Impact of By-product Feedstuffs on *Escherichia coli* O157:H7 and *Salmonella* Typhimurium in Pure and Mixed Ruminal and Fecal Culture *in Vitro*†

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† Proprietary or brand names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies neither approval of the product, nor exclusion of others that may be suitable. USDA is an equal opportunity provider and employer.

ABSTRACT

The use of by-product feedstuffs and prebiotics in animal diets has increased in recent years. The present study was undertaken to determine what effects novel by-product feedstuffs, including prebiotics, have on survival of the important foodborne pathogenic bacteria *Escherichia coli* O157:H7 and *Salmonella* enterica Typhimurium in pure and mixed ruminal and fecal culture fermentations from cattle and swine. By-product feedstuffs utilized in this study included: hyperimmunized whole egg, lysine biomass, lysine biomass (spray dried), threonine biomass (drum dried), threonine biomass (spray dried), beer well yeast (drum dried), beer well yeast (spray dried), ethanol yeast (pan dried) and corn meal as a control to simulate normal dietary conditions. Prebiotics examined included: PremiDex ™, CitriStim™, a CitriStim:PremiDex blend (50%:50%), and a commercial oligosaccharide source feedstuff. Pure culture populations of *E. coli* O157:H7 were reduced (P < 0.05) by 2% w/v of each of spray dried threonine, drum-dried threonine, ethanol yeast, hyper-immunized whole egg, and a blend of CitriStim:PremiDex. No effects on *Salmonella* populations were observed in pure cultures. Fermentations of mixed ruminal microorganisms from cattle fed a forage based diet demonstrated that 2% PremiDex reduced (P < 0.05) *E. coli* O157:H7 populations compared to controls and the CitriStim:PremiDex blend reduced *E. coli* O157:H7 and *Salmonella* populations (P < 0.05) in fermentations from cattle fed high grain diets. The anti-foodborne pathogen effects appear to be an indirect effect mediated by the microbial population of the intestinal tract, such as has been reported previously for prebiotics.

Keywords: by-products, prebiotics, probiotic, feedstuff, *E. coli* O157:H7, *Salmonella*, food safety

INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC), such as *E. coli* O157:H7, and *Salmonella enterica* are two of the most critical foodborne pathogenic bacteria and are often found asymptomatically in the gastrointestinal tract of farm animals (Ferens and Hovde, 2011; Scallan, et al., 2011). Both pathogens can survive undetected within the gastrointestinal microbial population of cattle (Berg, et al., 2004; Callaway, et al., 2006; Kunze, et al., 2008), and *Salmonella* is often found in cattle, swine and poultry (Borland, 1975; Davies, et al., 1999). When these pathogens are transmitted to a human they cause severe illness or even death, the combined yearly cost of these pathogens to the U.S. economy is in excess of $15 billion (Scharff, 2010).

Dietary effects on gastrointestinal pathogen populations have been examined extensively, and the effects have been extremely variable depending on the specific feedstuff utilized and the production situation (Callaway, et al., 2009; Diez-Gonzalez, et al., 1998; Kudva, et al., 1995). In recent years there has been a dramatic increase in the use of corn based by-product feedstuffs in food animal diets (Richman, 2007). This growth has been especially notable in the ethanol fermentation by products, such as distillers grains (DG). However, research has shown that the inclusion of DG in cattle diets can increase fecal prevalence and shedding of *E. coli* O157:H7 (Jacob, et al., 2009; Jacob, et al., 2010; Jacob, et al., 2008c; Wells, et al., 2009). The present study was designed to examine a variety of novel corn-derived feedstuffs and selected commercially available prebiotic products (PremiDex, CitriStim) in regard to their ability to provide a selective effect on *E. coli* O157:H7 and *Salmonella* Typhimurium populations in pure and mixed culture in vitro.

MATERIALS AND METHODS

Bacterial strains and culture conditions

*Escherichia coli* O157:H7 strain 933 (ATCC 43895) was originally isolated from a human hemorrhagic colitis outbreak, and the *Salmonella enterica* Typhimurium isolates that were used in this study were originally isolated from cattle and swine. All isolates were obtained from the Food and Feed Safety Research Unit (USDA/ARS, College Station, TX) culture collection. Both *E. coli* O157:H7 strain 933 and *S. Typhimurium* were selected for resistance to novobiocin (No) and nalidixic acid (NA; 20 and 25 μg/mL, respectively) by repeated transfer and selection in the presence of sub-lethal concentrations of each antibiotic. This resistant phenotype was stable through multiple unselected transfers in batch culture and through repeated culture vessel turnovers in continuous culture (data not shown).

