Effect of Nitrate Adaptation on the Bactericidal Activity of an Experimental Chlorate Product Against *Escherichia coli* in Cattle

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ABSTRACT

An experimental chlorate product (ECP) developed by the United States Department of Agriculture has been shown to have bactericidal effects against enteropathogens such as *Escherichia coli*. In studies with broilers and pigs, the bactericidal activity of ECP was enhanced by prior adaptation of gastrointestinal microflora to nitrate. The objective of this study was to examine the effects of nitrate adaptation on the bactericidal activity of ECP against *E coli* in Holstein steers. Results indicate that ECP was effective at reducing *E coli*. However, ECP did not reduce *E coli* in a dose-dependent manner, indicating that the highest ECP dose provided in this study exceeded that needed to be efficacious. Adapting gastrointestinal microflora with nitrate prior to feeding ECP did not improve efficacy of ECP against *E coli.* Rapid reduction of nitrate in the rumen is implicated as a possible explanation for why adaptation to nitrate did not enhance the bactericidal effects of ECP in cattle.

INTRODUCTION

Escherichia coli O157:H7 is a food-borne pathogen that causes hemorrhagic colitis, hemolytic uremic syndrome, and thrombo-

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cytopenic purpura in humans.[1] The gastrointestinal tract of beef cattle is a known reservoir of *E coli* O157:H7.[2,3] Elder et al.[4] reported contamination prevalence of 11% to 28% of cattle feces and 11% of cattle hides. Despite post-harvest efforts to reduce contamination of meat products by the meat-packing industry, food-borne illnesses due to *E coli* O157:H7 still occur in the United States (1.73 cases per 100,000 persons in 2002[5]). These occurrences have been estimated to cost the US beef industry $2.7 billion since the *E coli* O157:H7 outbreak at a Jack in the Box restaurant in 1993.[6] Consequently, pre-harvest interventions are needed to complement the industry's multi-hurdle approach to pathogen control, particularly since quantitative risk assessments indicate that such interventions may reduce human exposure to this pathogen.[7] An experimental chlorate product (ECP) that targets *E coli*'s respiratory nitrate reductase enzyme, which also reduces chlorate to cytotoxic chlorite, has shown promising results in reducing fecal *E coli* O157:H7 in ruminants.[8,9] Because the enzyme is induced by nitrate, we conducted a study to see if a short-term, low-level nitrate adaptation period would enhance expression of the enzyme and thus its *E coli* killing activity in cattle.

MATERIALS AND METHODS

Experimental Animals and Treatments

Holstein steers, averaging 210 ± 5.09 kg, were trained to use Calan gate feeders and adapted to a basal diet which consisted of 66%, 15%, and 19% corn, alfalfa hay, and supplement, respectively, for 21 days. Following this adaptation, a 5-day experimental period was initiated to examine the bactericidal effects of ECP against generic *E coli*, with and without prior adaptation to nitrate. Steers were blocked by body weight (BW) and randomly assigned (within block) to 1 of 6 treatments in a 2 x 3 factorial arrangement that included three ECP doses (0 g, 1.2 g, or 2.3 g chlorate ion equivalents/kg diet), with or without nitrate adaptation (0.95 g nitrate ion equivalents/kg diet). The nitrate level, at nearly 0.1% of the diet, is about one-tenth the level generally considered to be safe for healthy cattle.[10] The nitrate product containing 0% or 11% nitrate ion equivalents was fed for 3 days immediately preceding feeding of the ECP, which was formulated with an inert carrier to contain 0%, 12.5%, or 25% chlorate ion equivalents (all 3 ECP products also contained 4% nitrate ion equivalents). The ECP treatments were fed 1 day prior to termination of the study. All steers were fed twice daily, at 0800 h and at 1600 h throughout the 28-day study. (The proprietary nitrate and ECP preparations were provided by EKA Chemicals, Inc., Marietta, GA, USA.)

