Research Note

Effect of bismuth citrate, lactose, and organic acid on necrotic enteritis in broilers

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ABSTRACT *Clostridium perfringens*-associated necrotic enteritis causes significant economic losses. The objective of this study was to evaluate the effect of bismuth citrate, lactose, and organic acid on the development of necrotic enteritis in broilers. The first study was a dose response that evaluated bismuth citrate at 50, 100, or 200 ppm on bacterial intestinal colonization and lesion development associated with our *C. perfringens* challenge model. The second study evaluated bismuth citrate, lactose, and citric acid on intestinal pH and lesion development. For the third study, we determined if lactose would enhance the efficacy of bismuth citrate against intestinal colonization and lesion development associated with *C. perfringens*. In study 1, intestinal lesion scores at the 50, 100, and 200 ppm bismuth citrate treatment level were reduced ($P \leq 0.05$) when compared with the birds fed 0 ppm bismuth citrate. Intestinal *C. perfringens* colonization of the 100 and 200 ppm bismuth citrate treatment group was significantly reduced when compared with birds fed 0 ppm bismuth citrate. In study 2, we found no significant differences in lesion development, after *C. perfringens* challenge, between birds fed 100 ppm bismuth citrate or fed a combination of 100 ppm bismuth citrate with dietary lactose or citric acid relative to the controls. The intestinal pH of birds fed 100 ppm bismuth citrate or fed a combination of 100 ppm bismuth citrate with dietary lactose or citric acid was not significantly reduced when compared with the controls. However, a significant reduction in pH was observed in birds fed a combination of 100 ppm bismuth citrate and lactose relative to the negative controls. In study 3, a decrease ($P \leq 0.05$) in intestinal lesion scores occurred in birds fed lactose with 100 ppm bismuth citrate, compared with the positive controls. There were no significant differences in intestinal bacterial colonization. These preliminary data suggest that bismuth citrate may reduce intestinal lesion development and *C. perfringens* colonization in broilers infected with necrotic enteritis.

Key words: *Clostridium perfringens*, bismuth citrate, necrotic enteritis, lactose, citric acid

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INTRODUCTION

The incidence of necrotic enteritis (**NE**), commonly caused by *Clostridium perfringens*, has recently increased because of the withdrawal of in-feed antibiotic growth promoters (**AGP**) with anticlostridial activity (Knarreborg et al., 2002). Disease occurs when high numbers of *C. perfringens* adhere to damaged intestinal mucosa, proliferate, and produce toxins, resulting in lesions of the small intestine (Van Immerseel et al., 2004). Outbreaks of this disease may result in downgraded or rendered carcasses, and mortality rates may reach up to 1% per day (Kaldhusdal and Lovland, 2000). One strategy that may be worth pursuing to reduce the incidence of NE would be to modify the avian gastrointestinal mucosal environment in which *C. perfringens* flourish with bismuth compounds.

Bismuth compounds have been used to treat gastric disorders in humans for over 300 yr (Marshall, 1991). These compounds have treated duodenal ulcers, gastritis, and acute diarrhea in young children, and they have also been shown to inhibit growth of *Escherichia coli*, *Salmonella*, *Shigella*, and *Campylobacter* (Manhart, 1990; Steffen, 1990). The administration of bismuth compounds have also been reported to protect the gastric mucosa in humans. In past investigations, colloidal bismuth citrate reduced *Helicobacter pylori* by altering mucin characteristics in humans (Fraser, 2004). In poultry, colloidal bismuth citrate and bismuth citrate have been demonstrated to reduce cecal colonization

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by *Campylobacter jejuni* (Farnell et al., 2006). We hypothesize that the modification of chicken mucin with bismuth compounds may also reduce *C. perfringens* colonization in chickens.

In addition to bismuth compounds, lactose has been used to reduce pathogens in poultry. Lactose, or milk sugar, is a naturally occurring disaccharide found in mammalian milk. Lactose fed to broilers at an inclusion rate of 2.5% has significantly reduced intestinal lesions and mortality rates associated with NE (McReynolds et al., 2007). Along with this protective effect in the gut, milk sugars have changed the pH of the avian intestinal tract from a relatively neutral pH (6.0 to 7.4) to an acidic one (4.4 to 5.6; Hinton et al., 1990). Feeding lactose to chickens resulted in an increase in bacteriostatic acetic and propionic acids, resulting in a decrease in cecal pH (Corrier et al., 1990). Organic acids may be another method of lowering the pH of the avian gut environment and offering protection against *C. perfringens* colonization.

