at necropsy administered either control (n = 8) or PrimaLac® probiotic (n = 7) treatments.**




*** Indicates statistical significance ($P < 0.05$) between PrimaLac and control treatment groups**

## Table A.1. Ingredient Composition of Finishing Diet[1]

| Ingredient | As-Fed % |
|---|---|
| 15-4 | 5.6 |
| (15% protein, 4% fat) | |
| Cracked Corn | 30.3 |
| Whole Corn | 32.3 |
| Dried Distiller's Grains | 6.2 |
| Soyhull Pellets | 9.9 |
| Loose Cottonseed Hulls | 7.9 |
| Liquid Molasses | 2.7 |
| Limestone | 0.1 |
| Salt | 0.2 |
| Wildcat 60/45 | 5.0 |
| Urea based, 60% protein | |
| Rumensin | |

[1] Diets were obtained fromWildcat Feeds, LLC

**Table A.2 The presence of Nal$^{R}$ E. coli O157:H7 in necropsy gut contents of calves administered control (no probiotic) or PrimaLac® 454 F/G probiotic treatments.**

| | | Gut Contents | | | | | |
|---|---|---|---|---|---|---|---|
| Calf No. | Treatment | Rumen | Cecum | Colon | Rectum | Recto-anal Mucosa | Total no. (%) of sites positive |
| 1 | | - | - | - | - | - | |
| 2 | | - | - | - | - | - | |
| 3 | | - | - | - | - | - | |
| 4 | Control | - | + | - | - | - | |
| 5 | | - | - | - | - | - | 2 of 40 (5) |
| 6 | | - | - | - | - | - | |
| 7 | | + | - | - | - | - | |
| 8 | | - | - | - | - | - | |
| 9 | | + | + | + | + | + | |
| 10 | | - | + | + | + | - | |
| 11 | | - | - | - | + | - | |
| 12 | PrimaLac® | - | - | + | - | - | 12 of 35 (34) |
| 13 | | - | - | - | + | - | |
| 14 | | - | - | - | - | - | |
| 15 | | - | - | - | + | - | |

# Appendix B - Evaluating the Effect of Feeding MSE Probiotic (Natur's Way, Inc.) on the Fecal Shedding of *Escherichia coli* O157:H7 in Feedlot Cattle

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## Abstract

*Escherichia coli* O157:H7 is a foodborne pathogen that causes gastroenteritis, hemolytic uremic syndrome and occasionally death in humans. Cattle shed the organism in their feces where it can be a source of contamination for food, water, and the environment. Effective pre-harvest intervention strategies may significantly reduce the risk of human infection by lowering the pathogen load and ultimately contamination of food and water products. Probiotics have been previously identified as one such mitigation strategy. The objective of this study was to evaluate the effect of MSE probiotic (Natur's Way, Inc.), included during the final 15 days of the finishing phase, on fecal shedding of *E. coli* O157:H7 in feedlot cattle. Ninety-six finishing steers, prescreened to be positive for *E. coli* O157:H7 were enrolled in the study and randomly allocated to one of sixteen pens (n = 6 steers per pen). Pens were fed high-concentrate finishing diets and were allocated to one of two treatments, control (no probiotic; n = 8 pens) or MSE probiotic (n= 8 pens) top-dressed on daily rations. Fecal samples were collected from each animal on day 0, 7, and 15 and tested for *E. coli* O157:H7. In addition, the number of super-shedders was determined on day 15 by direct plating. The mean prevalence of *E. coli* O157:H7 over the duration of the study was reduced in cattle fed the MSE probiotic (10.3%) compared to control animals (22.2%; $P < 0.05$). On day 15, the control treatment had 3 steers classified as

super- shedders, whereas none of the MSE treatment steers were super-shedders. The results from this study indicate that MSE probiotic may be a successful pre-harvest intervention strategy for reducing *E. coli* O157:H7 in cattle when fed 15 days prior to harvest.