Pure culture studies

Feedstuffs were added to 16 x 100 mm tubes containing anoxic Trypic Soy Broth (TSB, (cooled after autoclaving under anoxic conditions [90% N₂, 5% H₂, 5% CO₂]; Difco Laboratories; Detroit, MI) to reach final concentrations of 2% wt/vol. Feedstuffs utilized in this study were: PremiDex, CitriStim, a CitriStim:PremiDex, commercial oligosaccharide, hyperimmunized whole egg, lysine biomass (drum dried), lysine biomass (spray dried), threonine biomass (drum dried), threonine biomass (spray dried), beer well yeast (drum dried), beer well yeast (spray dried), ethanol yeast (pan dried) and corn meal as a control. Corn meal was used as a control to simulate the typical ingredients in a ruminant diet that would be replaced by the by-product feedstuffs. Feedstuffs were added into tubes immediately before the pathogens were inoculated. *Escherichia coli* O157:H7 strain 933 (ATCC 43895) or *S. Typhimurium* were grown anaerobically and aseptically inoculated into feedstuff containing tubes at approximately 6 x 10⁵ CFU/mL of *E. coli* O157:H7 or 3 x 10⁵ CFU/mL of *S. Typhimurium* at t = 0. Tubes in triplicate (n = 3) were incubated at 39 °C for 24 h, when 1 mL aliquots were removed for enumeration (described below).
Animal diets

All animals were maintained in accordance with a protocol approved by the Southern Plains Agricultural Research Center Animal Care and Use Committee (ACUC No 06002). Holstein cattle (n = 4) were provided *ad libitum* access to water and minerals at all times. Ruminal fluid was collected from cattle grazing bermudagrass pasture (n=2) and a high-grain diet (n=2). Cattle in the pasture-fed group were grazed on an early vegetative stage ryegrass pasture at the time of ruminal fluid collection. The grain-fed cattle were fed a commercial feedlot ration (corn soybean mix; Producer’s Co-op, Bryan, TX) and was maintained on this feedlot diet for 10 d prior to ruminal or fecal collection. Crossbred pigs (n=4) were provided *ad libitum* access to water and minerals at all times. Pigs were fed a commercial finishing diet (Producer’s Co-op, Bryan, TX ) comprised of soybean meal and corn twice daily. Animals were maintained on this diet for 14 d prior to fecal fluid collection.

Ruminal and fecal fluid collection

Ruminal contents were collected by hand from the ventral sac of two ruminally cannulated Holstein cows on each diet (n = 2/diet group). The ruminal contents were collected from all cattle at approximately the same time (between 0800 and 0900 h). Immediately after removal from the rumen, the contents from each cow were strained via a fine mesh nylon strainer (Reaves and Co., Durham, NC) and pooled. Ruminal fluid was transported to the laboratory and incubated for 30 min at 39 °C to allow gas production to buoy large particles to the top of the flasks. Fresh ruminal fluid contained approximately 10¹⁰ cells/ml of total culturable anaerobes, as determined by serial dilution in anaerobic reinforced clostridial broth in triplicate tubes.

Fecal samples from cattle were collected directly per rectum by digital grab. Feces were collected from all cattle at the same time. Immediately upon collection, the feces were strained via a fine mesh nylon strainer to obtain a fecal fluid which was then pooled. Fecal fluid was transported to the laboratory as described above. Fresh fecal fluid contained approximately 10¹⁰ cells/ml of total culturable anaerobic bacteria as determined by serial dilution as described above.

Swine feces were collected directly per rectum by digital grab from all pigs at the same time, pooled equally by weight, and was added directly to the fecal fermentation media (described below) at a 33% w/v final concentration. Fresh swine feces contained approximately 10⁹ cells/g of total culturable anaerobic bacteria.

In vitro mixed ruminal microbial fermentations

Incubations of cattle ruminal and fecal fluids were performed by combining the gastrointestinal fluid (33% vol/vol) with an anoxic basal medium containing (per liter): 292 mg K₂HPO₄, 202 mg KH₂PO₄, 436 mg NH₄SO₄, 480 mg NaCl, 100 mg MgSO₄ • 7H₂O, 64 mg CaCl₂ • H₂O, 4,000 mg Na₂CO₃, 600 mg cysteine hydrochloride (Cotta and Russell, 1982) supplemented with 1 g/L glucose. Swine feces was added to the same basal medium in a 33% w/v concentration. Approximately 10⁴-5 CFU/mL *E. coli* O157:H7 strain 933 or *S. Typhimurium* were added to the buffered gastrointestinal fluid fermentations in all experiments. The resultant suspensions were anaerobically transferred to 18 × 150 mm Balch tubes (Bellco Glass, Vineland, NJ; 10 ml per tube). Feedstuffs described above were added to each tube to reach final concentrations of 2 % wt/vol under an anoxic gas phase. Tubes in triplicate (n = 3) were then sealed using rubber stoppers with aluminum crimps and incubated for 24 h at 39°C under a N₂, CO₂, H₂ (90:5:5 v/v) gas phase. Samples were removed after 24 h of incubation and centrifuged (10,000 × g, 5 min, 24°C) to remove particulate matter.

