Citrus Products Decrease Growth of *E. coli* O157:H7 and *Salmonella* Typhimurium in Pure Culture and in Fermentation with Mixed Ruminal Microorganisms *In Vitro*


Abstract

Orange peel and orange pulp are by-products that are included in feedlot and dairy cattle diets because of their low cost and high nutritional quality. The antimicrobial activity of citrus oils has been reported previously. The present study was carried out to determine whether these citrus by-products exert antimicrobial effects on *Escherichia coli* O157:H7 and *Salmonella* Typhimurium populations that are found in cattle gastrointestinal tracts. The growth of pure cultures (*n* = 3) of *E. coli* O157:H7 and *Salmonella* Typhimurium were reduced (*p* < 0.05) by addition of 2% (w/v) orange pulp and orange peel. Ruminal fluid was collected from cattle (*n* = 2) and *E. coli* O157:H7 or *Salmonella* Typhimurium were added. The addition of orange pulp and peel to *in vitro* mixed ruminal microorganism fermentations (*n* = 3) demonstrated that both orange pulp and peel reduced *E. coli* O157:H7 and *Salmonella* Typhimurium populations at least 2 log₁₀ in mixed ruminal fluid fermentations. Addition of orange pulp reduced (*p* < 0.05) *E. coli* O157:H7 populations from 10⁵ to 10² colony-forming units (CFU)/mL and *Salmonella* Typhimurium populations (*p* < 0.05) from 10⁴ to 10² CFU/mL. These results indicate that orange pulp and/or peel included in ruminant diets could decrease ruminal populations of foodborne pathogenic bacteria. Further research is needed to determine whether the antimicrobial activity of orange products against *E. coli* O157:H7 or *Salmonella* Typhimurium is expressed in the lower gastrointestinal tract.

Introduction

Anually, enterohemorrhagic *Escherichia coli* (EHEC; such as *E. coli* O157:H7) and *Salmonella* spp. infections cause more than 1.4 million illnesses at a cost to the U.S. economy of more than $3.4 billion (ERS/USDA, 2001). In recent years, several intervention strategies have been implemented in cattle packing plants (e.g., organic acid rinses, steam treatment) to decrease bacterial contamination on carcasses (Elder et al., 2000; Barkocy-Gallagher et al., 2003). Despite these effective interventions, foodborne bacterial illnesses linked to meat products and large-scale product recalls still occur far too frequently.

Much of the carcass and meat product contamination is a result of the carriage of foodborne pathogenic bacteria in the intestinal tract of cattle (Losinger et al., 1997; Low et al., 2005).
Foodborne pathogenic bacteria can be introduced into the abattoir in the feces of cattle or attached to their hide (Elder et al., 2000; Reid et al., 2002; Aslam et al., 2003; Barkocy-Gallagher et al., 2003), providing a direct route into the food chain. Additionally, illnesses caused by foodborne pathogenic bacteria have been carried to humans by other animal vectors, water, and direct animal contact (Anonymous, 2000; Pritchard et al., 2000). Thus, as a logical measure to decrease human illnesses, several strategies have been investigated that decrease foodborne pathogen carriage in cattle and other food animal species before slaughter (Callaway et al., 2004; Loneragan and Brashears, 2005; Doyle and Erickson, 2006; Sargeant et al., 2007).

Many plants demonstrate some antimicrobial activity that can alter the microbial ecology of the ruminal or intestinal population, and some of these are included in cattle diets as by-product feeds in least-cost formulations (Dorman and Deans, 2000; Hristov et al., 2001; Nam et al., 2006). Oranges and other citrus products contain essential oils (e.g., limonene, linalool) that are toxic to bacteria (Kim et al., 1995; Fisher and Phillips, 2006) and exhibit antioxidant effects in host animals (Dusan et al., 2006; Deyhim et al., 2007). Orange peel and dried orange pulp are a by-product of orange juice production that has a relatively high nutritive value and is available at low prices in citrus-producing regions (e.g., Florida and California). Because this by-product has natural antimicrobial effects, it has been proposed that this low-cost feed ingredient could decrease pathogenic bacterial populations in food animals. In the present study we examined the effects of raw orange peel and dried orange pulp against *E. coli* O157:H7 strains and *Salmonella* Typhimurium populations in vitro in pure and in mixed rumen bacterial culture to determine whether these feedstuffs could be included in animal diets to decrease populations of pathogens in live animals.

