December 2005

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Effects of Common Forage Phenolic Acids on *Escherichia coli* O157:H7 Viability in Bovine Feces

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Received 5 May 2005/Accepted 27 August 2005

Ruminant animals are carriers of *Escherichia coli* O157:H7, and the transmission of *E. coli* O157:H7 from cattle to the environment and to humans is a concern. It is unclear if diet can influence the survivability of *E. coli* O157:H7 in the gastrointestinal system or in feces in the environment. Feces from cattle fed bromegrass hay or corn silage diets were inoculated with *E. coli* O157:H7, and the survival of this pathogen was analyzed. When animals consumed bromegrass hay for <1 month, viable *E. coli* O157:H7 was not recovered after 28 days postinoculation, but when animals consumed the diet for >1 month, *E. coli* O157:H7 cells were recovered for >120 days. Viable *E. coli* O157:H7 cells in feces from animals fed corn silage were detected until day 45 and differed little with the time on the diet. To determine if forage phenolic acids affected the viability of *E. coli* O157:H7, feces from animals fed corn silage or cracked corn were amended with common forage phenolic acids. When 0.5% *trans*-cinnamic acid or 0.5% *para*-coumaric acid was added to feces from silage-fed animals, the *E. coli* O157:H7 death rate was increased significantly (17-fold and 23-fold, respectively) compared to that with no addition. In feces from animals fed cracked corn, *E. coli* O157:H7 death rates were increased significantly with the addition of 0.1% and 0.5% *trans*-cinnamic acid (7- and 13-fold), 0.1% and 0.5% *p*-coumaric acid (3- and 8-fold), and 0.5% ferulic acid (3-fold). These data suggest that phenolic acids common to forage plants can decrease viable counts of *E. coli* O157:H7 shed in feces.

*Escherichia coli* O157:H7 is a food-borne pathogen associated with hemorrhagic colitis and hemolytic-uremic syndrome in humans, and outbreaks are commonly associated with contaminated meat products from cattle (35). Numerous studies have demonstrated the shedding of *E. coli* O157:H7 from cattle in feces (5, 11, 23, 29, 38) and have reported the bovine gastrointestinal system to be a reservoir for the pathogen (24, 32). Improved meat-processing techniques and testing procedures appear to have decreased recent incidents of contaminated beef products reaching the public (4), but outbreaks associated with fecal contamination of water and produce have been documented (31).

Beef animal production typically involves the concentration of animals in feedlot pens and the provision of energy-rich diets (grain and/or grain silage) to maximize animal performance. Manure collected from cattle operations is often stored and later applied to the land as a means of disposal as well as a source of nutrients for agricultural crops. However, studies have shown that *E. coli* O157:H7 can survive in manure for >3 months after defecation (1, 2, 21, 26). The ability of this bacterium to survive in animal manure is both an environmental and a food safety concern.

Efforts to decrease the shedding and persistence of *E. coli* O157:H7 in cattle manure are ongoing, but few viable interventions have been identified. The feeding of hay to feedlot cattle may decrease bacteria such as *E. coli* O157:H7 from being shed (3, 7), but a specific mechanism to explain a decrease has not been documented. Treatment of manure or animal waste during storage or prior to land application has the potential for decreasing manure-borne pathogens, thereby reducing the risk of water and crop contamination (18, 31, 37). However, concerns pertaining to the cost and environmental impact of treatments are important considerations prior to their implementation by producers. Therefore, a variety of both economical and effective manure treatments need to be developed and tested.

Plant carboxylic phenols have been shown to inhibit certain pathogens (9, 28, 33, 39), and in grasses used as cattle forages, their concentrations range from 2 to 10 g/kg (6, 19). These compounds are synthesized from phenylalanine via a cinnamic acid intermediate to phenylpropanoid compounds (19), and the predominant phenylpropanoid compounds in many forages are *para*-coumaric acid and ferulic acid (12, 25, 30). The phenolic acids are often conjugated to sugars in plants and may play a role in protecting plants from pathogens (12, 30). However, the antimicrobial effects of these plant compounds against pathogens in the complex fecal or manure environment are unknown. The objectives of this study were to evaluate the survival of *E. coli* O157:H7 in feces from cattle fed different types of diets and to determine if amendments with *trans*-cinnamic, coumaric, or ferulic acid affected the survival of this pathogen in cattle feces. Information garnered from this study will identify candidate plant compounds with antimicrobial activity in the fecal environment that may be useful as dietary additives or manure treatments.

