Journal of Food Protection, Vol. 74, No. 11, 2011, Pages 1917–1921
doi:10.4315/0362-028X.JFP-11-234

Research Note

Escherichia coli O157:H7 Populations in Ruminants Can Be Reduced by Orange Peel Product Feeding†

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MS 11-234: Received 12 May 2011/Accepted 7 July 2011

ABSTRACT

Foodborne pathogenic bacteria such as *Escherichia coli* O157:H7 are threats to the safety of beef. Citrus peel and dried orange pulp are by-products from citrus juice production that have natural antimicrobial effects and are often incorporated into least-cost ration formulations for beef and dairy cattle. This study was designed to determine if orange peel and pulp affected *E. coli* O157:H7 populations in vivo. Sheep ($n = 24$) were fed a cracked corn grain–based diet that was supplemented with a 50-50 mixture of dried orange pellet and fresh orange peel to achieve a final concentration (dry matter basis, wt/wt) of 0, 5, or 10% pelleted orange peel (OP) for 10 days. Sheep were artificially inoculated with 10^{10} CFU of *E. coli* O157:H7 by oral dosing. Fecal shedding of *E. coli* O157:H7 was measured daily for 5 days after inoculation, after which all animals were humanely euthanized. At 96 h postinoculation, *E. coli* O157:H7 shedding was reduced ($P < 0.05$) in sheep fed 10% OP. Populations of inoculated *E. coli* O157:H7 were reduced by OP treatment throughout the gastrointestinal tract; however, this reduction reached significant levels in the rumen ($P < 0.05$) of sheep fed 10% OP diets. Cecal and rectal populations of *E. coli* O157:H7 were reduced ($P < 0.05$) by inclusion of both 5 and 10% OP diets. Our results demonstrate that orange peel products can be used as a preharvest intervention strategy as part of an integrated pathogen reduction scheme.

In spite of the numerous hurdles that have been implemented throughout the food production chain to ensure food safety, foodborne illnesses still affect more than 48 million Americans each year *(27)*. One of the most significant foodborne pathogenic bacteria is *Escherichia coli* O157:H7, which is typically associated with beef due to its natural reservoir in the gastrointestinal tract of cattle and other ruminant animals *(6, 11)*. Each year, *E. coli* O157:H7 and other related enterohemorrhagic *E. coli* strains cause 93,000 human illnesses at a cost to the U.S. economy of more than US$1 billion per year *(27, 28)*. Since *E. coli* O157:H7 is an asymptomatic resident of the gastrointestinal tract of cattle, reducing *E. coli* O157:H7 populations in the live animal can have a significant impact with regard to improving human health and safety *(7, 24, 26)*.

Phytochemicals, such as essential oils, are naturally occurring antimicrobials found in many plants that show promise in a variety of applications by decreasing bacterial growth and survival *(15, 16, 30)*. The addition of >1% orange peel and pulp to mixed ruminal fluid fermentations reduced populations of *E. coli* O157:H7 and *Salmonella* Typhimurium *(9, 22)*. Further studies have demonstrated that feeding orange peel and pulp reduced intestinal populations of diarrheagenic *E. coli* in weaned swine *(12)* and of *Salmonella* in experimentally infected sheep *(8)*. Orange peel and pulp are by-product feedstuffs commonly fed to cattle and have a good nutritive value for ruminants *(2)*. Therefore, the present study was designed to determine if using fresh and preserved orange peel and pulp as a dietary component could reduce gastrointestinal populations of *E. coli* O157:H7 in an experimentally inoculated sheep model.

MATERIALS AND METHODS

Bacterial cultures. *E. coli* O157:H7 strain 43895, taken from the Food and Feed Safety Research Unit culture collection, was repeatedly grown by 10% (vol/vol) transfer in anoxic (85% N_2, 10% CO_2, 5% H_2 atmosphere) tryptic soy broth (TSB) medium at 37°C. This strain was selected for resistance to novobiocin and nalidixic acid (20 and 25 μg/ml, respectively) by repeated transfer and selection in the presence of sublethal concentrations of each antibiotic. This resistant phenotype was stable through multiple unselected transfers in batch culture and through repeated culture

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† Proprietary or brand names are necessary to report factually on available data; however, the U.S. Department of Agriculture 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.

vessel turnovers in continuous culture (data not shown). Overnight cultures (1 liter) were harvested by centrifugation (7,500 × *g*, 10 min), and cell pellets were resuspended in TSB medium (total volume, 150 ml). Populations of *E. coli* O157:H7 in these cell suspensions were determined to be approximately 3×10^9 CFU/ml, by serial dilution and plating as described below.