**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 linked to ground beef consumption, and the *Salmonella* Typhimurium used in this study was originally isolated from cattle; both were obtained from the Food and Feed Safety Research Unit (U.S. Department of Agriculture-Agricultural Research Service [USDA/ARS], College Station, TX) culture collection. Both *E. coli* O157:H7 strain 933 and *Salmonella* Typhimurium were naturally resistant to 25 μg/mL novobiocin (NO) and were made resistant to 20 μg/mL of nalidixic acid (NA).

**Ruminal fluid collection**

Ruminal contents were collected by hand from the ventral sac of three ruminally cannulated Holstein cows (*n* = 2/diet group). Cattle were maintained in accordance with a protocol approved by the Southern Plains Agricultural Research Center Animal Care and Use Committee (ACUC No 06002). The ruminal contents were collected from all cattle at approximately the same time (between 0800 and 0900 hours). 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 minutes at 39°C to allow gas production to buoy large particles to the top of the flasks. Microscopic examination of diluted ruminal fluid revealed the presence of few feed particles or protozoa, and an abundance of bacteria with different morphological shapes. Fresh ruminal fluid contained approximately 10^11 cells/mL of total culturable anaerobes.

**Cattle diets**

Cattle were provided *ad libitum* access to water and minerals in all studies. Ruminal fluid was collected from cattle fed 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 group was adapted to a high-grain diet (Table 1) in a step-wise fashion (five steps over a 21-day period) and was maintained on this feedlot diet for 10 days prior to ruminal fluid collection.

**In vitro mixed ruminal microbial fermentations**

Incubations of ruminal fluid were performed by combining the ruminal fluid (1:3) 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, 4000 mg Na₂CO₃, 600 mg cysteine hydrochloride (Cotta and Russell, 1982). Approximately 10⁴ colony-forming units [CFU]/mL E. coli O157:H7 strain 933 or Salmonella Typhimurium was added to the buffered ruminal fluid fermentations in experiments using pasture-fed cattle, and 10⁵ CFU/mL of each pathogen was used in fermentations that used grain-fed cattle ruminal fluid. The resultant suspensions were transferred to 18×150 mm Balch tubes (Belco Glass, Vineland, NJ; 10 mL per tube) containing 0.2 g of ground hay (ground to pass a 1-mm Wiley mill screen) or commercial starch in the pasture- and grain-fed studies, respectively.

Orange peel was chopped using a hand chopper to be approximately 2 mm³ in size, dried orange pulp was included as fed, which was primarily comprised of pieces approximately 2 mm³. Orange peel or dried pulp were added to each tube to reach final concentrations of 0%, 0.125%, 0.25%, 0.5%, 1%, or 2% w/v. Tubes were then sealed using rubber stoppers with aluminum crimps and incubated for 24 hours at 39°C under a N₂, CO₂, H₂ (90:5:5 v/v) gas phase. Samples removed after 24 hours of incubation were centrifuged (10,000×g, 5 minutes, 24°C) to remove particulate matter. Supernatant fluids and cell pellets were separated and supernatants were acidified and frozen for later volatile fatty acid analysis. Final pH was measured in remaining ruminal fluid using an Orion 2 Star meter (Thermo Scientific, Waltham, MA).

**Pure culture studies**

*Escherichia coli* O157:H7 strain 933 (ATCC 43895) or *Salmonella Typhimurium* were anaerobically (90% N₂, 5% H₂, 5% CO₂ atmosphere) incubated at 39°C in anoxic tryptic soy broth (cooled after autoclaving under anoxic conditions [90% N₂, 5% H₂, 5% CO₂]; Difco Laboratories, Detroit, MI) for 24 hours. Dried orange pulp was added to each tube to reach final concentrations of 0%, 0.125%, 0.25%, 0.5%, 1%, or 2% w/v.

**Bacterial enumeration.** Samples were taken from all *in vitro* fermentations at 24 hours to determine the effect of citrus products on populations of E. coli O157:H7 and *Salmonella Typhimurium*. Samples were serially diluted (in 10-fold increments) in phosphate-buffered saline (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 *Salmonella 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. Student t-test was used to determine significance of differences between means of each treatment.

