Novel Preharvest Strategies Involving the Use of Experimental Chlorate Preparations and Nitro-Based Compounds to Prevent Colonization of Food-Producing Animals by Foodborne Pathogens

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ABSTRACT Foodborne diseases caused by enterohemorrhagic *Escherichia coli*, *Salmonella*, and *Campylobacter* species are of public health and economic significance. Shedding of these pathogens during production and slaughter are risks for contamination of products for human consumption. Consequently, strategies are sought to prevent or reduce the carriage of these pathogens in food animals before slaughter. Experimental products containing chlorate salts have been proven efficacious in reducing concentrations of *E. coli* and *Salmonella* Typhimurium in the gut of cattle, sheep, swine, and poultry when administered as feed or water additives. Mechanistically, chlorate selectively targets bacteria expressing respiratory nitrate reductase activity, such as most members of the family *Enterobacteriaceae*, as this enzyme catalyzes the reduction of chlorate to lethal chlorite. Most beneficial gut bacteria lack respiratory nitrate reductase activity, and thus the technology appears compatible with many bacteria exhibiting competitive exclusion capabilities. More recently, select nitrocompounds have been investigated as potential feed additives, and although these nitrocompounds significantly reduce pathogens on their own, evidence indicates that they may most effectively be used to complement the bactericidal activity of chlorate. A particularly attractive aspect of the nitrocompound technology is that, as potent inhibitors of ruminal methanogenesis, they may allow producers the opportunity to recoup costs associated with their use. At present, neither chlorate nor the nitrocompounds have been approved as feed additives by the US Food and Drug Administration, and consequently they are not yet available for commercial use.

(*Key words*: chlorate, food safety, foodborne pathogen, nitrocompound)

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INTRODUCTION

The gut of food-producing animals can be a reservoir for human pathogens such as enterohemorrhagic *Escherichia coli*, *Salmonella*, and *Campylobacter* species (Menning, 1988; Roberts, 1989; Altekruse et al., 1997; Mead et al., 1999). A number of technologies have been developed to reduce contamination of carcasses by these pathogens during and after slaughter (Castell-Perez and Moreira, 2004; Keeton and Eddy, 2004), but none are infallible because product recalls and outbreaks of human foodborne disease continue to occur. Consequently, there is a need to reduce the incidence and concentrations of foodborne pathogens in food animals during on-farm rearing particularly because quantitative risk assessments have indicated that such interventions, when applied just prior to slaughter, would reduce human exposure to pathogens (Hynes and Wachsmuth, 2000; Vugia et al., 2003). The present review discusses strategies using experimental chlorate preparations (ECP) as well as several novel nitrocompounds that, although not yet commercially available, are being investigated as potential interventions to mitigate the ability of foodborne pathogens to colonize the gut of food-producing animals.

CHLORATE AS A PREHARVEST FEED SUPPLEMENT

Many *Enterobacteriaceae*, including *E. coli* and *Salmonella* species, can metabolize nitrate using an inducible respiratory (also called a dissimilatory) nitrate reductase enzyme, which coincidentally converts inorganic chlorate to cytotoxic chlorite (Pichinoty and Piéchaud, 1968; Brenner, 1984; Gennis and Stewart, 1996). Because most,

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Abbreviation Key: ECP = experimental chlorate product.

but not all, strict anaerobic gut bacteria lack respiratory nitrate reductase activity (Stewart, 1988), it was proposed that chlorate may selectively target those bacteria possessing a respiratory nitrate reductase enzyme but not beneficial anaerobes lacking the enzyme (Anderson et al., 2000) thereby conserving the competitive exclusion potential of the host's normal flora. The selective bactericidal activity of ECP was subsequently demonstrated in laboratory studies (Anderson et al., 2000) as well as studies with ruminants (Anderson et al., 2002; Callaway et al., 2002, 2003; Edrington et al., 2003) and monogastrics (Anderson et al., 2001a,b, 2004a; Byrd et al., 2003; Jung et al., 2003a) thus supporting its conceptual use as a feed additive to reduce gut concentrations of foodborne pathogens. A particularly attractive feature of the ECP technology is that its activity is sufficient to achieve significant reductions (greater than 100-fold) in fecal *E. coli* and *Salmonella* Typhimurium concentrations in as little as 24 h. From a food safety point of view, ECP may best be administered in the feed or water during the last few days of production or just before animals are sent to slaughter.