Citric acid is a weak organic acid and has been speculated to cause a decrease in the pH of intestinal contents by contributing hydrogen ions to the intestinal environment in chickens (Brown and Southern, 1985). In an evaluation of potential disinfectants for preslaughter broiler crop decontamination, researchers found that citric acid at or greater than 10% concentration caused a reduction in *Salmonella* in a simulated crop environment, suggesting that citric acid may have antimicrobial affects (Barnhart et al., 1999).

Bismuth compounds form an active metabolite at a pH of less than 5, facilitating the infiltration of bismuth into human microvilli (Wagstaff et al., 1988). It is possible that the addition of lactose or citric acid may reduce intestinal pH to an optimal range in the avian gut, enhancing bismuth efficacy in broilers. The objective of this study was to determine if bismuth citrate can reduce gut colonization by *C. perfringens* and reduce intestinal lesion development in broilers challenged with our NE model. We also determined if the addition of lactose or citric acid enhanced the efficacy of bismuth citrate.

MATERIALS AND METHODS

Experimental Birds

One-day-old Cobb × Ross straight-run broiler chicks were obtained from a local commercial hatchery and were placed on new pine shavings. Birds were reared in 2.4 × 1.2 m pens, allowing 0.12 m^2 of pen space per bird. Chicks were provided with water and a uniform basal diet consisting of a 55% wheat-based broiler starter diet ad libitum that met or exceeded the NRC (1994) guidelines. Elevated concentrations of wheat in the diet have been shown to intensify the occurrence of NE (Riddell and Kong, 1992).

Experimental Design

Three independent studies were conducted to evaluate the effect of bismuth citrate (Sigma Chemical Co., St. Louis, MO), 2.5% lactose (Sigma Chemical Co.), or 0.04% citric acid (Agri Laboratories Ltd., St. Joseph, MO) on NE-infected broilers. In each study, birds were assigned to treatment groups in a completely randomized block design. Birds were fed their respective diets from day of hatch until termination of each study (25 d of age). Mortality, weight gain, and feed efficiency were not measured in this experiment. Due to the limited size of challenge facilities, we were not able to house a large number of birds to gather useful data on production parameters.

Study 1. This study was a dose response to determine the optimum concentration of bismuth citrate in broilers challenged with NE. In 2 trials, 50 birds were placed in 1 pen for each treatment group (0, 50, 100, or 200 ppm bismuth citrate) on day of hatch and fed the respective treatments until the termination of the study. To evaluate the effect of these treatments on NE, at 25 d of age, a subset of birds from each treatment group was killed by cervical dislocation and the small intestines were collected and analyzed for *C. perfringens* colonization (n = 20 birds) and lesion development (n = 50 birds).

Study 2. This individual study evaluated the effect of gut acidifiers and 100 ppm bismuth citrate in broilers challenged with NE. In one trial, 50 birds were placed in 1 pen for each group on day of hatch. The groups consisted of a negative control (no *C. perfringens* challenge), bismuth citrate negative control (no *C. perfringens* challenge), or one of the challenged groups. Challenged groups consisted of a positive control, lactose, citric acid, bismuth citrate, a combination of bismuth citrate with lactose, and a combination of bismuth citrate with citric acid. To evaluate the effect of these treatments on NE, at 25 d of age, a subset of birds from each treatment group was killed by cervical dislocation and the small intestines were collected and analyzed for lesion development (n = 20 birds) and pH modification (n = 5 birds).

Study 3. This individual study evaluated the effects of 100 ppm bismuth citrate with lactose in broilers challenged with NE. In one trial, 50 birds were placed in 1 pen for each group on day of hatch. The groups consisted of a negative control (no *C. perfringens* challenge), bismuth citrate negative control (no *C. perfringens* challenge), or one of the challenged groups. Challenged groups consisted of a positive control, lactose, citric acid, bismuth citrate, and a combination of bismuth citrate with lactose. To evaluate the effect of these treatments on NE, at 25 d of age, a subset of birds from each treatment group was killed by cervical dislocation and the small intestines were collected and analyzed for *C. perfringens* colonization (n = 10 birds) and lesion development (n = 25 birds).

Immunosuppression Vaccine Administration

As described previously, a commercial infectious bursal disease vaccine (Schering-Plough Animal Health, Millsboro, DE) was used as an immunosuppressant (McReynolds et al., 2004). All experimental birds were administered the vaccine via an ocular route on d 14 at a level 10× the recommended dose.

C. perfringens Administration

Multiple isolates of *C. perfringens* (type A) obtained from active field cases in Virginia, North Carolina, and Georgia were used in this investigation (McReynolds et al., 2004). The isolates were grown in thioglycollate medium (Becton Dickinson Co., Sparks, MD) for 12 h. Birds were challenged once a day for 3 d beginning on d 17 by oral gavage (1.5 mL/bird) with a stock culture of 10^7 cfu of *C. perfringens*/mL.

