

Highly recommended books on Probiotics and Foodborne Pathogens

- **Foodborne Pathogens: Microbiology and Molecular Biology**

Edited by: Pina M. Fratamico, Arun K. Bhunia and James L. Smith

2005, ISBN 978-1-904455-00-4 [click here](#)

- **Probiotics and Prebiotics: Scientific Aspects**

Edited by: Gerald W. Tannock

2005, ISBN 978-1-904455-01-1 [click here](#)

- **Probiotics and Prebiotics: Where are We Going?**

Edited by: Gerald W. Tannock

2002, ISBN 978-0-9542464-1-9 [click here](#)

- **Probiotics: A Critical Review**

Edited by: Gerald W. Tannock

1999, ISBN 978-1-898486-15-2 [click here](#)

Effects of Sodium Chlorate on Toxin Production by *Escherichia coli* O157:H7

T. R. Callaway, R. C. Anderson, T. S. Edrington,
Y. S. Jung, K. M. Bischoff, K. J. Genovese, T. L. Poole,
R. B. Harvey, J. A. Byrd and D. J. Nisbet.

Feed and Food Safety Research Unit, Southern Plains
Agricultural Research Center, 2881 F & B Rd., College
Station, TX 77845, USA

Abstract

Chlorate kills *E. coli* O157:H7 and has been proposed as a feed additive to be included in cattle rations immediately prior to slaughter to reduce *E. coli* O157:H7 populations in the gut. Antibiotic usage is not recommended in cases of *E. coli* O157:H7-induced hemorrhagic colitis because some antibiotics stimulate increased toxin production. This study was undertaken to determine if chlorate treatment affected toxin production. Pure cultures of *E. coli* O157:H7 were treated with 1/4 MIC of antibiotics (ampicillin, tetracycline, ceftiofur, gentamicin, monensin, tylosin, penicillin, ciprofloxacin, and novobiocin); toxin production was significantly increased by some antibiotics, but not by chlorate. Studies with mixed fecal bacteria demonstrated that chlorate killed *E. coli* O157:H7, but again did not stimulate toxin production. Chlorate appears to be an effective method to reduce shiga toxin-producing *E. coli* (STEC) populations in food animals, but additional studies are warranted before it is used to control infections.

Introduction

Escherichia coli O157:H7 is a foodborne pathogenic bacteria that causes severe haemorrhagic colitis (bloody diarrhea) in humans, particularly in children and the elderly (Mead *et al.*, 1999). *Escherichia coli* (and other enterobacteria such as *Salmonella*) can respire under anaerobic conditions via a dissimilatory nitrate reductase that reduces nitrate to nitrite (Stewart, 1988). Intracellular nitrate reductase co-metabolically reduces chlorate to chlorite, which accumulates within the cell, killing the bacterium (Stewart, 1988; Stouthamer, 1969). Chlorate has been used successfully to reduce *E. coli* O157:H7 and *Salmonella* populations in cattle, sheep and swine (Anderson *et al.*, 2000b; Callaway *et al.*, 2002; Edrington *et al.*, 2003).

"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, and exclusion of others that may be suitable."

*For correspondence. Email callaway@ffsru.tamu.edu.

The severe haemorrhagic colitis caused by *E. coli* O157:H7 infections is catalyzed by the production of a potent shiga toxin similar that produced by *Shigella* (Law, 2000). Patients suffering from *E. coli* O157:H7 infections are generally not treated with antibiotics because some antibiotics stimulate shiga toxin release by *E. coli* O157:H7 (Grif *et al.*, 1998; Walterspiel *et al.*, 1992; Yoh *et al.*, 1997). Increased toxin production increases the risk of detrimental, or even fatal complications (e.g., hemolytic uremic syndrome [HUS]), therefore the use of antimicrobials in *E. coli* O157:H7 patients has been discouraged, especially in susceptible groups, such as children (Grif *et al.*, 1998). The present study was undertaken to determine if chlorate treatment had any effects upon toxin production by *E. coli* O157:H7 under a variety of environmental conditions.