In Vitro Evidence Consistent with the Proposed Mechanism of Action

Concentrations of *E. coli* O157:H7 were reduced in a dose-dependent manner during anaerobic incubation of buffered ruminal fluid supplemented with ECP (Anderson et al. 2000). After addition of 5 m*M* active ion to batch cultures of ruminal fluid, concentrations of *E. coli* O157:H7 decreased from an initial level of greater than 1 million cells/mL to concentrations below detection levels (10 cells/mL) within a 6-h incubation period. The bactericidal activity of ECP was dependent on anaerobic conditions (unpublished) because the respiratory nitrate reductase enzyme is not expressed during growth with oxygen (Gennis and Stewart, 1996). Consistent with a characteristic pH optimum near pH 6.8 for most respiratory nitrate reductases (Nicholas and Nason, 1955), the bactericidal effect of ECP was better at pH 6.8 than at pH 5.6 (Anderson et al. 2000). Also consistent with the characteristic of respiratory nitrate reductases is the observation that tungsten, a valence state analog of the critical nitrate reductase component molybdenum, reduced the bactericidal effect of ECP on *E. coli* O157:H7 (Anderson et al. 2000). Excess tungsten can cause the synthesis of inactive nitrate reductase due to its incorporation into the enzyme (Enoch and Lester, 1972; Stewart, 1988). *Salmonella* Typhimurium DT104 concentrations were also reduced during in vitro incubation of buffered ruminal fluid supplemented with ECP (Anderson et al., 2000). Addition of nontoxic amounts of sodium nitrate to the buffered ruminal fluid increased the bactericidal effect of the active ion on *Salmonella* Typhimurium DT104, thus suggesting that nitrate was needed to induce optimal expression of the nitrate reductase activity. On the contrary, addition of nitrate had little effect on the bactericidal effect of the active ion on *E. coli* O157:H7, suggesting that chlorate by itself was sufficient to induce expression of the enzyme by this bacterium without the addition of nitrate.

For comparison purposes, the bactericidal effect of other potential sources of chlorite ion on *E. coli* O157:H7 and *Salmonella* Typhimurium DT104 were also tested during their in vitro incubation in buffered ruminal fluid. Preparations containing chlorate were found to be superior to preparations containing hypochlorite or perchlorate when incubated with added carbohydrates. Under these conditions, it is likely that the abundant supply of readily fermentable carbohydrate caused extracellular chlorite to be rapidly reduced, essentially detoxifying the ion before it could exert its lethal effects. Intracellular consumption of reductant would be preferentially channeled to the reduction of chlorate to chlorite, resulting in rapid cell death.

Whereas most strict anaerobes lack respiratory nitrate reductase activity, notable exceptions include members of *Clostridium*, *Viellonella*, *Propionibacterium*, *Wollinella*, and some *Selenomonas* species (Alaboudi, 1982; Allison and Reddy, 1984). Because ECP has been shown to kill *Clostridium perfringens* (McReynolds, 2004), it is reasonable to suspect that other nitrate respiring anaerobes would be inhibited by chlorate as well. Even if these bacteria are inhibited, evidence indicates that at least in the short term, other nonnitrate-respiring, and thus nonchlorate-susceptible, bacteria are able to occupy the niche vacated by those nitrate-respiring bacteria killed by chlorate. For instance, although concentrations of total culturable anaerobes decreased from initial levels during the 24 h incubation of untreated and ECP-treated incubations, the decrease was not exacerbated but rather was moderated within the incubations containing added ECP (Anderson et al., 2000).

Campylobacter species also have respiratory nitrate reductase activity, but results from a preliminary experiment showed no significant reduction in gut concentrations of wild-type *Campylobacter* species following oral administration of ECP to broiler chicks (unpublished). It is possible that the nitrate reductase activity of *Campylobacter* species, which is conferred by a periplasmic enzyme (Richardson et al., 2001), may not catalyze the reduction of chlorate to chlorite. Alternatively, it may be that nitrate also contained within these treatments may have repressed the nitrate reductase activity of *Campylobacter* species, as some, but probably not all, periplasmic nitrate reductases may be repressed by increased nitrate concentrations (Wang et al., 1999). Additionally, the chicks in this study had been challenged with *Salmonella* Typhimurium, and it is possible that the nitrate reductase of *Salmonella* Typhimurium may have had a greater affinity for chlorate than the *Campylobacter* enzyme.