## Introduction

*Escherichia coli* O157:H7, a food-borne pathogen, is associated with gastroenteritis in thousands of Americans every year. Cattle are a major reservoir of the organism which resides in the gastrointestinal tract, particularly the hindgut, of healthy cattle and are shed in the feces. Feces are a major source of contamination of beef products, produce, and recreational and drinking water (Rangel et al., 2005). There has been increased interest in a subpopulation of cattle that appear to shed *E. coli* O157:H7 in feces at higher concentrations ($> 10^4$ CFU/g of feces; "super shedders") than typically expected ($< 10^3$ CFU/g of feces). These animals may impact the prevalence of *E. coli* O157:H7 within the pen, and ultimately on carcasses. Modeling efforts have predicted that the transmission of *E. coli* O157:H7 infection in cattle populations is primarily due to a small proportion of animals, which are shedding at high levels (Matthews et al., 2006). In addition, Fox et al. (2008) showed that loads of cattle at harvest with a super shedder were at a higher risk for carcass contamination compared to loads without a super shedder. Factors that contribute to the fecal shedding of *E. coli* O157:H7 in cattle and potential pre- and post-harvest intervention strategies to minimize human health risks are of primary concern for food safety.

Pre-harvest reduction of *E. coli* O157:H7 in cattle requires targeted intervention strategies in order to deliver cattle to the abattoir with a lower pathogen load so that carcass contamination is minimized or eliminated. Probiotics, also called direct-fed microbials, or in some cases competitive exclusion (CE) cultures, have previously been identified as a potential pre-harvest intervention strategy, where they can be included in cattle diets prior to shipment for processing (Brashears et al., 2003; Diez-Gonzales and Shamberger, 2004). The mechanism responsible for a reduction in *E. coli* O157:H7 is not know but may be either an indirect effect by altering microbial populations and products of fermentation in the gastrointestinal tract, or a direct effect on *E. coli* O157:H7, reducing the ability of *E. coli* O157:H7 to establish in the hindgut and shed in feces (Ohya et al., 2000, Diez-Gonzales and Shamberger, 2004; Krehbiel et al., 2003; LeJeune and Wetzel, 2007; Loneragan and Brashears, 2005). Our aim was to evaluate the efficacy of a

specific probiotic product (MSE, Natur's Way Inc., Horton, KS) in reducing *E. coli* O157:H7 in cattle feces.

## Methods and Materials

**Animals and Sample Collection.** Ninety-six crossbred yearling steers, previously shown to be fecal positive for *E. coli* O157:H7 were included as subjects in this study. Steers were randomly allocated to one of sixteen pens (6 steers/pen). Cattle were fed high concentrate finishing diets, and prior to initiating the project, cattle had not been fed any probiotic product. All pens had ad libitum access to water. Eight pens were fed the control diet with no MSE added (Control) while the treatment group (n = 8 pens) received a daily dose of MSE probiotic (Probiotic; product contains: *Lactobacillus acidophilus*, *L. casei*, *Saccharomyces cerevisiae*, *Enterococcus faecium*, and a fungal extract) top-dressed on their daily ration. Study animals were monitored daily and fecal grab samples were obtained by rectal palpation from each animal on days 0, 7, and 15. Samples were placed in a cooler with ice, transported to the Pre-harvest Food Safety Laboratory in the College of Veterinary Medicine at Kansas State University, and held at 4°C until processed within 24 hours.

**Isolation of *E. coli* O157:H7 from fecal samples.** Fecal samples, obtained from each animal by rectal grab, were kneaded and approximately 1 g of fecal material was placed in 9 mL of Gram Negative (GN) broth supplemented with cefixime (0.05mg/L), cefsulodin (10.0 mg/L), and vancomycin (8.0 mg/L; GNccv). Samples were vortexed for 1 min and incubated for 5 h at 37°C. Immunomagnetic separation (IMS; Dynal, Inc.) was performed following enrichment, and 50 µL of product was plated onto sorbitol MacConkey agar supplemented with cefixime (50 ng/mL) and potassium tellurite (2.5µg/mL; CT-SMAC). Plates were incubated overnight at 37°C and up to six sorbitol negative colonies from each sample were picked and streaked onto blood agar plates. Blood agar plates were incubated overnight at 37°C and colonies were tested for indole production, the presence of the O157 antigen using latex agglutination, and confirmation of species with PCR analysis of *eae*, *fliC, stx*1, *stx*2, *hylA*, and *rfb*E virulence genes (Bai et al., 2008; Bertrand et al., 2007; Fagan et al., 1999).