Results

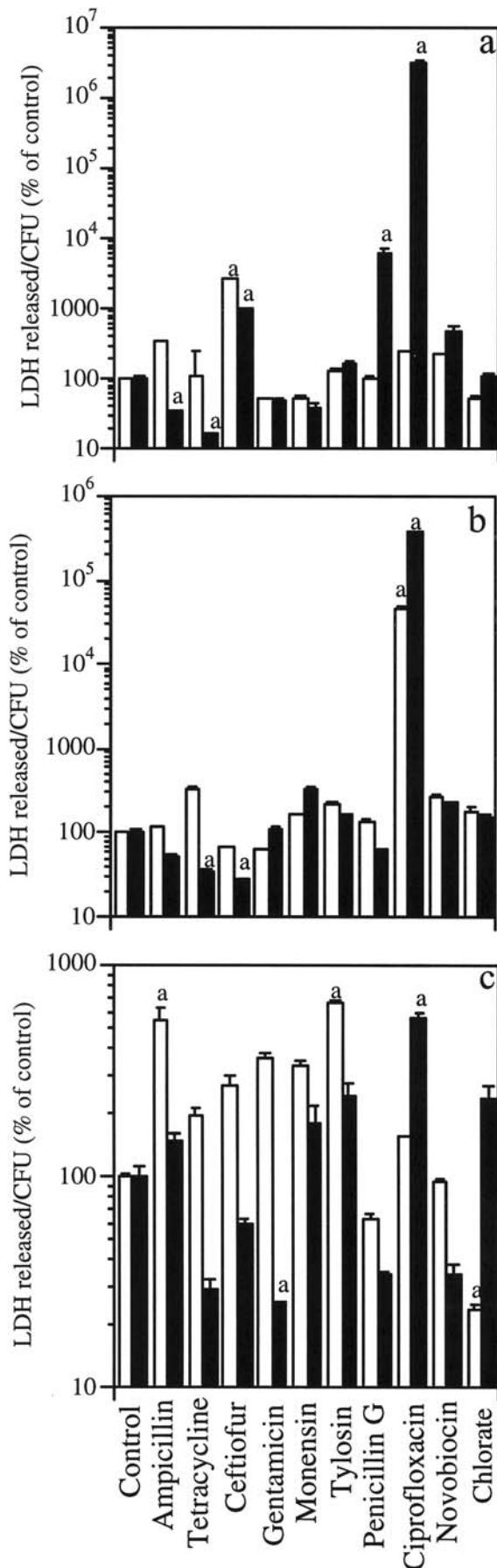
Minimum Inhibitory Concentrations (MIC) for eight medically-important antibiotics were determined via standard methods (NCCLS, 1999). Sub-lethal antibiotic concentrations (1/4 MIC) used to stimulate antibiotic effects on toxin production by *E. coli* O157:H7 strains 933 and 6058 are listed in Table 1. The amount of lactate dehydrogenase (LDH) released from Vero cells treated with culture supernatant containing shiga toxin was proportional to the amount of toxin added (data not shown). Sub-lethal doses (1/4 MIC) of antibiotics and feed additives did not reduce *E. coli* populations more than 10-fold in any culture (from 3×10^8 to 4×10^7 cells/ml), however sodium chlorate treatment reduced *E. coli* O157:H7 CFU/ml approximately 100-fold in mixed fecal cultures (data not shown).

Table 1. Antimicrobial concentrations (1/4 MIC) used to determine effect of sub-lethal doses on toxin production by *E. coli* O157:H7.

	6058	933
Ampicillin	0.5 µg/ml	1 µg/ml
Tetracycline	0.25 µg/ml	1 µg/ml
Ceftiofur	5 µg/ml	5 µg/ml
Gentamicin	0.125 µg/ml	0.125 µg/ml
Penicillin G	8 µg/ml	8 µg/ml
Ciprofloxacin	20 µg/ml	10 µg/ml
Novobiocin	4 µg/ml	4 µg/ml
Chlorate ^a	1.25 mM	1.25 mM
Monensin ^b	10 µM	10 µM
Tylosin ^b	2 µM	2 µM

^aChlorate concentration used is 1/4 that of the most efficacious chlorate dosage.