C. perfringens Colonization

The bacterial culture methodology was provided by McReynolds et al. (2007). Briefly, a 15.24-cm (6-in.) section of the small intestine cranial to Meckel's diverticulum was removed. The sample was placed in 10 mL of anaerobic thioglycollate (Becton Dickinson Co.), stomached for 30 s, and 0.5 mL of gut contents was removed and placed into 4.5 mL of thioglycollate medium (Becton Dickinson Co.). Three 10-fold serial dilutions were performed and plated onto thioglycollate agar (Becton Dickinson Co.) and incubated anaerobically (24 h at 37°C). Colonies exhibiting typical colony morphology were counted and recorded. Colony-forming units were transformed into $\log_{10}$ values.

NE Lesion Development

The jejunum and ileum were examined for lesion scores associated with NE. Lesion scores were recorded using the following criteria: 0 = no gross lesions, normal intestinal appearance; 1 = thin-walled or friable, gray appearance; 2 = thin-walled, focal necrosis, gray appearance, small amounts of gas production; 3 = thin-walled, sizable patches of necrosis, gas-filled intestine, small flecks of blood; and 4 = severe and extensive necrosis, marked hemorrhage, and much gas in intestine (Prescott et al., 1978).

pH Analysis

On the last day of the second study, upper ileum pH values were determined. Intestinal pH was determined by the insertion of a sterile glass pH electrode (Model 05669-20, Cole Palmer, Niles, IL) through an incision in the intestinal wall, ensuring that the electrode remained in contact with the gut contents.

Statistical Analysis

Statistical analysis was completed with the SPSS statistical software package (SPSS Inc., Chicago, IL). For each study, birds collected from each treatment served as the experimental unit. Data in all studies were analyzed via a 1-way ANOVA using the GLM procedure. Differences were deemed significant at $P \leq 0.05$ and means were separated using Duncan's multiple range test.

RESULTS AND DISCUSSION

Bismuth citrate has been used successfully in the treatment of *Helicobacter pylori*-induced ulcers in humans (Fraser, 2004) and has been demonstrated to reduce cecal *C. jejuni* colonization in broilers (Farnell et al., 2006). Research analyzing alternative methods to reduce the onset of NE in poultry has recently increased due to the concern of consumers regarding antimicrobial drug use in poultry and the perceived risk of drug resistance in humans (Angulo, 2004). As a result, there has been increasing pressure for some producers to voluntarily remove AGP from poultry feed, which are considered one of the most effective means of controlling NE (Williams, 2005).

In the present study, we used our NE model to investigate whether bismuth citrate would reduce intestinal *C. perfringens* colonization and lesion development.

Table 1. An evaluation of intestinal *Clostridium perfringens* colonization in broilers treated with 0, 50, 100, or 200 ppm bismuth citrate

	$\log_{10}$ values[2] of *C. perfringens*		
Study 1[1]	Trial 1	Trial 2	Mean[3]
0 ppm bismuth citrate	3.24 ± 2.4	3.93 ± 2.0	3.59^{A} ± 2.2
50 ppm bismuth citrate	3.59 ± 2.2	2.92 ± 1.9	3.25^{AB} ± 2.0
100 ppm bismuth citrate	2.45 ± 1.6	1.76 ± 1.2	2.10^{BC} ± 1.4
200 ppm bismuth citrate	2.20 ± 2.0	1.62 ± 1.4	1.92^{C} ± 1.7

[A-C]Means values within the same column with no common superscripts differ significantly ($P \leq 0.05$).

[1]Treatment groups represented by the concentration of bismuth citrate administered in a 55% wheat standard broiler diet from d 1.

[2]$\log_{10}$ values are represented by the mean of treatment subset (n = 20) ± the SD.

[3]Combined mean $\log_{10}$ values from trials 1 and 2.

Table 2. An evaluation of intestinal lesion development in broilers treated with 0, 50, 100, or 200 ppm bismuth citrate

	Intestinal lesion score[2]		
Study 1[1]	Trial 1	Trial 2	Mean[3]
0 ppm bismuth citrate	2.34 ± 0.97	1.38 ± 0.85	1.86^{A} ± 1.02
50 ppm bismuth citrate	1.50 ± 0.94	1.00 ± 0.56	1.25^{B} ± 0.81
100 ppm bismuth citrate	1.12 ± 0.66	0.91 ± 0.92	1.02^{BC} ± 0.80
200 ppm bismuth citrate	1.12 ± 0.67	0.47 ± 0.59	0.80^{C} ± 0.71

[A–C]Means values within the same column with no common superscripts differ significantly ($P \leq 0.05$).