Sheep, rations, and experimental design. All procedures in this study were approved by the Institutional Animal Care and Use Committee (IACUC protocol 05-001). Rambouillet/Suffolk sheep (average body weight, 60 kg) were purchased from a commercial feedlot and were transported to the laboratory. Sheep were fed a commercial high-grain ration composed of (on a dry matter [DM] basis) cracked corn (74.4%), soybean meal (9.2%), urea (0.7%), and trace mineral salts (0.4%) and supplemented with coastal Bermuda grass hay (15.3%). The diet was formulated according to National Research Council recommendations, and sheep were allowed ad libitum access to water.

Sheep were housed in environmentally controlled facilities, and feces from each sheep were sampled on arrival and each subsequent day ($n = 7$) during the dietary and facility adaptation period to verify that no organisms capable of growth on MacConkey's agar plates supplemented with novobiocin (20 μg/ml) and nalidixic acid (25 μg/ml) (Mac_{NN}) were present in the sheep. During this period no colonies grew on any of the Mac_{NN} plates.

Sheep ($n = 24$) were randomly assigned to one of three treatment groups ($n = 8$ in each group) that were fed diets supplemented with a 50-50 (DM basis, wt/wt) mixture of dried orange pellet and fresh orange peel to obtain final concentrations (DM basis, wt/wt replacement of concentrate portion of the ration) of 0, 5, or 10% orange product (OP; Texas Citrus Exchange, Mission, TX). Sheep were housed in enclosed facilities and were maintained on their respective diets for 10 days prior to the initiation of the study and throughout the entire study. Sheep were artificially inoculated with *E. coli* O157:H7 (3×10^{10} CFU per sheep) via oral gavage (total volume per sheep, 10 ml) at 0 h. Every 24 h for 96 h after inoculation, fecal samples were collected via rectal grab from individual sheep, and populations of inoculated *E. coli* O157:H7 were determined.

Gastrointestinal sample collection. Sheep were humanely euthanized at 96 h after the *E. coli* O157:H7 inoculation. Contents and tissues from the rumen, distal end of the cecum, and the terminal rectum next to the anal sphincter were aseptically collected upon necropsy. Digesta were serially diluted and plated as described below for quantitative enumeration of inoculated *E. coli* O157:H7 populations. Tissues and digesta samples were enriched for inoculated *E. coli* O157:H7 via a two-step process described below for detection. Gastrointestinal content pH was determined immediately upon return to the laboratory by a Corning 430 pH meter equipped with a calomel pH meter (Acton, MA). Intestinal contents were analyzed for volatile fatty acid concentrations *(13)*.

Bacterial enumeration. Ruminal, cecal, and rectal contents (10 to 20 g) as well as excreted feces were serially diluted (10-fold increments) in phosphate-buffered saline (pH 6.8) and directly plated on Mac_{NN} plates. Colonies that grew on agar plates after 24 h of incubation were directly counted (quantitative enumeration with a lower limit of detection of 10 CFU/ml). To qualitatively confirm the presence of inoculated *E. coli* O157:H7, intestinal contents and epithelial tissue samples as well as feces were enriched in GN Hajna broth at 39°C for 24 h with final plating on Mac_{NN} plates. Plates that contained typical *E. coli* O157:H7 colonies after 24 h of incubation were classified as positive for experimentally introduced *E. coli* O157:H7. Random colonies were picked during the course of the study and examined via dilution and spotting onto O157 identity sticks (Neogen Corp., Lansing, MI) to verify that the colonies growing on the Mac_{NN} plates were indeed *E. coli* O157:H7.