^bMonensin and tylosin concentrations used are equivalent to estimated concentrations *in vivo*.



When grown in tryptic soy broth (TSB), sub-lethal doses of ceftiofur increased toxin production (expressed as a percentage of untreated control LDH released/CFU *E. coli* O157:H7) significantly ($P < 0.05$) by both strains of *E. coli* O157:H7 (Figure 1a). Penicillin G and ciprofloxacin increased toxin production significantly ($P < 0.05$). However toxin production by strain 6058 was reduced significantly ($P > 0.05$) by ampicillin and tetracycline treatment. Chlorate treatment did not affect shiga toxin concentrations when *E. coli* O157:H7 was grown in TSB.

Ciprofloxacin increased toxin production ($P < 0.05$) when *E. coli* O157:H7 strains 933 and 6058 were grown in sterilized fecal fluid (Figure 1b). Toxin production by strain 6058 was decreased significantly ($P < 0.05$) by both tetracycline and ceftiofur treatment in sterilized fecal fluid.

When cultures were grown in fresh fecal fluid containing 10^{11} MPN/ml total culturable anaerobic bacteria, the effect of sub-lethal antibiotic doses on toxin production was dramatically reduced (Figure 1c). The ruminant feed additive tylosin and ampicillin caused an increase ($P < 0.05$) in toxin production by strain 933. Ciprofloxacin caused a significant increase ($P < 0.05$) in toxin production by strain 6058; conversely, gentamicin caused a significant decrease ($P < 0.05$) in toxin production by strain 6058. Sodium chlorate caused a significant decrease ($P < 0.05$) in toxin production by strain 933.

Discussion

The deleterious effects of *E. coli* O157:H7 infection in humans is due to potent toxins produced by this bacterium (Acheson *et al.*, 1998). These toxins share a large degree of homology with the toxin produced by *Shigella dysenteriae* and are consequently known as shiga-like toxins (or Stx). These toxins are taken up by mammalian epithelial cells where they bind to the 28S component of the 60S ribosomal subunit, resulting in an inhibition of protein synthesis (Acheson *et al.*, 1998). The inhibition in the intestinal epithelium leads to the onset of severe haemorrhagic colitis, a hallmark of *E. coli* O157:H7 infection in humans.

Sub-lethal doses of antibiotics often have effects quite different than do lethal doses; effects that are different in form, not just severity (Lorian, 1980). In previous research, sub-lethal doses of antimicrobials caused decreased, increased, or unchanged toxin production (Grif *et al.*, 1998; Kohler *et al.*, 2000; Yoh *et al.*, 1997). For example, exposure of *E. coli* O157:H7 to sublethal doses of cotrimoxazole or trimethoprim resulted in a 4- and 8-fold increase in toxin production, respectively (Karch *et al.*, 1985). In the current study, ciprofloxacin significantly increased toxin production when cultures were grown in

Figure 1. Toxin production (24 h) by *E. coli* O157:H7 strains 933 and 6058 as determined by lactate dehydrogenase (LDH) release from Vero cells/CFU, presented as a percentage of untreated controls. Cultures were grown in TSB (a), sterilized fecal fluid (b), or fresh fecal fluid (c). Open columns represent strain 933, shaded columns represent strain 6058. Columns marked with a superscript differ from control toxin concentrations ($P < 0.05$). Error bars indicate standard deviations.

TSB, sterilized fecal fluid, and mixed bovine fecal culture; agreeing with results from previous researchers (Grif *et al.*, 1998; Walterspiel *et al.*, 1992).

Antibiotic treatment of patients suffering from *E. coli* O157:H7 has been correlated with an increased risk of negative outcomes (e.g., death, HUS) (Grif *et al.*, 1998). This has been linked to the increased toxin production caused by antibiotic treatment (Butler *et al.*, 1987). This correlation led to the recommendation that antibiotics not be used to treat human patients with haemorrhagic colitis, but this topic remains highly controversial (Neill, 1998).