**Results**

The addition of both orange peel and dried orange pulp decreased (p < 0.05) populations of *E. coli* O157:H7 and *Salmonella Typhimurium* in our pasture-fed *in vitro* ruminal fluid fermentations (Fig. 1). Populations were significantly decreased by citrus product additions of ≥ 1%. Because dried pulp showed largely similar (or greater) effects against pathogens than did fresh chopped peel, the remaining studies focused on the use of the prepared feedstuff of dried orange pulp in order to more closely approximate what occurs in animal diets.

In the *in vitro* fermentations using ruminal fluid from cattle fed a high-grain diet, *E. coli* O157:H7 populations were decreased (p < 0.05)
FIG. 1. Effect of increasing concentrations (w/v) of orange peel or orange pulp on populations of *E. coli* O157:H7 strain 933 and *Salmonella* Typhimurium grown in mixed ruminal bacteria fermentations from cows fed a pasture diet. *E. coli* O157:H7 strain 933 cultures are represented by circles (○, ○), and *Salmonella* Typhimurium cultures are indicated by triangles (△, △). Fermentations that included orange pulp are indicated by open symbols (○, △), and fermentations that included orange peel are depicted by closed symbols (●, ▲). Error bars indicate standard deviations.

by dried orange pulp concentrations ≥0.05% (w/v). Similarly, *Salmonella* Typhimurium populations were decreased (*p* < 0.05) by dried orange pulp concentrations ≥1% (w/v) (Fig. 2). Volatile fatty acid concentrations and profiles and final pH were not significantly changed by addition of up to 2% w/v of dried orange pulp, indicating that feeding orange pulp or peel would not likely have a negative impact on ruminal fermentation (data not shown). While *E. coli* O157:H7 and *Salmonella* Typhimurium were added at the same initial concentration (10⁴ CFU/mL in pasture-fed cattle, and 10⁵ CFU/mL in grain-fed cattle) to ruminal fluid fermentations, *Salmonella* Typhimurium populations were always less (approximately 1 log₁₀ CFU/mL) than *E. coli* O157:H7 populations given the same treatments (Figs. 1 and 2). In pure culture studies, dried orange pulp concentrations decreased (*p* < 0.05) populations of *E. coli* by concentrations ≥1%, *Salmonella* Typhimurium were decreased (*p* < 0.05) by ≥0.05% (w/v) (Fig. 3).

Discussion

The primary route of infection by *E. coli* O157:H7 in humans is consumption of improperly cooked or handled ground beef (USDA:APHIS, 1997), although serious waterborne outbreaks have occurred (USDHHS, 1999; Anonymous, 2000). Recent (2006) human outbreaks of *E. coli* O157:H7 were transmitted to spinach consumers via swine as intermediate vectors, highlighting the role that intestinal populations of pathogens play in food safety (Jay et al., 2007). *Salmonella* species can be spread
to humans via similar routes from cattle or other food animals. Although slaughter plants do a great job of reducing pathogens on carcasses and meat products (Koohmaraie et al., 2005), too many illnesses associated with meat and environmental exposures occur. To decrease the total exposure of humans to these pathogens via all sources, it is therefore logical to attempt to reduce pathogens on the farm, prior to entry to the food chain. Therefore, non-antibiotic anti-pathogen strategies have been sought.

Cattle, swine, and poultry are fed a wide variety of low-cost by-product feeds in an effort to obtain a least-cost ration. One by-product feed that is widely used in citrus-producing regions is orange peel or dried orange pulp, which can be stored and shipped long distances (Volanis et al., 2006). Dried orange pulp has a relatively high nutritive value (total digestible nutrients = 82%, net energy maintenance = 86 Mcal/cwt; net energy gain = 56 Mcal/cwt) for cattle (NRC, 2000). Orange pulp is typically fed in cattle at levels up to 2–8% of the total diet, depending on cost (relative to cost of other feedstuffs), availability, and palatability (NRC, 2000). Cattle fed at these levels would have a ruminal concentration of orange pulp of approximately 0.5–1% w/v, suggesting that the pathogen-inhibiting effects observed in this study in vitro, could be possible in the rumen, and that as the orange pulp (or essential oil) concentration in the diet is increased, more pathogen inhibition could be gained.