**Semi-quantification of *E. coli* O157:H7 from feces.** A semi-quantitative method was employed to categorize fecal culture positive cattle into low shedders and super-shedders (Sanderson et al., 2007; Fox et al., 2008) on day 15 only. Briefly, a swab of 1:10 diluted fecal suspension in GNccv broth before enrichment was plated onto a CT-SMAC plate and incubated for 16 to 18 h at 37°C. From direct streaked CT-SMAC plates, up to six sorbitol negative colonies were transferred to a blood agar plate and evaluated for indole production, latex agglutination for the O157:H7 antigen, and PCR as described above.

**Statistical Analysis.** The prevalence of fecal *E. coli* O157:H7 was compared between the two treatment groups (Control and Probiotic). Data were analyzed using generalized estimated equations for binomial outcomes (PROC GLIMMIX of SAS; v. 9.1), and a correlation structure to account for the lack of independence between animals within pens was included. Treatment, sampling day, and the treatment × sampling day interaction were the fixed effects in the analysis.

## Summary of Results

In order to obtain a high prevalence of *E. coli* O157:H7, cattle were prescreened for fecal shedding of *E. coli* O157:H7 and 96 positive cattle were enrolled in the study. However, one week later (day 0) the average prevalence of *E. coli* O157:H7 was 22.9% in both control and probiotic-fed groups. The overall prevalence of *E. coli* O157:H7 throughout the fifteen day trial was 17.4% (50 of 287). Across sampling days, the average estimated prevalence of *E. coli* O157:H7 in fecal samples from cattle given the control treatment was significantly higher (22.2%) than the prevalence from cattle given the MSE probiotic treatment (10.3%, $P < 0.05$). The prevalence of *E. coli* O157:H7 in fecal samples was lower in cattle given the MSE probiotic on days 7 and 15 (Fig. B.1). Although sampling day tended to impact *E. coli* O157:H7 prevalence ($P = 0.07$), there was no treatment by sampling day interaction ($P = 0.12$). The extent of reduction in the prevalence of *E. coli* O157:H7 on sampling days 7 and 14 in the probiotic group compared to the control was 54 and 80%, respectively. Table B.1 lists the number (and %) of cattle in each pen positive for *E. coli* O157:H7 on each collection day. The results from this study show a negative association between cattle administered MSE probiotic and fecal shedding of *E. coli* O157:H7, when compared to cattle not administered the probiotic.

In addition, on day 15, samples were evaluated by semi-quantification method to determine the number of super (~$10^4$ or higher) and low shedders (~$10^3$ or lower) in each treatment. There were three super shedders (representing two pens) in the control treatment (6%; 3 of 48), while no super shedders were found in the MSE probiotic treatment group (0%).

In conclusion, cattle that received the MSE probiotic, as a top-dress in the final finishing diet, for 15 days had a significantly lower prevalence of fecal *E. coli* O157:H7 than control cattle which did not receive the product. It is unknown if the product reduced the number of cattle shedding high levels of *E. coli* O157:H7 (initial measurements were not taken to evaluate this), however, no cattle were found to be super-shedders in the group of cattle receiving the MSE product at the final collection. Overall, MSE appears to be an effective pre-harvest intervention strategy to reduce *E. coli* O157:H7 in cattle feces. The mechanism responsible for this negative association remains unknown; several possibilities include a change in the physiology of the hindgut ecosystem, competition for nutrients or space by the organisms in the MSE product, direct inhibition of *E. coli* O157:H7 by products secreted from the organisms in the MSE product, or others. Further work is needed to confirm this association and determine the mode of action responsible for a decrease in *E. coli* O157:H7 prevalence.

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**Figure B.1 Mean prevalence of *Escherichia coli* O157:H7 in fecal samples collected on days 0, 7, and 15 from pens of cattle administered MSE probiotic (Probiotic) or a control diet with no probiotic (Control).**