Monensin is an ionophore that is often included in cattle rations to improve production efficiency; however, monensin is excluded from reaching the cell membrane by the outer membrane of gram-negative species (Ahmed and Booth, 1981). However, recent studies have indicated that certain lipophilic compounds (e.g., monensin, tylosin) may diffuse through the outer membrane and reach the inner membrane of gram-negative bacteria, causing non-growth energy dissipation (Lewis *et al.*, 1994), which could stimulate up-regulation of toxin production by *E. coli* O157:H7. Recent research has indicated that some feed grade antimicrobials may increase phage and toxin release from EHEC, however monensin treatment decreased phage induction and toxin production (Kohler *et al.*, 2000). Our results demonstrated that toxin production by *E. coli* O157:H7 was not stimulated in the current culture conditions by monensin.

The incidence of *E. coli* O157:H7 in live food animals on-farm highlights the need to reduce pathogen concentrations within the animal prior to entering the food chain, potentially reducing human illnesses (Hynes and Wachsmuth, 2000). Our laboratory has developed a strategy that could potentially reduce *E. coli* O157:H7 populations in cattle prior to harvest by specifically targeting an important metabolic pathway of enterobacteria (Anderson *et al.*, 2000a). Sodium chlorate specifically kills bacteria equipped with nitrate reductase, including *E. coli* O157:H7; but does not affect the end products of the ruminal or intestinal fermentations (Callaway *et al.*, 2002). Although cattle lack shiga toxin receptors (Pruimboom-Brees *et al.*, 2000), factors that alter toxin production could affect the competitive fitness of *E. coli* O157:H7 in the gastrointestinal tract; or could cause more severe illnesses in humans infected with this critical pathogen. Our results indicate that sub-lethal doses of chlorate do not affect toxin production *in vitro*. However further research is warranted to determine if the effects of sodium chlorate on *E. coli* O157:H7 on the gastrointestinal microecology.

Experimental Procedures

Cultures and Minimum Inhibitory Concentrations (MIC) determination

Escherichia coli O157:H7 strain 933 (ATCC 43895) and 6058 were cultivated in anoxic Tryptic Soy Broth (TSB) (Difco Laboratories; Detroit, MI) incubated at 39°C. Antibiotic sensitivities (MIC's) of cultures were determined via standard antibiotic sensitivity panels (Texas Veterinary Medical Diagnostic Laboratory, College Station, TX) using methods set by the National Committee on Clinical

Laboratory Standards (NCCLS, 1999). Sensitivities to the antibiotics ampicillin, tetracycline, ceftiofur, gentamicin, penicillin G, ciprofloxacin, tylosin, and novobiocin were used to determine 1/4 MIC. Monensin does not inhibit the growth of *E. coli* O157:H7, but is an ionophore commonly included in cattle rations; concentrations used in this study were equivalent to estimated ruminal concentrations. Sodium chlorate concentrations >5 mM inhibited growth of *E. coli* O157:H7 (data not shown) and was designated as the "MIC" for this study.

Fecal fluid collection

Feces were obtained via rectal grab from two Holstein cows and strained through a fine mesh nylon strainer. The fecal fluid was returned to the laboratory and one subsample was used as fresh fecal fluid (contained > 10¹¹ cells/ml total culturable anaerobic bacteria as determined by most probable number [MPN] estimates), and another subsample was sterilized (autoclaved 121°C, 18 psi, 20 min). Most probable number (MPN) estimates of total culturable anaerobes from fecal fluid were determined by a 3-tube MPN test using anoxic reinforced clostridial agar supplemented with 1.67 mM xylose, 0.73 mM cellobiose and 40% filter-sterilized ruminal fluid.

