Escherichia coli O157:H7 Populations in Sheep Can Be Reduced by Chlorate Supplementation†


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MS 02-134: Received 23 April 2002/Accepted 18 August 2002

ABSTRACT

Ruminant animals are a natural reservoir of the foodborne pathogen Escherichia coli O157:H7. Some foodborne pathogens (e.g., E. coli) are equipped with a nitrate reductase that cometabolically reduces chloride. The intracellular reduction of chloride to chlorite is found in the gastrointestinal tracts of both monogastric and ruminant animals (15); however, E. coli O157:H7 can cause severe hemorrhagic colitis in humans (29). Ruminant animals, such as cattle, sheep, and goats, are a natural reservoir of this pathogenic bacterium (21, 27, 31), and recent evidence has shown that approximately 28% of all cattle in the United States (17) and approximately 20% of feedlot cattle in Europe are carriers of E. coli O157:H7 (35). Several human outbreaks of hemorrhagic colitis have been linked to E. coli O157:H7 as ‘‘feedlot’’ or ‘‘barbecue’’ disease (28).

Some bacteria can respire anaerobically by reducing nitrate to nitrite via the intracellular enzyme nitrate reductase (1). However, nitrate reductase cometabolically reduces chloride to chlorite, a cytotoxic end product (32). Nitrate reductase–equipped bacteria (e.g., E. coli, Salmonella) die when exposed to chlorite (2, 4, 6, 9, 32). Chlorite supplementation in cattle was shown to significantly reduce experimentally inoculated E. coli O157:H7 populations, E. coli populations, and total coliform populations in the rumen, ileum, cecum, colon, and rectum (10). On the basis of these results, it was suggested that chloride supplementation could be an effective strategy to reduce E. coli O157:H7 populations in ruminants prior to harvest (3). The present study was designed to examine the effect of an experimental sodium chlorate product (XCP, EKA Chemicals, Marietta, Ga.) on gastrointestinal populations of E. coli O157:H7, as well as those of generic E. coli, total coliforms, and total culturable anaerobic bacteria, in ruminants.

MATERIALS AND METHODS

Sheep rations and experimental design. All procedures in this study were approved by the Institutional Animal Care and Use Committee (IACUC protocol 01-002). Fourteen Suffolk sheep (with an average body weight of 45 kg) were adapted in a stepwise fashion to a high-grain ration composed of (dry matter basis) 74.4% cracked corn, 9.2% soybean meal, 0.7% urea, 0.4% trace mineral salts, and 15.3% coastal bermudagrass hay. This diet was formulated according to National Research Council recommendations (30), and sheep were allowed ad libitum access to water. Seven sheep were randomly assigned to each treatment group (control or XCP-treated). Sheep were housed in environmentally controlled facilities and were screened for the presence of E. coli capable of growth on antibiotic-supplemented agar prior to experimental infection with E. coli O157:H7.

Bacterial cultures. E. coli O157:H7 strain BDMS T4169 (ATCC 700728) was obtained from the American Type Culture Collection (Manassas, Va.) and was repeatedly grown by 10% (vol/vol) transfer in anoxic (85% N₂, 10% CO₂, 5% H₂ atmosphere) tryptic soy broth (TSB) at 37°C. This strain was resistant to novobiocin and nalidixic acid (at 20 and 25 μg/ml, respective-
ly). This resistant phenotype was stable through multiple unselected transfers in batch culture and through repeated culture vessel turnovers in continuous culture (data not shown). Overnight cultures (1,000 ml) were harvested by centrifugation at 7,500 × g for 10 min, and cell pellets were resuspended in TSB (150-ml total volume). Each sheep was inoculated with E. coli O157:H7 (3 × 10^9 CFU) via oral gavage (10-ml total volume). Fecal samples were collected via rectal grab 6 h after inoculation and subsequently at 12-h intervals. Fecal and intestinal populations of inoculated E. coli O157:H7, as well as those of generic E. coli and total coliforms, were enumerated as described below.