The pathogen-inhibiting effects of orange pulp in the present study appeared to directly affect the pathogens in our study based upon the pure culture inhibition of Salmonella Typhimurium and E. coli O157:H7. Our data further demonstrate that the low-cost by-product feed of orange peels and dried orange pulp exerts effects on the microbial population in a mixed ruminal fluid in vitro fermentation. Populations of two foodborne pathogens known to reside within the rumen were decreased significantly by addition of orange peel and pulp in mixed ruminal fluid incubations from animals fed both a pasture- and grain-based diet. These results indicate that orange pulp can be anti-pathogenic in cattle fed pasture or high-grain diets, and that it likely can be applied in both dairy and beef production systems.

Essential oils (e.g., limonene) are natural antimicrobials that comprise a significant proportion of citrus-based feedstuffs (Matlack, 1940). Essential oils exert their toxic effects at the membrane level (Dusan et al., 2006; Di Pasqua et al., 2007) where they can cause increased permeability of the cell membrane (Gill and Holley, 2006). The most well-characterized of the essential oils from citrus products include citrullene and limonene, which can exert potent antimicrobial activity (Di Pasqua et al., 2006). It has been suggested to use essential oils as feed additives to alter the intestinal fermentation or to decrease pathogens from the intestinal ecosystem (Barnhart et al., 1999; Shin, 2005; Nam et al., 2006). The known antimicrobial activity of the essential oils has been shown to encompass E. coli strains (Dusan et al., 2006), some Salmonella spp. (Kim et al., 1995; Parish et al., 2003; Nam et al., 2006) and other foodborne pathogenic bacteria (Megias et al., 1997; Fisher and Phillips, 2006). Other benefits to animals have been attributed to feeding essential oils because they have been shown to act as oxygen scavengers or antioxidants (Huang et al., 2007). The antioxidant benefits of feeding an orange-pulp diet have been linked to decreased tumorigenesis and increased immune responses in rats (Kossoy et al., 2001). Although the level of orange-pulp in the diet that was anti-tumorigenic is well above those tested in the present study (15% vs. 2%), the antioxidant and immunostimulatory activity is likely to be of benefit to animal producers.

The inclusion of orange pulp or peel into animal diets to decrease pathogenic bacteria is a simple concept that would likely be viewed by consumers as a “green” solution to enhance food safety. In the present study, we have focused on two foodborne pathogens which often reside in the rumens of cattle. Although the most direct route of environmental and carcass contamination is through the feces, the rumen is a reservoir for pathogens that can recolonize the intestinal tract (Rasmussen et al., 1993; Laven et al., 2003). Thus if ruminal populations of E. coli O157:H7 and Salmonella can be reduced in the rumen, then intervention strategies (e.g., phage, chlo- rate, probiotics) that reduce intestinal populations of pathogens have a chance to be more successful due to the reduced re-inoculation of
the lower gut. However, it is unclear from the literature how much, if any, of the dried orange pulp or the active essential citrus oils reaches the lower gut where pathogens are commonly associated with the epithelial tissues. Thus studies that examine the passage of orange pulp and essential oils through the rumen and abomasum of cattle are critical to understand what role these compounds can play in altering pathogen shedding by cattle. Furthermore, the use of specific antimicrobial essential citrus oils targeted for release in the lower gut is an obvious refinement of the simple concept presented in this study that is being currently examined.

Conclusions

Foodborne pathogens can live within the gut of food animals, and non-antibiotic methods to decrease pathogen populations in the live animal are critical to improving food safety. Citrus by-products are often included in animal diets due to their low cost and these contain essential oils that can kill *E. coli* O157:H7 and *Salmonella*. In our *in vitro* mixed ruminal microbial fermentations *E. coli* O157:H7 and *Salmonella* populations were reduced by orange pulp addition, but the end-products of the fermentations were not altered by orange pulp inclusion at levels up to 2% w/v. Based on our data, it appears that orange pulp provides an anti-pathogenic vehicle that is amenable to current cattle production practices. Further research is needed to determine whether the antimicrobial activity of orange products against *E. coli* O157:H7 or *Salmonella* Typhimurium continues in the lower gastrointestinal tract and in monogastric animals.

Acknowledgments

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 no approval of the product, or exclusion of others that may be suitable.

References

ORANGE PULP DECREASES PATHOGENS


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