Fecal Samples

Freshly voided fecal samples collected prior to and after the 3-day nitrate administration period and 1 day following ECP administration were cultured quantitatively for generic *E coli* via plating of serial 10-fold dilutions to *E coli*/coliform Petrifilm (3M Inc., Minneapolis, MN, USA) and assayed for respiratory nitrate reductase activity via anaerobic incubation (24 h at 39°C under 100% CO_2) of freshly collected fecal contents in anaerobic buffer[11] containing 10 mM added sodium nitrate and 60 mM sodium formate.

Hide Swabs

All cattle were swabbed on the left rump just below the pelvic bone (an approximately 100-cm^2 area) while restrained in a squeeze chute prior to nitrate administration and 1 day following administration of the ECP. Swabs were collected using Speci-Sponges (Nasco, Fort Atkinson, WI, USA) hydrated with 20 mL Butterfield's buffer and cultured for generic *E coli* as described above.

Statistical Analysis

Feed intake, average daily gain (ADG), feed efficiency, nitrate reductase activity, and log transformations of fecal and hide swab *E coli* concentrations were analyzed using the Mixed procedure of SAS (SAS Institute, Cary, NC, USA) with nitrate adaptation (0 g or 0.95 g nitrate ion equivalents/kg diet), ECP (0 g, 1.2 g, or 2.3 g chlorate ion equivalents/kg diet),

Table 1. Generic *E coli* Concentration of Feces Following Nitrate Adaptation Period, but Prior to Administration of Experimental Chlorate Product (ECP)

ECP dose[a]	*E coli* Concentration ± SE (log_{10} colony forming units (CFU)/g feces)	
	No nitrate adaptation	Nitrate adaptation
0.0 g	5.59 ± 0.29[d]	5.18 ± 0.29[bcd]
1.2 g	4.58 ± 0.31[b]	4.74 ± 0.29[bc]
2.3 g	5.25 ± 0.29[bcd]	5.49 ± 0.29[cd]
Nitrate effect	$P = 0.99$	
ECP effect	$P = 0.03$	
Interaction	$P = 0.49$	

[a]Grams of chlorate ion equivalents/kg of diet.
[bcd]Values not sharing common superscripts differ ($P < 0.05$).

Table 2. The Effects of Nitrate Adaptation on the Bactericidal Activity of an Experimental Chlorate Product (ECP) Fed 1 Day Prior to Study Termination

ECP dose[a]	*E coli* Concentration ± SE (log_{10} colony forming units (CFU)/g feces)	
	No nitrate adaptation	Nitrate adaptation
0.0 g	5.33 ± 0.43[d]	4.98 ± 0.43[d]
1.2 g	2.57 ± 0.46[bc]	2.89 ± 0.43[bc]
2.3 g	1.98 ± 0.43[b]	3.20 ± 0.43[c]
Nitrate effect	$P = 0.23$	
ECP effect	$P < 0.001$	
Interaction	$P = 0.14$	

[a]Grams of chlorate ion equivalents/kg of diet.
[bcd]Values not sharing common superscripts differ ($P < 0.05$).

and the 2-way interaction as fixed effects and weight block as a random effect.

RESULTS

One steer died early in the study prior to administration of nitrate or ECP. The interaction between nitrate adaptation and ECP was not significant ($P > 0.10$) for any of the variables evaluated. Fecal and hide swab *E coli* concentrations were similar ($P > 0.30$) among cattle from all treatment groups when sampled prior to the nitrate adaptation. Supplementation of ECP did yield a significant ($P < 0.05$) reduction in fecal *E coli* concentrations, but the reduction was not in a dose-dependent manner. *Escherichia coli* concentrations 1 day following ECP supplementation were 5.15 log_{10} colony forming units (CFU)/g feces, 2.73 log_{10} CFU/g feces, and 2.59 log_{10} CFU/g feces for cattle receiving 0.0 g, 1.2 g, and 2.3 g ECP, respectively. The lack of a dose-dependent response could be attributed to the fact that *E coli* concentration of the feces sampled after nitrate adaptation, but before ECP administration, was lower ($P < 0.05$) in cattle assigned to the 1.2-g ECP supplementation than in cattle assigned to 0.0 g or 2.3 g ECP supplementation (Table 1). Nitrate adaptation had no effect ($P > 0.20$) on fecal *E coli* concentration on any of the sampling days (Table 2). However, *E coli* concentration in hide swab samples taken 1 day following ECP supplementation (2.54 log_{10} CFU/g) were higher ($P < 0.05$) in cattle fed nitrate for 3 days than in control animals not fed nitrate (1.91 log_{10} CFU/g). Average daily gain, dry-matter intake, and gain:feed ratio during the 28-day study were similar ($P > 0.50$) among the 6 treatments, which averaged 1.28 ± 0.10 kg/d, 6.36 ± 0.30 kg/d, and 0.20 ± 0.02 kg gain/kg feed, respectively. Moreover, nitrate adaptation and ECP supplementation did not alter ($P > 0.50$) feed intake on the nitrate adaptation or ECP feeding days.