**Chlorate treatment.** Feed was provided to animals at 95% of their ad libitum intake for 3 days prior to inoculation and for 72 h following inoculation. At 48 h after inoculation, a control (containing the equivalent of 2.5 mM KNO_3_ and 100 mM NaCl) or XCP treatment (containing the equivalent of 2.5 mM KNO_3_ and 100 mM NaClO_3_) was provided in the drinking water for 24 h. Feed and water were withdrawn from sheep 72 h after inoculation to simulate transport to slaughter facilities.

**Gastrointestinal sample collection.** Sheep were humanely euthanatized and exsanguinated at 96 h postinoculation. Intestinal contents and epithelial tissues from the rumen, cecum, and rectum were aseptically collected upon necropsy. Samples were diluted as described below for quantitative enumerations of E. coli O157: H7, total E. coli, and total coliforms. Sample aliquots and epithelial tissues were added to TSB for qualitative enrichment of inoculated E. coli O157:H7. The pHs of gastrointestinal contents were determined immediately upon the return to the laboratory with a Corning 430 pH meter equipped with a calomel pH meter (Corning, Acton, Mass.).

**Bacterial enumeration.** Feces and ruminal, cecal, and rectal contents were serially diluted (in 10-fold increments) in sterile phosphate-buffered saline. Quantitative dilutions were plated on MacConkey agar supplemented with novobiocin (20 μg/ml) and nalidixic acid (25 μg/ml) (for the enumeration of inoculated E. coli O157:H7), MacConkey agar (for the enumeration of total coliforms), and M-Endo agar LES (for the estimation of total E. coli on the basis of colony color [metallic sheen] and morphology); plates were incubated overnight at 37°C. Colonies that grew on agar plates after 24 h of incubation were directly counted (quantitative enumeration). To qualitatively confirm the presence of inoculated E. coli O157:H7, intestinal contents and epithelial tissue samples were incubated overnight (for 12 h) in TSB at 37°C and were streaked on MacConkey agar supplemented with novobiocin and nalidixic acid. Colonies that grew on MacConkey agar supplemented with novobiocin and nalidixic acid after 24 h of incubation tested positive for inoculated E. coli O157:H7 (quantitative enumeration). A nonselective enrichment medium was used to recover E. coli O157:H7 in order to alleviate the potential underestimation of chloride-injured E. coli O157:H7 populations arising from the use of a highly selective medium during quantitative analysis. Intestinal contents were analyzed for pH and volatile fatty acid concentrations as previously described (14).

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

Statistics. Data were compared by Student’s t test by using GraphPad Prism (Version 3.00 for Windows, GraphPad Software, San Diego, Calif.).

![Figure 1](http://example.com/fig1.png)  
**FIGURE 1.** E. coli O157:H7 populations (CFU/g) in feces from sheep (a) and numbers of sheep shedding inoculated E. coli O157:H7 in feces (b). E. coli O157:H7 populations from sheep (n = 7) treated with the equivalent of 100 mM sodium chloride (control) are indicated by open symbols, and populations from sheep that received XCP treatment (the equivalent of 100 mM sodium chloride) (n = 7) are indicated by closed symbols. The commencement of XCP supplementation is indicated by the vertical dashed line. Standard errors are indicated by error bars.

**RESULTS**

Prior to inoculation with E. coli O157:H7, sheep did not contain ruminal or fecal E. coli populations capable of growth on MacConkey agar supplemented with novobiocin and nalidixic acid, and these sheep did not contain Salmonella (data not shown). Following inoculation with E. coli O157:H7, populations of inoculated E. coli O157:H7 ranged from 10^5 to 10^7 CFU/g of feces (Fig. 1a). Populations of E. coli O157:H7 remained stable in ruminal fluid and feces until XCP treatment commenced (at 48 h). Fecal populations of inoculated E. coli O157:H7 in control sheep remained constant (at approximately 10^5 CFU/ml) throughout the study, but XCP treatment significantly reduced fecal populations (P < 0.05; Fig. 1a). Water intake levels were not significantly different between control and XCP groups (4.39 versus 4.8 liters per sheep, respectively). The number of sheep shedding inoculated E. coli O157:H7 in their feces
was sharply reduced after the commencement of XCP treatment (Fig. 1b).