Incubation Conditions

Cultures of *E. coli* O157:H7 strains 933 and 6058 were grown in TSB (2 + 4 x 10⁷ CFU/ml), in sterilized fecal fluid (diluted 1:1 with anoxic 50 mM Na₂HPO₄ supplemented with [1% wt/vol each] cellobiose, glucose, soluble starch and xylose) and fresh fecal fluid suspensions (diluted same as sterilized fecal fluid). Cultures were anaerobically incubated at 39°C for 24 h and were treated with 1/4 MIC of each antibiotic or a concentration of feed antimicrobial or chlorate as described above. All incubations were performed in duplicate (n = 2). Colony forming units (CFU/ml) of *E. coli* O157:H7 were determined at 24 h of incubation from each control and antibiotic-treated culture to determine specific toxin concentrations on a per CFU basis. Pure and mixed cultures containing *E. coli* O157:H7 were serially diluted (10-fold increments) in phosphate buffered saline (PBS, pH 7.0), plated on MacConkey's agar (supplemented with 20 and 25 µg/ml novobiocin and nalidixic acid for strain 933; or with 25 µg/ml rifampicin for strain 6058) and incubated at 37°C overnight for direct counting of colonies.

Toxin Production

Culture supernatants were collected after 24 h of incubation and were filter sterilized through 0.22 µm pore-size membranes prior to analysis for shiga toxin concentrations. Vero cells were grown in Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum (FBS) and an antibiotic/antimycotic mix (containing 100 units penicillin, 100 µg streptomycin, and 0.25 µg amphotericin B) as monolayers in 96-well microtiter tissue culture plates. Serial dilutions (100 µl of a 10- or 2-fold dilution) of each supernatant were added to wells that contained 100 µl of Eagle's medium with 10% FBS. Microtiter plates were incubated at 37°C in a 5% CO₂ atmosphere for 24 hours, Vero cell lysis was determined via measurement of

Lactate Dehydrogenase (LDH) activity (Roche Molecular Biochemicals; Indianapolis, IN) in triplicate (n=3) wells.

Statistics

All incubations were performed in duplicate (n=2). Treatment differences were analyzed by Students' t-test.