DISCUSSION

In agreement with earlier studies,[8,9,12] the ECP treatment effectively reduced fecal *E coli* concentrations within 24 h of feeding. However, in contrast to earlier studies with broilers and pigs,[13,14] the bactericidal effect of ECP treatment was not enhanced by nitrate adaptation. The most likely explanation for the lack of an effect of nitrate adaptation on the killing of *E coli* by ECP was that the nitrate was rapidly reduced by bacteria within the rumen, thus precluding its delivery to the lower gut. In support of this, fecal nitrate reductase activity measured after the nitrate adaptation period, but before feeding of ECP, was not affected ($P > 0.05$) by nitrate adaptation, with mean ±

standard error (SE) rates of enzymatic nitrate reduction being essentially the same for control animals fed no nitrate and animals fed the nitrate-supplemented diets (0.08 ± 0.02 vs 0.09 ± 0.03 μmol nitrate/g fecal contents per hour, respectively). These rates were lower than rates measured before feeding the nitrate supplemented diet (0.20 ± 0.01 μmol nitrate/g).

Reductions in fecal *E coli* concentrations were not statistically different between those animals that were fed diets supplemented with either 1.2 g or 2.3 g chlorate ion equivalents per kilogram of diet, suggesting that the lower ECP treatment level was near or above the minimum effective dose needed to achieve optimum pathogen control. Results from the present experiment provide additional evidence that ECP can be practically, easily, and safely fed to cattle the day prior to harvest in order to reduce *E coli* concentrations in feces. However, unlike results observed in studies with poultry and swine, low-level nitrate adaptation in this study did not enhance the bactericidal activity of ECP against *E coli*. It is possible that chlorate by itself may have been sufficient to induce respiratory nitrate reductase activity by *E coli*, thus making addition of nitrate to the diet redundant. Alternatively, however, rapid reduction of nitrate in the rumen may have prevented delivery of nitrate to the lower gut. The rumen ecosystem has long been known to be able to reduce nitrate, and considering that this activity can be markedly increased via gradual adaptation to higher nitrate levels,[15] it is questionable whether simply adding more nitrate to the diet may allow sufficient quantities to reach the lower gut. Moreover, since the ECP product was quite efficacious by itself, it may not be worth having to adapt ruminants to high—possibly approaching toxic—levels, concentrations of nitrate likely needed to escape rumen degradation. Consequently, the preferred methodology to achieve the marked enhancement in the *E coli*- and *Salmonella*-killing activity of chlorate may be to supplement cattle diets with other innocuous inducers that are more resistant to degradation in the rumen and thus able to achieve the desired enhancement of the respiratory nitrate reductase activity in the lower gut. Potential candidates are presently being tested in the laboratory and initial results showed that these inducers enhanced the bactericidal activity of chlorate as good or even more strongly then nitrate.[14] A potential advantage to using these alternative inducers is that some also inhibit ruminal methane production, which represents an efficiency in ruminal gross energy utilization.[16] A potential disadvantage of the inducers is that they may require extensive regulatory review and thus may not be as near to market as the ECP product. For instance, while evidence suggests that these inducers are not used as substrates by the nitrate reductase enzyme, it is unknown whether they are absorbed or not within the rumen. Thus, further research with these compounds will reveal whether they may persist long enough to reach the lower gut to enhance the bactericidal activity of chlorate.