[1]Treatment groups represented by the concentration of bismuth citrate administered in a 55% wheat standard broiler diet from d 1.

[2]Lesion score is represented by the mean of treatment subset (n = 50) ± the SD.

[3]Combined mean lesion scores from trials 1 and 2.

Additionally, due to reports of bismuth compounds increasing in binding affinity in an acidic environment (Wagstaff et al., 1988), we also investigated whether acidifiers would enhance the efficacy of bismuth citrate. In the first study, to determine the optimal concentration of bismuth citrate on intestinal *C. perfringens* colonization and lesion development, chicks were fed 0, 50, 100, or 200 ppm bismuth citrate and were challenged using our NE model. Feeding 100 or 200 ppm bismuth citrate reduced ($P \leq 0.05$) intestinal *C. perfringens* colonization and lesion development when compared with feeding 0 ppm bismuth citrate (Tables 1 and 2). When birds were fed 50 ppm bismuth citrate, the intestinal lesions were reduced ($P \leq 0.05$) when compared with feeding 0 ppm bismuth citrate (Table 2); however, the 50-ppm treatment did not reduce *C. perfringens* colonization (Table 1).

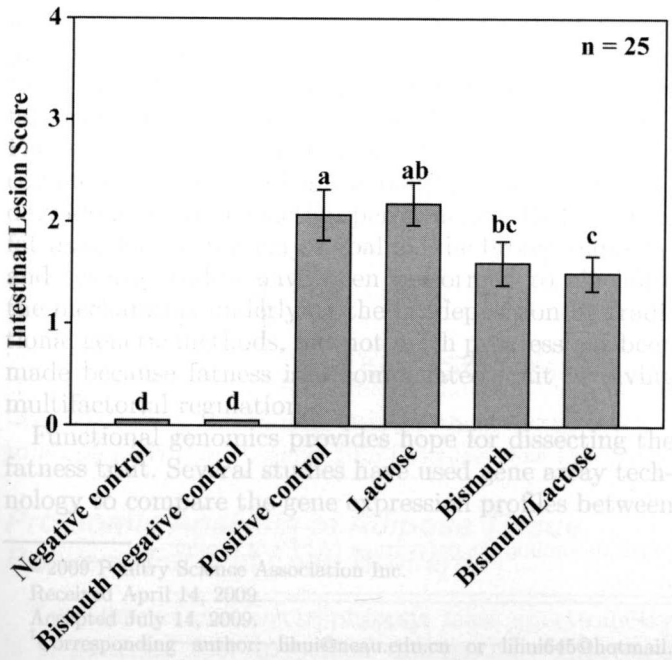

Figure 1. Evaluation of 2.5% lactose and 100 ppm bismuth citrate on intestinal lesion development. [a–d]Means with different letters differ significantly ($P \leq 0.05$).

In study 2, to evaluate whether acidifiers increased the efficacy of bismuth citrate, lactose or citric acid were included in the bismuth diet and intestinal *C. perfringens* colonization and pH were evaluated, after challenge with our NE model. Including acidifiers in the bismuth diet failed to contribute any added effect on reducing *C. perfringens* intestinal colonization or pH in challenged birds when compared with the challenged birds that were fed bismuth citrate alone (data not shown). However, when lactose was included in the bismuth diet of challenged birds (5.2 ± 0.21), a significant reduction in intestinal pH was observed when compared with the bismuth citrate negative controls (5.79 ± 0.01). No significant interactions were found in this study.

In study 3, to further evaluate the effect of lactose in combination with bismuth citrate, broilers were fed bismuth citrate and lactose to determine their combined effect on intestinal *C. perfringens* colonization and lesion development associated with NE. Lactose fed to broilers has been shown to significantly reduce intestinal lesions associated with NE (McReynolds et al., 2007). Similarly, we demonstrated that challenged birds fed bismuth citrate alone or with lactose significantly reduced intestinal lesion development relative to the challenged birds receiving the control diets (Figure 1). A significant interaction between lactose and bismuth citrate was not observed when *C. perfringens* colonization was evaluated (data not shown).

In conclusion, bismuth citrate treatments of 100 ppm and 200 ppm significantly reduced *C. perfringens* colonization and intestinal lesion development. Due to the reduction of AGP in the commercial poultry industry, alternative feeding strategies need to be investigated to mitigate the incidence of NE. Bismuth citrate treatments may provide a cost-effective approach in controlling this disease.

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