**MATERIALS AND METHODS**

**Fecal sample collection.** Fecal samples were collected at 4-week intervals from beef steers fed growing diets as part of a separate feed efficiency study. The growing diets were either bromegrass hay (100% ground bromegrass hay [as fed]) or corn silage (88% corn silage, 9% cracked corn, 3% soybean meal [as fed]) fed ad libitum. Mineral blocks and water were accessible at all times. The

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animals were composite animals from Angus, Hereford, Pinzgauer, and Red Poll cattle bred from populations at the U.S. Meat Animal Research Center, USDA-ARS (MARC; Clay Center, NE). Prior to the provision of experimental diets, animals were adapted to outdoor concrete-surfaced pens and fed corn silage for 4 weeks. Following the adaptation period, the animals were blocked by weight (range, 150 to 250 kg) into dietary treatment groups. Rectal fecal samples were collected from steers on both diets at 4, 8, 12, and 16 weeks, using sterile gauntlet gloves. After collection, the fresh fecal samples were transported to the lab, and feces from six animals (N = 200 g/animal) were collected, with little soil contamination, and were compiled for each experiment.

In subsequent studies to determine the potential for forage phenolic acids to decrease the viability of E. coli O157:H7 in bovine feces, fresh feces were collected from cattle feedlot pens located at MARC. The pens have packed dirt floors with concrete aprons extending from the feed trough, where most fecal samples were collected. The beef steers (approximately 245 to 455 kg, or 9 to 10 months of age) were being fed either corn silage (MARC feedlot growing diet; 87.5% corn silage and 12.5% cracked corn [base diet]) or cracked corn (MARC feedlot finishing diet; 69% cracked corn and 31% corn silage [base diet]). The corn silage and cracked-corn base diets were supplemented, as fed, with 2.5% or 4.5% complete liquid supplement to provide additional dietary nitrogen (as urea), vitamin A, vitamin E, calcium, phosphorus, and salt. Feces were collected on four occasions over an 8-week period (June to August) from representative pens. A minimum of eight undisturbed fecal samples (~2 kg of feces total) were collected, with little soil contamination, and were compiled for each experiment.

**Bacterial cultures.** Escherichia coli O157:H7 Str+ strains 43985 and MARC S-1 were maintained throughout the experimental periods on tryptic soy agar plates supplemented with 250 μg/ml streptomycin. Prior to inoculation, colonies were picked and grown overnight at 37°C in tryptic soy broth supplemented with 250 μg/ml streptomycin. The cultures were then removed from the incubator and allowed to sit at room temperature for at least 24 h to acclimate to laboratory temperatures.

**Fecal inoculations and incubations.** The fecal samples were analyzed prior to inoculation with the laboratory strains and did not contain indigenous Str+ bacteria at the lowest dilution. To determine the survival of E. coli O157:H7 in feces from cattle fed the growing diets, laboratory-acclimated cultures of E. coli O157:H7 Str+ strains 43985 and MARC S-1 were diluted in buffered peptone water to yield approximately 10^8 cells for each culture in a 2-ml cocktail, as described above with E. coli O157:H7 strains, and treated with phenolic acid compounds common to forages. Trans-cinnamic, para-coumaric, and ferulic acids were added individually as dry compounds to 200 g of inoculated feces at 0.1 or 0.5% of the wet fecal weight in separate plastic bags. Each bag with amended inoculated feces was mixed well by hand massage for 10 min. The fecal samples were then placed into deep culture dishes and maintained loosely covered in the laboratory at room temperature throughout the experiment. Incubations for each diet treatment were performed in duplicate.