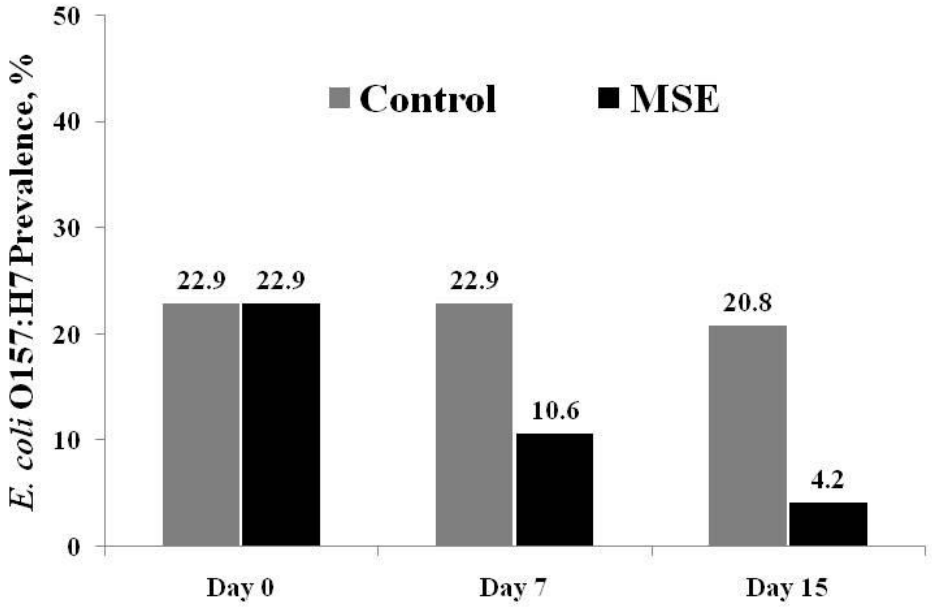

**Table B.1 Number of *Escherichia coli* O157:H7 positive fecal samples and total samples collected (% positive) from each pen given either the control or MSE probiotic treatment on each sampling day.**

| | | Sampling Day | | |
|---|---|---|---|---|
| **Treatment** | **Pen ID** | **Day 0** | **Day 7** | **Day 15** |
| Control | 101 | 1/6 (16.7) | 1/6 (16.7) | 3/6 (50) |
| | 105 | 1/6 (16.7) | 0/6 (0) | 3/6 (50) |
| | 111 | 2/6 (33.3) | 4/6 (66.7) | 2/6 (33.3) |
| | 115 | 0/6 (0) | 0/6 (0) | 0/6 (0) |
| | 117 | 1/6 (16.7) | 3/6 (50) | 1/6 (16.7) |
| | 121 | 2/6 (33.3) | 2/6 (33.3) | 1/6 (16.7) |
| | 127 | 2/6 (33.3) | 1/6 (16.7) | 0/6 (0) |
| | 131 | 2/6 (33.3) | 0/6 (0) | 0/6 (0) |
| Probiotic | 103 | 2/6 (33.3) | 2/6 (33.3) | 0/6 (0) |
| | 107 | 0/6 (0) | 1/5 (20) | 0/6 (0) |
| | 109 | 0/6 (0) | 0/6 (0) | 0/6 (0) |
| | 113 | 2/6 (33.3) | 0/6 (0) | 0/6 (0) |
| | 119 | 2/6 (33.3) | 1/6 (16.7) | 1/6 (16.7) |
| | 123 | 1/6 (16.7) | 0/6 (0) | 0/6 (0) |
| | 125 | 3/6 (50) | 1/6 (16.7) | 0/6 (0) |
| | 129 | 1/6 (16.7) | 0/6 (0) | 1/6 (16.7) |

# Appendix C - *In vitro* Evaluation of Nova Cell Probiotic (Nova Microbial Technologies™) for Anti-*E. coli* O157:H7 Activity

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## Abstract

Probiotic products have been proposed as a potential pre-harvest intervention strategy to reduce the prevalence of *E. coli* O157:H7 in cattle. Nova Cell is a *Lactobacillus*-based product marketed by Nova Microbial Technologies™ which has not been evaluated for anti-*E. coli* O157:H7 activity. Our objectives were to use *in vitro* batch culture fermentation and direct inhibition methodologies to determine the effectives of Nova Cell at inhibiting *E. coli* O157:H7. Using ruminal and fecal microbial batch culture fermentations inoculated with *E. coli* O157:H& and with added Nova Cell probiotic at a does of 0 (Control), $10^7$(Low), $10^9$(Medium), $10^{11}$(High) per animal, revealed no effect of the probiotic on *E. coli* O157:H7 concentration. Using direct inhibition, the Nova Cell *Lactobacillus* enrichment and supernatant produced 2 to 3 mm zones of inhibition in an *E. coli* O157:H7 bacterial lawn, however, once the pH was adjusted to near neutral there was no zone of inhibition. Results from this study indicate that there may be some anti-*E. coli* O157:H7 activity from Nova Cell probiotic, but the inhibition is likely due to production of acid which creates an environment inhospitable to the organism. The effect of Nova Cell in reducing *E. coli* O157:H7 in cattle is not known.