XCP supplementation reduced total coliform (Fig. 2a), wild-type E. coli (Fig. 2b), and E. coli O157:H7 populations (Fig. 2c) throughout the gastrointestinal tract. Total coliforms were significantly ($P < 0.01$) reduced in the rumen, the cecum, and the rectum ($P < 0.01$) in both the cecum and the rectum (Fig. 2b). Inoculated E. coli O157:H7 made up a small proportion of the total E. coli population in the rumen, the cecum, and the rectum (always <10%) (Fig. 2b versus 2c). XCP treatment decreased populations of inoculated E. coli O157:H7 in the rumen ($P < 0.05$), in the cecum ($P < 0.01$), and in the rectum ($P < 0.01$) (Fig. 2c). The number of XCP-treated sheep testing positive for inoculated E. coli O157:H7 in the rumen, in the cecum, and in the rectum upon enrichment was smaller (Fig. 3).

XCP treatment did not significantly impact gastrointestinal pH (Fig. 4a). The fermentation profile throughout the gastrointestinal tract was not significantly affected by XCP treatment, as indicated by total volatile fatty acid production (Fig. 4b) and the acetate/propionate ratio (Fig. 4c).

**DISCUSSION**

Ruminant animals can be asymptomatic carriers of E. coli O157:H7 and other enterohemorrhagic E. coli (8, 13, 31). Many human outbreaks of hemorrhagic colitis have been linked to direct contact with ruminant animals or to products derived from ruminants (19, 33). For many years research indicated that E. coli O157:H7 was found in only approximately 1 to 3% of cattle (20); however, as more sensitive isolation techniques (e.g., immunomagnetic separation) have become more widely used, the accepted incidence rate has steadily increased (8, 11, 17). Dramatic seasonal variation has made the estimation of the prevalence of E. coli O157:H7 in cattle difficult (20), but recent studies have indicated that 18 to 30% of cattle carry the pathogen (17).

As ruminants, sheep can also harbor enterohemorrhagic E. coli (24, 26, 35) and have been used as intestinal models
**FIGURE 4.** Ruminal, cecal, and rectal pHs of sheep (a); ruminal, cecal, and rectal total volatile fatty acid concentrations (b); and acetate/propionate ratios (c). Samples that were taken from sheep treated with the equivalent of 100 mM sodium chloride (control) (n = 7) are indicated by hatched bars, and samples from sheep treated with XCP (the equivalent of 100 mM sodium chlorate) (n = 7) are indicated by filled bars. Standard errors are indicated by error bars.

of colonization and infection (12, 13, 25, 34). Sheep have been shown to be colonized by *E. coli* O157:H7 populations comparable to those for cattle (35). Although fewer human cases of hemorrhagic colitis have been attributed to ovine sources than to bovine sources, sheep still serve as a useful model of *E. coli* O157:H7 colonization of the ruminant gastrointestinal tract (13, 25).

Although postharvest intervention methods have been shown to successfully reduce *E. coli* O157:H7 contamination of carcasses (17), more than 93,000 Americans are affected each year by enterohemorrhagic *E. coli*, resulting in a cost of more than $1 billion per year in the United States (16). With a shift in the focus of intervention strategies from the abattoir to the feedlot or farm, populations of enterohemorrhagic *E. coli* and other pathogens entering the abattoir could be reduced. Strategies that reduce specific foodborne pathogenic bacterial populations in animals prior to slaughter could produce “the most significant reduction in human exposures to the organism and therefore reduction in related illnesses and deaths” (22).

A preharvest intervention strategy that has recently been investigated is the use of sodium chlorate to reduce *E. coli* O157:H7 and *Salmonella* populations in food animals (2, 7). Some intestinal bacteria (e.g., *E. coli* and *Salmonella*) can respire under anaerobic conditions by reducing nitrate to nitrite via a dissimilatory nitrate reductase (23, 32). Chlorate is a valence state analog of nitrate that is cometabolically reduced to chlorite, which accumulates to toxic levels inside the bacterium via a dissimilatory nitrate reductase (32). The addition of chlorate has been shown to significantly reduce *E. coli* O157:H7 populations in ruminal fluid incubations (3, 9). The addition of chlorate directly into the rumen of cattle has been shown to significantly reduce fecal populations of wild-type *E. coli* in cattle (5).