To determine if phenolic acid compounds common to forage plants have an antimicrobial capacity, fecal composites were collected from pen-housed animals consuming either a corn silage or cracked-corn diet, inoculated (as described above) with E. coli O157:H7 strains, and treated with phenolic acid compounds common to forages. Trans-cinnamic, para-coumaric, and ferulic acids were added individually as dry compounds to 200 g of inoculated feces at 0.1 or 0.5% of the wet fecal weight in separate plastic bags. Each bag with amended inoculated feces was mixed well by hand massage for 10 min. The fecal samples were then placed into deep culture dishes and maintained loosely covered in the laboratory at room temperature throughout the experiment. Fecal samples were collected on four occasions for either the corn silage or corn diets, and incubations for each treatment were performed in duplicate.

**Viable cell counts.** Viable cell counts were determined by counting the CFU of streptomycin-resistant bacterial cells plated onto MacConkey sorbitol agar containing 250 μg/ml streptomycin. The CFU were determined at time zero with freshly inoculated feces and at the same relative time each assay day thereafter by serial dilution of a 1-g fecal sample. Aliquots (100 μl) of the appropriate dilutions were spread onto individual plates. Colonies were counted after the plates were incubated overnight at 37°C. Colonies were visually typical of E. coli O157:H7 and were confirmed to be E. coli O157 by agglutination using anti-O157 antibodies (Escherichia coli O157 test kit; Oxiid Ltd., Hampshire, England) if questionable. The inoculated feces were assayed until no cells were recovered by serial dilution (10^-1) for at least two consecutive assays. In cases where rapid death was observed, no viable cells were found for up to 14 days after death. The viable counts were transformed to log_{10} equivalents for analysis. The detection limit was 1.5 log_{10} CFU g per g feces.

**Chemicals and analyses.** Chemicals and antimicrobials were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Bacterial growth media and buffers were Difco brand (Becton Dickinson Company, Sparks, MD). The fecal pH was determined with 0.5 g of freshly composited feces suspended in 2.5 ml of distilled water.

**Statistics.** All incubations were performed in duplicate for each fecal collection or treatment, and the coefficients of variation for duplicate pairs were ~10%. Means and standard errors of the means are reported for all treatment effects. The statistical significance of treatment means were determined using Student's t test, and linear regressions were fit using least-square means by KaleidaGraph for the Macintosh, version 3.5x (Synergy Software, Reading, PA).

**RESULTS**

**Dietary effects.** Viable E. coli O157:H7 Str+ cells were recovered at >10^5 CFU per g feces directly following inoculation into feces from cattle fed either bromegrass hay or corn silage for 4 weeks. These viable counts declined with time in the feces but decreased faster in feces from animals fed bromegrass hay (bromegrass hay feces) (Fig. 1a). Viable E. coli O157:H7 cells were no longer detected after 28 days in the bromegrass hay feces, whereas the corn silage feces sustained viable counts for 42 days (data not shown).

Feces from animals on each diet for 8, 12, and 16 weeks were also collected and inoculated with E. coli O157:H7 Str+ cells. When animals were fed the same diets for 8 weeks (Fig. 1b), the viable counts in bromegrass hay feces decreased slowly (Fig. 1b) and were detected longer (over 100 days) than those in the corn silage feces (45 days). Similar results were observed in feces from animals consuming the diets for 12 and 16 weeks (data not shown). The fecal pH was always lower in feces from animals fed corn silage than in those from animals fed bromegrass hay (6.36 versus 7.31, respectively) and was not significantly affected by the time on the diet (data not shown).

The time-dependent decreases in viable cells were nearly
linear when expressed in log_{10} form \( (R^2 > 0.7). \) The slopes of the lines represent death rates for \( E. coli\) O157:H7 (log_{10} CFU/g feces/day) and are shown in Fig. 2. The death rate was 0.12 log_{10} CFU/g feces/day in feces from animals consuming bromegrass hay for only 4 weeks. In fecal samples from animals consuming bromegrass hay for 8, 12, and 16 weeks, the \( E. coli\) O157:H7 death rates were not significantly different from each other (average rate, 0.036 log_{10} CFU/g feces/day; \( P > 0.5 \)) but were significantly lower (\( P < 0.01 \)) than the \( E. coli\) O157:H7 death rate in feces collected on week 4. The \( E. coli\) O157:H7 death rates in feces from animals consuming corn silage were not significantly affected by the time on the diet (4, 8, 12, or 16 weeks), and the average death rate was 0.074 log_{10} CFU/g feces/day, a value significantly different from the observed rates for bromegrass hay feces (\( P < 0.05 \)).