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REFERENCES

1. Park S, Worobo RW, Durst RA. *Escherichia coli* 0157: H7 as an emerging food borne pathogen: A literature review. *Crit Rev Food Sci Nutr.* 1999;39:481–502.

2. Rasmussen MA, Casey TA. Environmental and food safety aspects of *Escherichia coli* O157:H7 infections in cattle. *Crit Rev Microbiol.* 2001;27:57–73.

3. Bach SJ, McAllister TA, Veira DM, Gannon VPJ, Holley RA. Transmission and control of *Escherichia coli* O157:H7: a review. *Can J Anim Sci.* 2002;82:475–490.

4. Elder RO, Keen JE, Siragusa GR, Barkocy-Gallagher GA, Koohmaraie M, Laegreid WW. Correlation of enterohemorrhagic *Escherichia coli* 0157 prevalence in feces, hides, and carcasses of beef cattle during processing. *Proc Natl Acad Sci USA.* 2000;97:2999–3003.

5. US Department of Health and Human Services-Centers for Disease Control and Prevention (USDHHS-CDC). Preliminary FoodNet data on

the incidence of food borne illnesses: selected sites, United States, 2002. *Morb Mort Weekly Rep*. 2003;52:340–343.

6. Kay S. The cost of *E coli* O157:H7, $2.7 billion. *Meat & Poultry*. 2003;(February):26–34.
7. Hynes NA, Wachsmuth K. Escherichia coli *O157:H7 Risk Assessment in Ground Beef: a Public Health Tool.* 4th International Symposium and Workshop on Shiga Toxin (Verocytotoxin)-Producing *Escherichia coli* Infections, Kyoto, Japan, October 29–November 2, 2000; p. 46.
8. Callaway TR, Anderson RC, Genovese KJ, et al. Sodium chlorate supplementation reduces *E coli* O157:H7 populations in cattle. *J Anim Sci.* 2002;80:1683–1689.
9. Edrington TS, Callaway TR, Anderson RC, et al. Reduction of *E coli* O157:H7 populations in sheep by supplementation of an experimental sodium chlorate product. *Small Rumin Res.* 2003;49:173–181.
10. Provin TL, Pitt JL. *Forage Nitrates and Prussic Acid in Forages: Sampling, Testing and Management Strategies.* L-5433 College Station, TX: Texas Cooperative Extension, Texas A&M University, January 2003. (Texas Documents Z TA265.7 L47 No. 5433.)
11. Bryant MP, Burkey LA. Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. *J Dairy Sci.* 1953;36:205–217.
12. Anderson RC, Callaway TR, Anderson TJ, Kubena LF, Keith NK, Nisbet DJ. Bactericidal effect of sodium chlorate on *Escherichia coli* concentrations in bovine ruminal and fecal contents in vivo. *Microbiol Ecol Dis.* 2002;14:24–29.
13. Jung YS, Anderson RC, Byrd JA, et al. Reduction of *Salmonella typhimurium in* experimentally challenged broilers by nitrate adaptation and chlorate supplementation in drinking water. *J Food Prot.* 2003;66:660–663.
14. Anderson RC, Jung YS, Genovese KJ, et al. *Effect of Select Nitrocompounds Against Salmonella in Porcine Fecal Suspensions.* Presented at the Proceedings of the 18th Congress of the International Pig Veterinary Society, Hamburg, Germany, 2004.
15. Allison MJ, Reddy CA. Adaptations of gastrointestinal bacteria in response to changes in dietary oxalate and nitrate. In: Klyg MJ, Reddy CA, eds. *Current Perspectives in Microbial Ecology. Proceedings of the 3rd International Symposium of Microbial Ecology,* American Society for Microbiology, Washington, DC, 1984.
16. Anderson RC, Callaway TR, Van Kessel JS, Jung YS, Edrington TS, Nisbet DJ. Effect of select nitrocompounds on ruminal fermentation; an initial look at their potential to reduce economic and environmental costs associated with ruminal methanogenesis. *Bioresource Technol.* 2003;90:59–63.