References

- Acheson, D.W.K., Lincione, L.L., Jacewicz, M.S., and Keusch, G.T. (1998). Shiga toxin interaction with intestinal epithelial cells. In *Escherichia coli* O157:H7 and other shiga-toxin producing *E. coli* strains, J. B. Kaper, and A. D. O'Brien, eds. (Washington, DC, ASM Press), pp. 140-147.
- Ahmed, S., and Booth, I.R. (1981). Quantitative measurements of the proton-motive force and its relation to steady state lactose accumulation in *Escherichia coli*. *Biochem. J.* **200**, 573-581.
- Anderson, R.C., Buckley, S.A., Kubena, L.F., Stanker, L.H., Harvey, R.B., and Nisbet, D.J. (2000a). Bactericidal effect of sodium chlorate on *Escherichia coli* O157:H7 and *Salmonella typhimurium* DT104 in rumen contents in vitro. *J. Food Prot.* **63**, 1038-1042.
- Anderson, R.C., Callaway, T.R., Buckley, S.A., Anderson, T.J., Genovese, K.J., Sheffield, C.L., and Nisbet, D.J. (2000b). Effect of sodium chlorate on porcine gut concentrations of *Escherichia coli* O157:H7 in vivo. Paper presented at: Procs. Allen D. Leman Swine Conference (Minneapolis, MN, Univ. Minnesota Coll. Vet. Med).
- Butler, T., Islam, M.R., Azad, M.A.K., and Jones, P.K. (1987). Risk factors for development of hemolytic uremic syndrome during shigellosis. *J. Pediatr.* **110**, 894-897.
- Callaway, T.R., Anderson, R.C., Genovese, K.J., Poole, T.L., Anderson, T.J., Byrd, J.A., Kubena, L.F., and Nisbet, D.J. (2002). Sodium chlorate supplementation reduces *E. coli* O157:H7 populations in cattle. *J. Anim. Sci.* **80**, 1683-1689.
- Erdington, T.S., Callaway, T.R., Anderson, R.C., Genovese, K.J., Jung, Y.S., Elder, R.O., Bischoff, K.M., and Nisbet, D.J. (2003). Reduction of *E. coli* O157:H7 populations in sheep by supplementation of an experimental sodium chlorate product. *Small Ruminant Res.* **49**, 173-181.
- Grif, K., Dierich, M.P., Karch, H., and Allerberger, F. (1998). Strain-specific differences in the amount of shiga toxin released from enterohemorrhagic *Escherichia coli* O157 following exposure to subinhibitory concentrations of antimicrobial agents. *Eur. J. Clin. Microbiol. Infect. Dis.* **17**, 761-766.
- Hynes, N.A., and Wachsmuth, I.K. (2000). *Escherichia coli* O157:H7 risk assessment in ground beef: A public health tool. Paper presented at: Proc. 4th Int. Symp. on Shiga Toxin-Producing *Escherichia coli* Infections (Kyoto, Japan).
- Karch, H., Goroncy-Bermes, P., Opferkuch, W., Kroll, H.P., and O'Brien, A. (1985). Subinhibitory concentrations of antibiotics modulate amount of shiga-like toxin produced by *Escherichia coli*. In *The influence of antibiotics on the host-parasite relationship II*, D. Adam, H. Hahn, and W. Opferkuch, eds. (Springer-Verlag Berlin, Heidelberg), pp. 239-245.
- Kohler, B., Karch, H., and Schmidt, H. (2000). Antibacterials that are used as growth promoters in animal husbandry can affect the release of Shiga-toxin-2 converting bacteriophages and Shiga toxin 2 from *Escherichia coli* strains. *Microbiology* **146**, 1085-1090.
- Law, D. (2000). The history and evolution of *Escherichia coli* O157 and other shiga toxin-producing *E. coli*. *World J. Microbiol. Biotechnol.* **16**, 701-709.
- Lewis, K., Naroditskaya, V., Ferrante, A., and Fokina, I. (1994). Bacterial resistance to uncouplers. *J. Bioenerg. Biomemb.* **20**, 639-646.
- Lorian, V. (1980). Effects of subminimum inhibitory concentrations of antibiotics on bacteria. In *Antibiotics in laboratory medicine*, V. Lorian, ed. (Baltimore, Williams & Wilkins), pp. 342-403.
- Mead, P.S., Slutsker, L., Dietz, V., McCraig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M., and Tauxe, R.V. (1999). Food-related illness and death in the United States. *Emerg. Infect. Dis.* **5**, 607-625.
- NCCLS (1999). Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard. (Wayne, PA, National Committee for Clinical Laboratory Standards), pp. 36-40.
- Neill, M.A. (1998). Treatment of disease due to shiga toxin-producing *Escherichia coli*: Infectious disease management. In *Escherichia coli* O157:H7 and other shiga toxin producing *E. coli* strains, J. B. Kaper, and A. D. O'Brien, eds. (Washington, DC, ASM Press), pp. 357-363.
- Pruimboom-Brees, I.M., Morgan, T.W., Ackermann, M.R., Nystrom, E.D., Samuel, J.E., Cornick, N.A., and Moon, H.W. (2000). Cattle lack vascular receptors for *Escherichia coli* O157:H7 shiga toxins. *Proc. Nat. Acad. Sci. (USA)* **97**, 10325-10329.
- Stewart, V.J. (1988). Nitrate respiration in relation to facultative metabolism in enterobacteria. *Microbiol. Rev.* **52**, 190-232.
- Stouthamer, A.H. (1969). A genetical and biochemical study of chlorate-resistant mutants of *Salmonella typhimurium*. *Antoine van Leeuwenhoek* **35**, 505-521.
- Walterspiel, J.N., Ashkenazi, S., Morrow, A.A., and Cleary, T.G. (1992). Effect of subinhibitory concentrations of antibiotics on extracellular shiga-like toxin I. *Infection* **20**, 25-29.
- Yoh, M., Frimpong, E.K., and Honda, T. (1997). Effect of antimicrobial agents, especially fosfomycin, on the production and release of vero toxin by enterohaemorrhagic *Escherichia coli* O157:H7. *FEMS Immun. Med. Microbiol.* **19**, 57-64.