Forage phenolic acid treatments. In experiments with inoculated feces from animals fed corn silage, the loss in viable \( E. coli\) O157:H7 Str^+ cells in untreated feces (no phenolic acid addition) was similar to the previous results (Fig. 3 versus Fig. 1), and viable cells were recovered for at least 40 days. The loss of viable \( E. coli\) O157:H7 cells was greater in the presence of 0.5% trans-cinnamic acid or 0.5% para-coumaric acid, and no viable cells were detected after approximately 4 days (Fig. 3). The \( E. coli\) O157:H7 death rates were determined from the linear decreases in viable counts over time, and 0.5% trans-cinnamic and para-coumaric acids increased the \( E. coli\) O157:H7 death rate 13-fold (\( P < 0.01 \)) and 8-fold (\( P < 0.01 \)), respectively (Fig. 4). A lower level (0.1%) of trans-cinnamic or para-coumaric acid or ferulic acid at 0.5% was not as effective, and these amendments did not significantly increase (\( P > 0.1 \)) the \( E. coli\) O157:H7 death rate (fourfold, less than twofold, and less than twofold, respectively) in experiments with corn silage feces. The addition of trans-cinnamic, para-coumaric, or ferulic acid at 0.1 or 0.5% to bovine feces did not affect the fecal pH in any of the treatments (data not shown).

Viable \( E. coli\) O157:H7 cells in inoculated, untreated feces from animals fed cracked corn were not recovered after 20 days and decreased in number faster than cells in corn silage feces (Fig. 5 versus Fig. 3). In the presence of 0.5% trans-cinnamic or 0.5% para-coumaric acid, the decrease in viable \( E. coli\) O157:H7 cells in feces from animals fed cracked corn was rapid, and in the presence of 0.5% trans-cinnamic acid, no viable cells were observed after 2 days (Fig. 5). The \( E. coli\) O157:H7 death rates were determined from the decreases in linear viable counts over time, and the addition of 0.5% trans-cinnamic acid and 0.5% para-coumaric acid increased the death rate 13-fold (\( P < 0.01 \)) and 8-fold (\( P < 0.01 \)), respectively, compared to that in inoculated, untreated feces (Fig. 6).
The _E. coli_ O157:H7 viable counts decreased with a lower level (0.1%) of *trans*-cinnamic or *para*-coumaric acid, and the apparent death rates were significantly higher (threefold \( P < 0.01 \) and sevenfold \( P < 0.01 \), respectively) than that in inoculated, untreated feces. The addition of 0.5% ferulic acid also significantly increased the _E. coli_ O157:H7 death rate nearly threefold \( (P < 0.01) \) compared to that in inoculated, untreated feces. The addition of *trans*-cinnamic, *para*-coumaric, or ferulic acid at 0.1 or 0.5% to bovine feces did not affect the fecal pH in any of the treatments (data not shown).

**Fecal pH and _E. coli_ O157:H7 viability.** The fecal pH was lowest with the cracked-corn diet and highest with the brome-grass hay diet (data not shown). Lower fecal pHs were associated with higher death rates, but overall the fecal pH had a minimal effect on the death of _E. coli_ O157:H7 cells in inoculated, untreated feces (Fig. 7). In treatments with phenolic acids, the initial fecal pH affected the antimicrobial activities of *trans*-cinnamic and ferulic acids. The effect of fecal pH was greatest with 0.5% *trans*-cinnamic acid (Fig. 7), suggesting that the bactericidal effect of *trans*-cinnamic acid on _E. coli_ O157:H7 is influenced by the fecal pH. Similar, albeit weaker, death rate/pH relationships were observed with 0.1% *trans*-cinnamic acid and 0.5% ferulic acid additions, whereas no apparent death rate/pH relationship was observed with 0.1 or 0.5% *para*-coumaric acid (data not shown).