In studies in which swine were used as a simple gastrointestinal tract model, experimentally inoculated *E. coli* O157:H7 and *Salmonella* populations were significantly reduced by chlorate treatment (2, 7).

In an in vivo study involving cattle experimentally inoculated with three different *E. coli* O157:H7 strains, it was observed that chlorate treatment significantly reduced populations of inoculated O157:H7 strains as well as those of total coliforms and wild-type *E. coli* (10). On the basis of these results, it has been suggested that ruminant food animals should be treated with sodium chlorate immediately prior to transport for slaughter in order to reduce *E. coli* O157:H7 populations in their intestinal tracts (3).

Elder et al. (17) demonstrated a direct correlation between fecal populations of *E. coli* O157:H7 and carcass contamination levels. In the present study, XCP treatment significantly reduced inoculated *E. coli* O157:H7, generic *E. coli*, and total coliform populations throughout the intestinal tracts of experimentally infected sheep within 24 h. These results indicate that chlorate treatment immediately prior to slaughter could reduce subsequent carcass contamination and human illnesses by reducing fecal populations of pathogens in live animals.

Not all intestinal bacteria are equipped with nitrate reductase, and nitrate reductase-negative species are chlorate insensitive (1). However, some important intestinal bacterial species (e.g., *Selenomonas, Wolinella*) can reduce nitrate to nitrite and are killed by chlorate. It was previously demonstrated that the addition of chlorate did not alter the gastrointestinal volatile fatty acid profile in cattle (10). Likewise, in the present study, the fermentation profiles and pHs throughout the gastrointestinal tract were not significantly affected by XCP treatment.

*E. coli* is able to become chlorate-resistant under in vitro conditions (32). However, recent research involving mixed cattle fecal incubations has indicated that chlorate-
resistant *E. coli* O157:H7 mutants cannot persist in competition with the native chloride-insensitive intestinal microbial population (9). Further evidence has indicated that chloride-resistant *E. coli* was not isolated from swine treated with chloride in a terminal fashion (24 h prior to slaughter) (9). Therefore, while the possibility of the development of chloride resistance cannot be completely discounted, it appears that a short-term chloride treatment strategy, such as treatment 24 h prior to slaughter (chlorate activity in the gut while the animals are in transit to the abattoir), could mitigate this concern.

Chlorate is toxic to mammals (50% lethal dose \([LD_{50}]\) in rats >1.2 g/kg of body weight) (Material Safety Data Sheets [MSDS], Sigma), but the relative toxicity of chloride is low. The \([LD_{50}]\) for NaCl in rats is 3 g/kg of body weight, and the \([LD_{50}]\) of acetylsalicylic acid (aspirin) is 200 mg/kg of body weight (MSDS, Sigma). The effective dose used in this study was approximately 0.4 g of NaClO per kg of body weight. Chlorate absorbed into the plasma is rapidly eliminated (6-h half-life) and accumulated in tissues at concentrations of <1 ng chlorate per g (18). These data indicate that toxicity issues do not preclude the use of chloride as a feed additive for the reduction of *E. coli* O157:H7 populations in ruminants immediately prior to harvest; however, more research is needed to determine the toxicity of chlorate and the extent of its accumulation in the tissues of ruminant animals.

Although postharvest intervention strategies do effectively reduce carcass contamination in the abattoir, foodborne pathogenic bacteria still enter the food chain. *E. coli* O157:H7 and other enterohemorrhagic *E. coli* are threats to public health and confidence in the safety of the food supply and can be found in the intestinal tracts of food animals prior to slaughter. The results of this study indicate that XCP supplementation reduced *E. coli*, total coliform, and *E. coli* O157:H7 populations in ruminant animals at a potential preharvest critical control point. Chlorate kills bacteria equipped with a nitrate reductase, but the gastrointestinal fermentation profile was not altered by chlorate supplementation. Therefore, it appears that a chlorate product could be used to improve the safety of ruminant-derived foods, but further studies are needed to determine the most effective treatment methods.

REFERENCES

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CHLORATE REDUCES E. COLI O157:H7 POPULATIONS IN SHEEP