Bacterial enumeration

Samples were taken from all pure and mixed culture in vitro fermentations at 24 h to determine the effect of feedstuffs on populations of *E. coli* O157:H7
and S. Typhimurium. Samples were serially diluted (in 10-fold increments) in phosphate buffered saline (PBS, pH 7.0), and subsequently plated on MacConkey’s agar (supplemented with 25 µg/mL NO and 20 µg/mL NA) and incubated at 37°C overnight for direct counting of E. coli O157:H7 CFU/ml. To determine populations of S. Typhimurium, samples were serially diluted as described above and plated on Brilliant Green Agar (supplemented with 25 µg/mL NO and 20 µg/mL NA) and incubated at 37°C overnight for direct counting.

Statistical analysis
Pure culture experiments were performed with (n=3) tubes on consecutive days. Mixed ruminal bacteria experiments were performed in duplicate tubes (n=2) on consecutive days, and the values presented are means. Students’ t-test was used to determine significance of differences between means of each treatment.

RESULTS
In pure culture studies, none of the feedstuffs used in this study affected Salmonella Typhimurium populations (Figure 1) compared to controls using corn meal. However, populations of E. coli O157:H7 in pure cultures were reduced (P < 0.05) when: spray dried threonine biomass, drum-dried threonine biomass, ethanol yeast, hyper-immunized whole egg, and a blend of CitriStim:PreMiDex were included; although it must be noted that this statistically sig-

Figure 1. Effects of feedstuffs on populations of E. coli O157:H7 (light bars) and Salmonella Typhimurium (dark bars) in pure cultures. Feedstuffs were included at 2% w/v, and bars represent the mean of 3 fermentations, and error bars indicate standard deviation. Bars marked with superscript a indicate differences from control of P < 0.05.
significant reduction was less than a 10-fold decrease in *E. coli* O157:H7 populations (Figure 1).

When pathogens were added to *in vitro* mixed ruminal microorganism fermentations of cows fed high forage diets, the inclusion of 2% Premidex reduced (*P* < 0.05) *E. coli* O157:H7 and reduced (*P* < 0.06) *Salmonella* Typhimurium populations compared to controls (Figure 2). However, when cows were fed high grain diets (data not shown) there was no effect of feedstuff addition on pathogen populations in ruminal fluid fermentations. In *in vitro* mixed fecal microorganism fermentations of cattle fed high grain diets (Figure 3), *E. coli* O157:H7 populations were numerically reduced in hyperimmunized whole egg and beer well yeast (spray dried) and was significantly reduced (*P* < 0.05) in fermentations containing the CitriStim:PremiDex blend. *Salmonella* Typhimurium populations in these *in vitro* fermentations were also reduced (*P* < 0.05) by addition of the CitriStim:PremiDex blend.

When these by-product feedstuffs were included in *in vitro* pig fecal fermentations, PremiDex and the CitriStim:PremiDex blend reduced (*P* < 0.05) *E. coli* O157:H7 populations more than 10-fold compared to corn meal controls (Figure 4). However, the addition of the commercial oligosaccharide product and hyperimmunized whole egg increased (*P* < 0.05) populations of *Salmonella* Typhimurium more than 1 log$_{10}$ CFU/ml compared to corn meal controls.

![Figure 2. Effects of feedstuffs on populations of *E. coli* O157:H7 (light bars) and *Salmonella* Typhimurium when inoculated into mixed ruminal microorganism fermentations from cattle fed a forage-based diet. Feedstuffs were included at 2% w/v, and bars represent the mean of 3 fermentations, and error bars indicate standard deviation. Bars marked with superscript a indicate differences from control of *P* < 0.05.](image-url)
DISCUSSION

In recent years, food safety has become an increasingly important issue to animal producers (Davies, 2011; Sargeant, et al., 2007) and to the national economy as a whole (Scharff, 2010). Research has shown that diet composition and feeding methods can affect intestinal microbial populations of cattle, including populations of foodborne pathogenic bacteria, such as *E. coli* O157:H7 and *Salmonella* (Buchko, et al., 2000; Diez-Gonzalez, et al., 1998; Fox, et al., 2007; Jacob, et al., 2008b). Distillers grains (DG) have seen a rapid increase in inclusion in animal diets (Klopfenstein, et al., 2008; Richman, 2007), following the increase in the production of ethanol from corn fermentation (Richman, 2007). Distillers grains as well as other by-products have some broad impacts on some members of the microbial ecosystem (Callaway, et al., 2010; Fron, et al., 1996; Williams, et al., 2010). Recently, the inclusion of DG in cattle diets was shown to increase fecal shedding of *E. coli* O157:H7 (Jacob, et al., 2008b; Jacob, et al., 2008c; Wells, et al., 2009). In in vitro studies, it was found that ruminal fluid from cattle fed DG supported a higher level of *E. coli* O157:H7 growth, and that the inclusion of DG in fermentations also increased *E. coli* O157:H7 populations (Jacob, et al., 2008a). However, results were variable based largely on the type of feedstuff used.