**DISCUSSION**

_Escherichia coli_ O157:H7 is a zoonotic pathogen commonly found in the feces of farm animals, in particular ruminant animals (14). Ruminants are grazing animals, but in confinement they are fed a variety of diets, such as hays (dried grass or legumes), corn silage (chopped and ensiled corn plants), and/or grain (corn or barley). In experiments with confined cattle fed bromegrass hay or corn silage, we not only observed significant differences among diets with regard to the death rates of _E. coli_ O157:H7 in the feces from these animals, but we also saw differences among times on the diets with feces from cattle fed hay. When animals were fed corn silage, the death rates of _E. coli_ O157:H7 in feces were similar regardless of the time on the diet (up to 16 weeks) (Fig. 2). However, in feces from cattle fed bromegrass hay, _E. coli_ O157:H7 death rates were associated with the time on the diet. In feces from animals consuming bromegrass hay for <1 month, the death rate was nearly fourfold higher than the observed death rate in feces when animals were on the bromegrass hay diet for >1 month.

The feeding of hay (timothy-grass hay) was proposed early as a dietary intervention to possibly control _E. coli_ O157:H7 shedding (7). Reductions in acid-resistant _E. coli_ levels in feces were noted when animals were fed hay for <2 weeks, but no specific mechanism to explain these effects has been reported (3). Other reports did not observe a benefit in feeding hays...
eral microorganisms capable of partially degrading these phenolic acids in the feces. Nevertheless, these compounds still had significant antimicrobial capabilities in the feces.

*Escherichia coli* O157:H7 is generally recognized as a problem in feces from concentrated animal operations where animals are fed low-forage/high-energy diets. In our work, the death of *E. coli* O157:H7 was fastest in the feces of animals fed a high-corn diet (Fig. 4 and Fig. 6), and this effect seemed to be associated with a lower fecal pH (Fig. 7). Nonetheless, viable counts were recovered from the feces of animals on the diets for appreciably long periods of time (>20 days), and this would be a problem for manure management. Considering that *E. coli* O157:H7 can persist as a contaminant in soil and on crops for months after manure application or irrigation (16, 17, 27), the reduction or elimination of *E. coli* O157:H7 in production animal systems would be ideal.

To our knowledge, the plant phenolic acids have not been associated with decreased pathogen survival in feces. In our initial study with bovine diets and the viability of *E. coli* O157:H7 in the feces, we did not anticipate that the viability would be affected by the time on the diet, and as a consequence, we did not sample these feces for phenolic acids. Recognizing the differences with the time on the diet, the adaptation of ruminants to forages, and the ability of plant phenolic acids to affect pathogens in vitro studies (13, 20, 28, 39) led us to analyze the effects of phenolic acids in bovine feces. The fact that we observed significant effects with concentrations as low as 0.1% of the phenolic acids, an amount less than that found in many forages, suggests a potential role for controlling pathogens in the gastrointestinal system.

The treatment of stored animal manures with plant essential oils has decreased the amounts of pathogens (37), and our work suggests that phenolic acids may also be useful for similar treatments. In addition, extrapolation of our work with bovine feces to the gastrointestinal system suggests that supplementation of plant phenolic acids to the diets of feedlot animals may be an exploitable intervention. However, more research needs to be done to determine the feasibility of this approach. Recent works by Duncan et al. (9, 10) have demonstrated the potential of dietary esculin and esculitin, which are plant coumarin compounds, to decrease viable *E. coli* O157:H7 in rumen fluid and colonic contents. The study of plant phenolic acid metabolism in ruminants has been limited to forage-adapted animals (6, 25), and limited information is available about their metabolism in animals fed feedlot diets. Future studies will need to determine the stability of phenolic acids in animals fed grain and then determine their dietary potential to reduce pathogen shedding from feedlot cattle.

**ACKNOWLEDGMENTS**

We acknowledge the secretarial assistance of Jackie Byrkit and the technical assistance of Dee Kucera, Cindy Felber, and Jane Long. We also acknowledge Calvin Ferrell for allowing access to animals from the feed efficiency study.

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