## Introduction

*Escherichia coli* O157:H7 is a human foodborne pathogen associated with gastroenteritis, hemolytic uremic syndrome and rarely death. Cattle are a primary reservoir for the organism which can colonize the lower gastrointestinal tract and be shed in the feces. Cattle feces can

serve as a major source of contamination for food and water products, as well as the environment (Rangel et al., 2005). Pre-harvest intervention strategies that minimize the pathogen load and ultimately limit contamination of food and other products may reduce the human health risk of *E. coli* O157:H7. Probiotics have previously been identified as a potential pre-harvest intervention strategy, where they can be included in cattle fed and reduce prevalence prior to harvest (Brashears et al., 2003; Diez-Gonzales and Shamberger, 2004). The proposed mechanism of *E. coli* O157:H7 reduction includes altered microbial populations and products of fermentation in the bovine hindgut which indirectly effect *E. coli* O157:H7, or a direct effects which reduce the ability of *E. coli* O157:H7 to colonize (Ohya et al., 2000, Diez-Gonzales and Shamberger, 2004; Krehbiel et al., 2003; LeJeune and Wetzel, 2007; Loneragan and Brashears, 2005). Nova Microbial Technologies™ markets a *Lactobacillus acidophilus* product (Nova Cell) for use as a cattle feed additive. The ability of this product to reduce *E. coli* O157:H7 or display anti-*E. coli* O157:H7 properties has not been evaluated. Our objectives were to determine the anti-*E. coli* O157:H7 properties of Nova Cell using *in vitro* batch culture fermentation and direct inhibition methods.

## Methods and Materials

### *Study 1*

Batch culture fermentations were set up in 70-ml serum bottles sealed with butyl rubber stoppers fitted with Bunsen valves. Each bottle contained 50 ml of a fermentation mixture composed of 33 ml of McDougall's buffer and 17 ml of ruminal fluid or fecal microbial inoculum (2:1 buffer to inoculum ratio) from cannulated Holstien steers fed a forage-based diet. An equal volume of Ringer's solution was added to each fecal sample so that the dry matter content was approximately the same as that of ruminal fluid. The ruminal fluid and fecal inoculums were strained through cheesecloth and added with buffer under flowing oxygen-free $CO_2$ gas maintain an anaerobic environment to fermentation bottles. Treatments were arranged in a 2 × 3 factorial with the first factor being 0 (Control), $10^7$(Low), $10^9$(Medium), $10^{11}$(High) Nova-Cell probiotic calculated per animal. The second factor was inclusion of 0 or 500 mg gluconic acid (used to stimulate *E. coli* O157:H7 growth; Fox et al., 2009). Each treatment was duplicated once within animal and three fecal and ruminal fluid sources were used. Fermentations were then inoculated anaerobically (under flowing $O_2$ –free $CO_2$) with 100 µl

(approximately $10^3$ CFU/ml of fermentation) of a five-strain mixture of *E. coli* O157:H7 resistant to 50 µg/ml nalidixic acid (*Nal*$^R$). Fermentations were kept at 37°C in an orbital incubator (80 rpm) and sampled anaerobically (under flowing $O_2$ –free CO2) at 0, 6, 12, and 24 h to determine concentrations of *Nal*$^R$ *E. coli* O157 and record the fermentation pH. Samples were serially diluted in buffered peptone water (Sigma-Aldrich, St. Louis, MO) and 0.1 ml of appropriate dilution was spread, in triplicate, to ct-SMAC plates with nalidixic acid (50 µg/ml). After incubation of plates for 24 hr, colonies were counted and the concentration was calculated. Ruminal fluid and fecal microbial fermentations were set up in duplicates. Statistical analyses were conducted with the MIXED procedure of SAS to determine differences in the $\log_{10}$ concentration of *Nal*$^R$ *E. coli* O157 for both ruminal fluid and fecal fermentations. Fixed effects included animal, gluconic acid treatment, probiotic treatment, hour, and all interactions. Results were considered significant at $P < 0.05$.