In vitro survival of inoculated *E. coli* O157:H7 in feces. Feces from sheep (per treatment, $n = 4$; total, $n = 12$) fed the three levels of orange peel (0, 10, and 20%) were collected prior to *E. coli* O157:H7 inoculation, homogenized, and diluted (33%, wt/vol) into an anaerobic growth buffer that contained 1 g of glucose per liter *(14)*. Fecal fermentations were incubated separately in sealed, crimped Balch tubes that were inoculated with 10^6 CFU of *E. coli* O157:H7 per g and were incubated at 39°C for 24 h. Fecal populations were determined by serial dilution and direct plating on Mac_{NN} at 24 h after inoculation.

Reagents and supplies. Unless otherwise noted, all media and agars were from Difco Laboratories, BD, Sparks, MD. Reagents and antibiotics were obtained from Sigma Chemical Co., St. Louis, MO.

Statistics. *E. coli* O157:H7 CFU per gram values were log transformed. Treatment groups were compared at each time point by the Mixed procedure of SAS (SAS Institute Inc., Cary, NC). The experimental unit was the individual sheep. Time × treatment interactions were discounted due to the natural decay of *E. coli* O157:H7 populations in this artificially inoculated model; therefore, only within-time comparisons were performed for rumen, cecal, and rectal samples. Significance was determined at P values of <0.05.

RESULTS

In this study, sheep in the 5 and 10% OP groups consumed all of the OP fed daily. No colonies grew from GN Hajna broth–enriched feces on the Mac_{NN} plates prior to the inoculation of the sheep with *E. coli* O157:H7; sheep never showed any clinical signs of infection following inoculation, based on observation. Populations of *E. coli* O157:H7 in the feces of sheep fed 10% dried orange pulp were reduced ($P < 0.05$) compared with controls and with those fed 5% OP 48 h after inoculation and were reduced ($P < 0.05$) in 5 and 10% OP compared with controls at 72 h (Fig. 1).

Ruminal *E. coli* O157:H7 populations at necropsy 96 h after inoculation were reduced ($P < 0.05$) by approximately 1 log CFU/g by 10% OP treatment (Fig. 2). Cecal and rectal populations of *E. coli* O157:H7 were decreased ($P < 0.05$) by both 5 and 10% OP feeding compared with controls (Fig. 2). Intestinal pH and volatile fatty acid concentrations were not different ($P > 0.05$) in sheep fed OP (data not shown).

When fecal fermentations from the sheep fed 0, 5, or 10% OP were inoculated with *E. coli* O157:H7 in vitro, increasing the levels of orange pulp inclusion in the diet reduced survival of *E. coli* O157:H7 in fecal in vitro fermentations (Fig. 3). Populations of *E. coli* O157:H7 declined in the fecal fermentations, likely due to low nutrient availability over the 24 h of incubation. Popula-

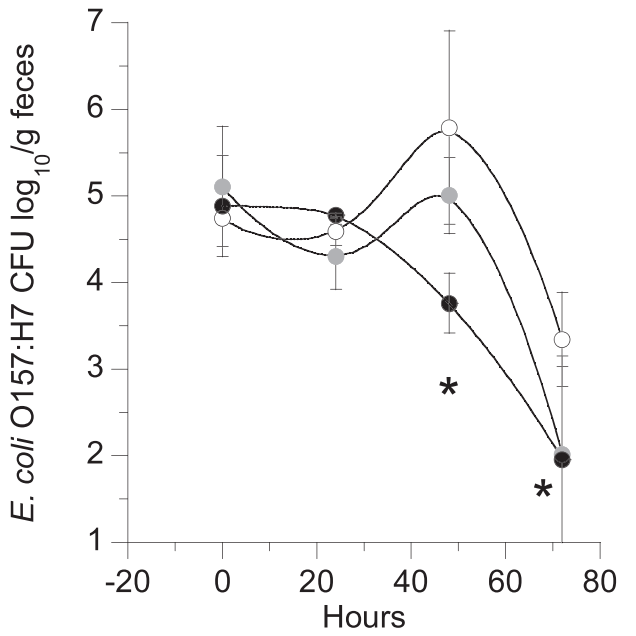

FIGURE 1. *Fecal populations (in log CFU per gram of feces) of* E. coli *O157:H7 in sheep fed 0, 5, or 10% OP. Open circles represent 0% OP, grey circles 5% OP, and black circles 10% OP. Error bars indicate standard deviations, and asterisks denote significant differences (*$P < 0.05$*) within each time point.*

tions of *E. coli* O157:H7 were approximately 1 and 1.3 log CFU/g lower in sheep fed 5 and 10% ($P < 0.05$) OP after 24 h of incubation, respectively, compared with control populations.