In Vivo Evidence and Proof of Concept

Studies have now demonstrated that practical administration protocols (via drinking water) of ECP success-

TABLE 1. Effects of drinking water administration of an experimental chlorate preparation (ECP) to broilers, turkeys, and pigs[1]

	Incidence (%) of *Salmonella*-positive contents						Concentration (log_{10} cfu) of *Salmonella*					
	Crop		Cecal		Fecal		Crop		Cecal		Fecal	
Species[2]	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Broilers	37	5*	53	53	ND[3]		0.9	0.2*	2.5	1.5*	ND	
Turkeys	80	20*	65	10*	ND		2.9	0.4*	1.9	0.3*	ND	
Pigs	—		100	83	100	50	—		3.8	1.8*	1.9	1.3

[1]Treatments were administered at least 24 h after initial *Salmonella* Typhimurium challenge with 10^8 to 10^9 cfu for broilers and turkeys and 10^{10} cfu for pigs, via ad libitum access to water (control) or water containing ECP (treated) as indicated for 24 h prior to necropsy.

[2]Broilers, n = 60 per treatment (Byrd et al., 2003); turkeys, n = 20 per treatment (Moore et al., 2002); and pigs, n = 6 per treatment (Anderson et al., 2004a).

[3]ND = not done.

*Denotes value is significantly different than control at $P < 0.05$.

fully reduced the incidences and concentrations of *Salmonella* Typhimurium colonization in crops and ceca of market age broilers and turkeys as well as gut concentrations of *Salmonella* Typhimurium in finished pigs (Table 1). Moreover, we observed significant reductions in fecal *E. coli* concentrations following intraruminal addition of ECP (5.4 vs 2.9 log_{10} cfu for control versus treated animals, respectively) to ruminally cannulated cattle (Anderson et al., 2002). Callaway et al. (2002) reported significant (greater than 100-fold) reductions in fecal *E. coli* O157:H7 concentrations in experimentally infected cattle following 24-h access to water containing ECP. Evidence from these and other studies with cattle and sheep (Anderson et al., 2003b; Edrington et al., 2003) indicate that strategies designed to optimize passage of the active ion through the rumen to the large intestine may be beneficial. For instance, drinking water administration of ECP to cattle significantly reduced ruminal but not fecal *E. coli* concentrations, whereas administration of a stabilized ECP product in the feed was effective in reducing fecal *E. coli* concentrations (Anderson et al., 2003b). Negative effects of ECP treatment on carcass quality were not observed (King et al., 2003), and at no time did ECP treatment adversely affect water or feed intake or animal well-being in any of our studies.

Consistent with our in vitro results, ECP treatment had little effect on the microbial fermentation in the gut of weaned or finished pigs as evidenced by few changes in gut volatile fatty acid concentrations (Anderson et al., 2001a,b, 2004a). Moreover, studies with cattle and sheep have revealed no adverse effect of ECP administration on accumulations of fermentation acids (Anderson et al., 2002; Callaway et al., 2002, 2003). Barry et al. (1978) have shown that intraruminal administration of up to 230 mg of potassium chlorate per kilogram of BW per day for 3 d to sheep increased propionate and correspondingly decreased ruminal acetate concentrations. From that study, it appears that under certain as yet ill-defined conditions, chlorate could affect the gut microflora, thereby perturbing propionate production. Regardless of any possible inhibition of specific groups of anaerobes, adverse effects of chlorate treatment on concentrations of total culturable anaerobes have not been observed in vivo (Anderson et al., 2001a,b, 2002; Callaway et al., 2002, 2003).

To assess the risk that *Salmonella* Typhimurium populations may develop resistance to ECP, an event that naturally would limit the practical effectiveness of this technology, a series of experiments were conducted to quantify the frequency at which resistance to ECP may develop and determine the effect of resistance on the competitive fitness of the affected bacteria (Anderson et al., 2001c, Callaway et al., 2001). The results demonstrated that resistance is unlikely to occur within mixed bacterial populations and that even when generated in pure culture, resistant bacteria are less healthy and do not persist in competitive environments (Anderson et al., 2001c; Callaway et al., 2001). Additionally, results from recent in vivo experiments with pigs and broilers have shown that the sensitivity of gut populations of *Salmonella* Typhimurium to ECP was enhanced if preceded by a short, low-level nitrate preconditioning period (Jung et al., 2003a; Anderson et al., 2004c). Thus, low-level nitrate selection is a tool that can be used to enrich for nitrate-respiring bacteria, which by definition are chlorate sensitive (Stewart, 1988). Unfortunately, rapid reduction of nitrate within the rumen may preclude the use of a nitrate preconditioning strategy to increase the chlorate susceptibility of targeted bacteria in the hindgut of ruminants (Fox et al., 2004).