Figure 3. Effects of feedstuffs on populations of *E. coli* O157:H7 (light bars) and *Salmonella Typhimurium* when inoculated into mixed fecal microorganism fermentations from cattle fed a grain-based diet. Feedstuffs were included at 2% w/v, and bars represent the mean of 3 fermentations, and error bars indicate standard deviation. Bars marked with superscript a indicate differences from control of *P* < 0.05.
upon source of DG (Jacob, et al., 2009; Jacob, et al., 2010; Wells, et al., 2009). Other researchers found that inclusion of high levels of corn or wheat DDGS in feedlot diets of cattle may allow E. coli O157:H7 improved survival in feces (Yang, et al., 2010).

Emerging microbial diversity data indicates that different gastrointestinal microbial populations are selected for by different feedstuffs, possibly due to the presence (or absence) of some limiting component in the diet, such as is found in prebiotic feedstuffs (Patra and Saxena, 2009; Tajima, et al., 2001). In the present study, a variety of novel by-product feedstuffs and prebiotic compounds were examined to determine their impact on foodborne pathogenic bacterial populations in pure and mixed cultures of bacteria from cattle and swine. In general, the novel by-product feedstuffs did not affect populations of E. coli O157:H7 or Salmonella in pure or mixed culture fermentations. In pure cultures, the direct effects of the different by-products were negligible, with no feedstuff causing as much as a 10-fold decrease in pathogen populations, though a blend of CitriStim:PremiDex (a prebiotic source of oligosaccharides such as maltooligosaccharides) did cause a significant reduction in E. coli O157:H7 populations.

When the prebiotic PremiDex was included in ruminal fluid fermentations from a cow fed a high forage diet, populations of both E. coli O157:H7 and

Figure 4. Effects of feedstuffs on populations of E. coli O157:H7 (light bars) and Salmonella Typhimurium when inoculated into mixed fecal microorganism fermentations from growing swine fed a commercial finishing diet. Feedstuffs were included at 2% w/v, and bars represent the mean of 3 fermentations, and error bars indicate standard deviation. Bars marked with superscript a indicate differences from control of P < 0.05.
Salmonella populations were decreased significantly; but this effect disappeared in ruminal fluid from cattle fed a high grain diet. However, when fecal bacteria from cattle fed high grain diets were fermented with by-products feeds, the CitriStim:PremiDex prebiotic blend reduced E. coli O157:H7 populations. Collectively, these results indicate that CitriStim and/ or PremiDex reduced E. coli O157:H7 populations and could be an effective dietary additive to reduce foodborne pathogenic bacteria prior to harvest in ruminant animals, although further research is needed to clarify the magnitude of the effect in further in vitro studies and in live animals during feeding trials.

In swine fecal fermentations, prebiotic addition had a greater impact than in the cattle ruminal or fecal fermentations. PremiDex and the CitriStim:PremiDex blend significantly decreased E. coli O157:H7 populations in swine fecal fermentations, while no novel by-product feedstuff affected E. coli O157:H7 populations. However, residual biomass and hyperimmunized whole egg increased S. Typhimurium populations more than 20-fold in these in vitro fecal fermentations. The difference in effects of these feedstuffs between the two pathogenic species is likely due to indirect effects on other members of the microbial population who either outcompete the pathogen (possibly mediated via a prebiotic-type effect), or alternatively creation of a competitive “vacuum” in the fecal microbial ecosystem that is consequently exploited by Salmonella. Further research is needed to understand the mechanism behind this impact on pathogen populations.

CONCLUSIONS

Collectively, it appears that PremiDex, and the CitriStim:PremiDex blend had the greatest impact on foodborne pathogenic bacterial populations in mixed gastrointestinal microbial fermentations in the feedstuffs examined presently. Populations of E. coli O157:H7 in fecal fermentations from cows fed high grain diets and swine fecal fermentations were most strongly affected by the CitriStim:PremiDex blend inclusion. The anti-foodborne pathogen effect appears to be indirectly mediated by prebiotic effects on the mixed gastrointestinal microbial consortium rather than by direct effects against the pathogens. Further research is needed to clarify the mode of action of these and other by-product feedstuffs and how much impact they have on the mixed microbial ecosystem when fed to live animals.

REFERENCES