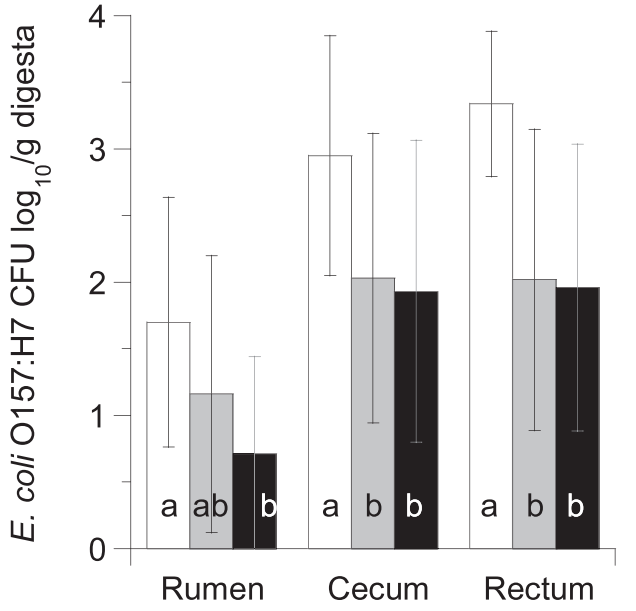

FIGURE 2. *Intestinal populations (in log CFU per gram of feces) of* E. coli *O157:H7 in sheep fed 0, 5, or 10% OP at 96 h after experimental inoculation. White bars represent 0% OP, grey bars 5% OP, and black bars 10% OP. Error bars indicate standard deviations. Different letters indicate statistical differences (*$P < 0.05$*) within a location.*

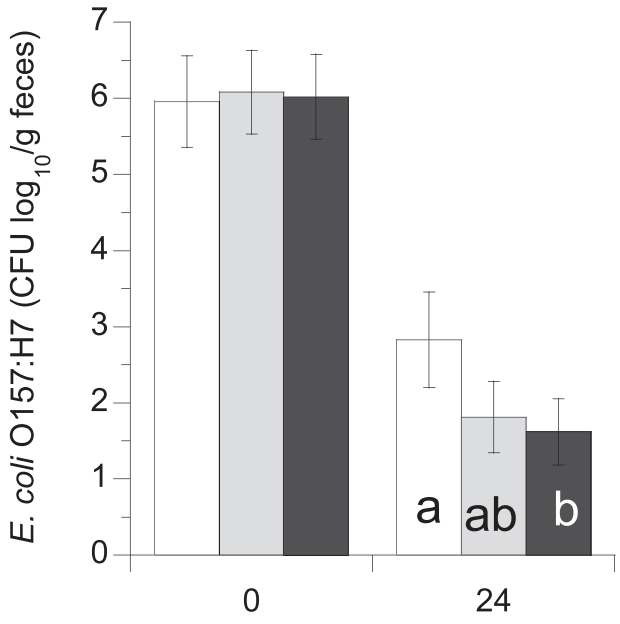

FIGURE 3. E. coli *O157:H7 populations (in log CFU per gram of feces) at inoculation (0 h) and after 24 h of in vitro fermentation of feces from sheep fed 0, 5, or 10% OP diets. White bars represent 0% OP, grey bars 5% OP, and black bars 10% OP. Error bars indicate standard deviations. Different letters indicate statistical differences (*$P < 0.05$*) compared with controls.*

DISCUSSION

One of the largest food safety threats facing the cattle industry is the foodborne pathogenic bacteria *E. coli* O157:H7 and other enterohemorrhagic *E. coli* strains *(19, 20)*. Because this critical pathogen enters the food supply via live animals, many intervention strategies have been implemented in the processing plant *(5, 21)* at a cost of more than $2 billion. In addition to being spread via contaminated meat products, *E. coli* O157:H7 on the farm and feedlot can be spread by direct contact to farm workers and visitors and carried by water runoff to nearby water supplies or irrigated crops *(1, 10)*. Therefore, researchers and producers have increased their focus on the development of preharvest intervention strategies that reduce pathogenic bacteria in live food animals *(25, 26, 29)*. Lowering the *E. coli* O157:H7 burden entering the processing facility would enhance the effectiveness of current and future in-plant interventions, thereby reducing human foodborne illnesses.