Precedence exists for the use of chlorate salts in veterinary and human medicine (Cosmetic Ingredient Review Panel, 1995), but their proposed use as a preharvest pathogen reduction tool is a recent development and likely will require regulatory approval. For instance, potassium chlorate has reportedly been approved by the European Union for use in toothpastes at concentrations of up to 5% (Cosmetic Ingredient Review Panel, 1995). At sufficient concentrations; however, chlorate can be toxic, with a lethal dose of approximately 2,000 to 7,000 mg of sodium chlorate per kilogram of body weight for most animals (Frank, 1948, Radeleff, 1970). Common toxic effects associated with ingestion of chlorate and reduced ion chlorite include methemoglobinemia, erythrocyte fragility, and renal necrosis (Steffen and Wetzel, 1993). Evidence with rats revealed that [36–

Cl-labeled chlorate and chlorite do not bioaccumulate but rather appear to be ultimately reduced to chloride (Abdel-Rahman et al., 1985; National Research Council, 1987). Further studies are underway to determine the fate of chlorate in food animals (Smith et al., 2004).

NITROCOMPOUNDS AS POTENTIAL COST-RECOVERABLE PREHARVEST FEED SUPPLEMENTS

Although in vitro evidence suggests that low-level nitrate preconditioning may potentially support a temporary enrichment of *Salmonella* Typhimurium in suspensions incubated without chlorate, subsequent studies show that nitrate preconditioning can be safely managed by limiting the duration and amount of nitrate supplementation. For example, concentrations less than 2.5 m*M* do not enrich populations of *Salmonella* Typhimurium or *E. coli* in vitro and enrichment does not occur in vivo (Anderson et al., 2004c). Moreover, additional laboratory tests with electronegative nitrocompounds (i.e., 2-nitro-1-propanol, 2-nitro-1-ethanol, and nitroethane) showed that substitution of these compounds for nitrate into the fecal suspensions further enhances the bactericidal activity of chlorate against *Salmonella* Typhimurium (Anderson et al., 2004b). The nitrocompounds by themselves do not enrich for *Salmonella* Typhimurium but rather exhibit in their own right a more persistent bactericidal activity against *Salmonella* Typhimurium than does chlorate alone (Anderson et al., 2004b). After incubation of these compounds and chlorate, *Salmonella* Typhimurium populations are reduced by approximately 1 million-fold after 24 h. Results from laboratory studies further showed that the nitrocompounds may be preferable to nitrate as preconditioning agents because these exhibit bactericidal activity against *Salmonella* Typhimurium in their own right and enhance the bactericidal effect of chlorate in animals (Anderson et al., 2004c). In support of this, 2-nitropropanol has been shown to inhibit *Salmonella* Typhimurium, *E. coli* O157:H7 and *Enterococcus faecalis* in vitro (Jung et al., 2004a), and 2-nitropropanol and nitroethane have been shown to reduce *Salmonella* Typhimurium concentrations in the guts of pigs and broilers (Jung et al., 2003b; Anderson et al., 2004c; Jung et al., 2004b). We have also shown that nitrocompounds inhibited the growth of pure cultures of *Campylobacter jejuni* and *Yersinia enterocolitica* in vitro (unpublished), although in vivo reductions have been inconsistent (Jung et al., 2003b; unpublished). Considering that the activity of the nitrocompound used in these in vivo studies is markedly more effective at pH values greater than 8.0 (unpublished) it is possible that the pH of cecal and fecal contents may have been too acidic (pH less than 7.0) to allow the selected nitrocompound to consistently be effective against *Campylobacter* species in vivo.

Nitrate preconditioning appears not to be a variable strategy for enhancing the bactericidal activity of chlorate in ruminants (Fox et al., 2004), although 2-nitro-1-propanol and nitroethane are presently being investigated as potential alternatives to nitrate to enhance the bactericidal effect of ECP against *E. coli* and *Salmonella* Typhimurium. These compounds have the added benefits in that they are not substrates for the nitrate reductase enzyme, and they exhibit a more persistent bactericidal activity than chlorate. Moreover, because these nitrocompounds are also potent inhibitors of ruminal methanogenesis (Anderson et al., 2003a) they may prove cost-effective for producers because of potential improvements in gross energy use. Ruminal methanogenesis accounts for losses of 2 to 4% of gross energy intake by feedlot cattle and losses of as much as 16% for forage fed cattle (Johnson and Johnson, 1995). It is possible that the nitrocompounds could reduce ruminal methanogenesis by 50 to 90% (Anderson et al., 2003a) thereby improving productivity.

Whether the use of the nitrocompounds or nitrate as preconditioners for chlorate may require comprehensive review for approval by US Food and Drug Administration has yet to be determined. Research is underway to more fully examine the inhibitory activity of the nitrocompounds against these foodborne pathogens.

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