### *Study 2*

The Nova Cell probiotic product (100 mg) was inoculated into 10 mL of a lactobacilli-specific medium (MRS) and grown overnight at 37°C. After enrichment, 100 µL was plated onto MRS plates and grown at 37°C for 18 hr. One single, isolated colony was selected and plated onto a new MRS plate and again incubated at 37°C for 18 hr, after which the colony was Gram stained to confirm colony morphology typical of *Lactobacillus*. One colony representing a putative *Lactobacillus* was incubated in 10 mL of aerobic and anaerobic MRS broth and grown under both aerobic and anaerobic conditions at 37°C for 9 hr or until the growth reached a minimum spectrophotometer absorbance of 600 nm. The pH of each culture was recorded and aliquots (1 mL each) were centrifuged for 10 min at 15,000 RPM. After centrifugation, the pH of each supernatant was recorded. For one aliquot, the pH was adjusted with sodium hydroxide to a pH of at least 7.0, while the other sample remained at the original pH. Next, 100 µL of the original enrichment, the unadjusted supernatant, and the pH-adjusted supernatant was placed into wells in Luria-Bertani (LB) agar plates. The LB plates were first inoculated with *E. coli* O157:H7 (ATCC strain 43894) grown overnight in a non-selective LB broth and determined to be a McFarland standard of at least 0.5 to obtain a bacterial lawn. The wells were be made by removing plugs from the agar plates. Plates were be incubated for 24 h at 37°C. After

incubation, zones of inhibition were measured. Each colony enrichment (under both anaerobic and aerobic growth conditions) was repeated.

## Summary of Results

The concentration of *Nal*$^R$ *E. coli* O157:H7 and the final pH from rumen and fecal microbial batch culture fermentations did not differ based on Nova Cell probiotic treatment ($P > 0.10$). Gluconic acid was shown to stimulate growth of *E. coli* O157:H7 in fecal batch culture fermentation systems, however, this treatment effect was not observed in rumen fluid fermentations. Hour was significantly associated with *E. coli* O157:H7 concentration ($P < 0.05$), yet there were no interactions between hour or gluconic acid with the probiotic treatment. These results indicate that under this *in vitro* batch culture fermentation system, there does not appear to be any anti-*E. coli* O157:H7 effect of Nova Cell probiotic. From this system, however, we could not confirm that the Nova Cell *Lactobacillus* bacteria were active and therefore had the ability to exert any direct inhibition activity. Because of this, we used the direct inhibition method to evaluate the anti-*E. coli* O157:H7 activity with actively growing Nova Cell *Lactobacilli*. The results from the direct inhibition trial reveal that when the enrichment culture or the enrichment supernatant were inoculated into plugs and incubated with *E. coli* O157:H7 there was a 2 to 3 mm zone of inhibition (Table C.1). When the same samples had their pH adjusted to near neutral there was no effect of the probiotic on *E. coli* O157:H7 inhibition. It is unknown what significance this zone of inhibition would have in the complex ecosystem of the bovine hindgut, however, we hypothesize this inhibition is due to the production of lactic acid. Previous *in vitro* work has shown that lactic acid can inhibit *E. coli* O157, but only at low pH (Jordan et al, 1999). In conclusion, it appears that Nova Cell probiotic can produce a pH-mediated inhibition of *E. coli* O157:H7, however, futher work is needed to test the efficacy of this product in feedlot cattle.

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**Table C.1 pH and zone of *E. coli* O157 inhibition of enrichment, supernatant, and pH-adjusted supernatant samples of Nova Cell probiotic grown in MRS *Lactobacillus* broth**

| | | pH | | | Zone of inhibition (mm) | | |
|---|---|---|---|---|---|---|---|
| Growth Condition | Absorbance | Enrichment | Supernatant | pH-adjusted Supernatant | Enrichment | Supernatant | pH-adjusted Supernatant |
| Aerobic | 0.664 | 5.6 | 5.7 | 6.7 | 0 | 0 | 0 |
| Aerobic | 0.602 | 5.7 | 5.9 | 6.9 | 0 | 0 | 0 |
| Anaerobic | 1.40 | 4.4 | 4.4 | 8.7 | 3 | 2 | 0 |
| Anaerobic | 1.23 | 4.4 | 4.4 | 7.7 | 3 | 2 | 0 |