Citrus fruits contain a variety of compounds, including essential oils that exert antimicrobial activity and can alter the microbial ecology of the gastrointestinal tract *(18, 23, 30)*. Citrus peel and pulp have been included as low-cost ration ingredients at levels of 5 to 16% in dairy and beef cattle rations for many years *(2)*, and these products have a good nutritive value for ruminants (6.9% crude protein; total digestible nutrients, 82%; net energy for maintenance, 1.9 Mcal/kg; net energy for gain, 1.3 Mcal/kg). Research has indicated that citrus product feedstuffs can reduce foodborne pathogenic bacterial populations in both pure and mixed ruminal fluid cultures in vitro *(9, 22)*. Populations of *Salmonella* were reduced by >2 log CFU/g by inclusion of

up to 2% orange peel and pulp in the mixed ruminal microorganism fermentations *(9)*. In studies using swine as a gastrointestinal model, OP inclusion reduced ileal and cecal populations and completely eliminated rectal populations of the diarrheagenic *E. coli* strain Fl8 *(12)*.

Populations of *Salmonella* in sheep fed 10% OP were significantly reduced in the cecum and numerically reduced in the rectum; however, in that particular study, it was found that feeding above 10% OP resulted in reduced intake due to palatability issues *(8)*. In the present study, the inclusion of 5 and 10% OP decreased populations of *E. coli* O157:H7 in the cecum and rectum more than 1 log CFU/g and reduced ruminal populations in animals fed 10% OP. These results suggest that even during a relatively short exposure to orange peel products, foodborne pathogen populations can be reduced. It must be noted that in this study, the animals were being fed OP prior to pathogen challenge, and therefore, the effects of OP feeding on animals already colonized with *E. coli* O157:H7 are still unclear.

Survival of *E. coli* O157:H7 that was inoculated into the feces in in vitro fermentations was only statistically significant in feces from sheep fed 10% OP compared with control sheep, suggesting that the antibacterial component of OP passes through the intestinal tract. In previous studies, the limonene and terpeneless fractions were found to exert the antipathogenic activities *(22),* but in the present study the antimicrobial compounds were not identified. Results indicate that the feces of animals fed orange peel products can exhibit antipathogen activity, possibly reducing some of the hide contamination during shipping and lairage that is associated with contamination of carcasses and meat products *(3, 4, 17)*.

Orange peel and pulp do present some palatability issues that preclude its use as a sole ration ingredient. Our present results suggest that feeding citrus products as part of a diet can reduce carriage of *E. coli* O157:H7 in the intestinal tract of ruminants. It is important, however, to note that the present study utilized an experimentally inoculated animal model to demonstrate the efficacy of a citrus peel feeding treatment within a relatively narrow window of *E. coli* O157:H7 colonization. Following our inoculation of sheep with *E. coli* O157:H7, intestinal populations declined in a manner that is not identical to the colonization of the intestinal tract. Furthermore, the populations inoculated into these sheep (10^{10} CFU in total) are much higher than levels normally found in the gastrointestinal tract; therefore, products like citrus pulp and peel may have a greater impact in naturally infected animals.

On the basis of these data, we conclude that the inclusion of orange peel and pulp in ruminant rations is a viable strategy to reduce *E. coli* O157:H7 in the gastrointestinal tract before harvest. If we are to implement this pathogen intervention in the human food chain, further research must be performed to determine the most efficacious dosing strategies and levels of orange peel product feeding. Orange peel and pulp feeding will not prevent all human foodborne illnesses; however, including citrus oils and products in the diet can be utilized as a part of an integrated, multihurdle system aimed at reducing the passage of foodborne pathogens from farm to human consumers.

ACKNOWLEDGMENTS

Portions of this research were supported by the U.S. Department of Agriculture and by beef and veal producers and importers through their $1-per-head checkoff; this research project was produced for the Cattlemen's Beef Board and state beef councils by the National Cattlemen's Beef Association.

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