Evaluation of Potential Disinfectants for Preslaughter Broiler Crop Decontamination

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ABSTRACT The broiler crop has recently been implicated as a major source of *Salmonella* contamination at commercial processing. Furthermore, feed withdrawal has been positively correlated with increased *Salmonella* incidence in the crop, probably due to coprophagy. In the present study, a rapid screening assay was developed to evaluate several potential disinfectants in the presence of large quantities of organic matter, simulating the crop environment. An apparent synergistic combination of *d*-Limonene (DL) and citric acid (CA) was observed when evaluating the potential to eliminate *Salmonella* in the presence of organic material. A method of encapsulation of DL and CA was developed for voluntary consumption by broilers during feed withdrawal. During an 8-h feed withdrawal individual 8-wk-old broilers voluntarily consumed an average of 21.5 capsules (total of 3.44 g material). When eight capsules were force-administered to *Salmonella*-challenged 8-wk-old broilers during an abbreviated 4 h feed withdrawal, *Salmonella* was not recovered using selective enrichment. To evaluate the effect of voluntary capsule consumption, 8-wk-old broilers were challenged with 1×10^8 cfu of *Salmonella* 5 d prior to an 8 h feed withdrawal. When these broilers were allowed unlimited continuous access to capsules containing DL/CA during an 8 h feed withdrawal, 24.8 capsules per broiler were ingested without affecting *Salmonella* recovery from crops. When access to capsules containing DL/CA was limited to the final 45 min of an 8 h feed withdrawal in a similar experiment, an average of 22.2 capsules were consumed by each broiler, resulting in a significant decrease in the number of *Salmonella*-positive crops. Although a number of practical questions and considerations remain, these data suggest that appropriate disinfectants could be administered during preslaughter feed withdrawal for the purpose of reducing foodborne pathogens in crops.

(*Key words*: *Salmonella*, crop, citric acid, *d*-Limonene)

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INTRODUCTION

Salmonella is the most prevalent foodborne pathogen worldwide (Persson and Jendteg, 1992). The majority of research directed toward the source of *Salmonella* contamination at processing has centered around cecal and intestinal colonization (Fanelli *et al.*, 1971; Snoeyenbos *et al.*, 1982; Corrier *et al.*, 1990a,b). Recently, data published from our laboratory has implicated the broiler crop as a significant source of *Salmonella* at commercial processing (Hargis *et al.*, 1995; Ramirez *et al.*, 1997). Hargis *et al.* (1995) reported that the crops from market age broilers were frequently contaminated with *Salmonella* at a processing plant. Indeed, it was reported that the crop was 3.5 times more likely to be contaminated with *Salmonella* than the ceca. Furthermore, the crop was found to rupture 85 times more frequently than the ceca during commercial processing at one plant. In addition, the incidence of *Salmonella* in the crop has been reported to significantly increase following 8 h feed withdrawal in both experimental and commercial settings (Humphrey *et al.*, 1993; Ramirez *et al.*, 1997).

Presently, we evaluated the ability of several candidate compounds to kill *Salmonella* in a simulated crop environment *in vitro*, and selected one combination of compounds for further evaluation *in vivo*, at selected times during preslaughter feed withdrawal.

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Abbreviation Key: BGA = brilliant green agar; CA = citric acid; DL = *d*-Limonene; DSS = dioctylsulfosuccinate; NA = nalidixic acid; NO = novobiocin.

MATERIALS AND METHODS

Salmonella *Source*

A primary poultry isolate of *Salmonella enteritidis*, phage type 13A, was obtained from the National Veterinary Services Laboratory (Ames, IA 50010) for use in our laboratory. This isolate was selected for resistance to nalidixic acid[2] (NA). For these experiments, *Salmonella* was grown in tryptic soy broth[3] for approximately 8 h. The cells were washed 3 times with 0.9% sterile saline by centrifugation (3,000 × *g*) and the approximate concentration of the stock solution was determined spectrophotometrically. The stock solution was serially diluted and confirmed by colony counts of three replicate samples (0.1 mL per replicate) that were spread-plated on brilliant green agar (BGA) plates[4] containing 25 μg / mL novobiocin[2] (NO) and 20 μg / mL NA. The colony-forming units of *Salmonella* were determined by spread plating and reported as the concentration of *Salmonella* in colony-forming units per milliliter.

In Vitro Salmonella *Killing Determinations*

In order to evaluate the relative disinfectant properties of candidate compounds, an assay system was developed to simulate the organic matter found in the broiler crop. For this purpose, an autoclaved unmedicated corn-soybean meal-based broiler ration, formulated to meet or exceed the levels of critical nutrients for growing broilers (NRC, 1984), was used. The test system consisted of sterile 12 × 75 mm borosilicate tubes containing 0.5 g sterile feed, 0.4 mL test solution and 1.6 mL 0.9% sterile saline containing approximately 4 × 10^5 cfu *Salmonella.* For selecting compounds for screening in the present study, consideration was given to potential disinfectant efficacy, minimal toxicity, cost effectiveness and traditional use as disinfectants. The sampling size encompassed 15 different test solutions, with some treatments evaluated in combination. Compounds evaluated included acetic acid, ascorbic acid, chlorhexidine, citric acid (CA), dioctylsulfosuccinate (DSS), *d*-Limonene (DL), DL / CA, ethanol, formaldehyde, formic acid, hipuric acid / ethanol, iodine / ethanol, potassium iodide, quaternary ammonia, shikimic acid, sodium hypochlorite, and 2-imidazolidone. In all experiments, each milliliter of DL was solubilized with 1 g DSS.

For screening, the test tubes and all contents were vortexed for 2 s and incubated for 1 h at 37 C, after which the tubes were vortexed for 2 s and one loop (~20 μL) of the sample was streaked on BGA plates containing 25 μg / mL NO and 20 μg / mL NA. The plates were then incubated at 37 C for 24 h. Following incubation, each plate was evaluated for growth of lactose-negative, NA-resistant *Salmonella* colonies. Each concentration of test solution was streaked on five BGA plates. Plates were scored using a qualitative index of efficacy (Table 1) whereby the following categories were noted: 0 cfu (complete inhibition), 0 to 50 cfu (moderate inhibition), > 50 cfu (ineffective inhibition).

This assay system was modified by changes in incubation, with selected times and temperatures, for quantification of *Salmonella* recovered following exposure to 2% CA[2] and 0.5% DL[2] (Figure 1). In this experiment, the contents of each test tube were maintained at the appropriate temperature (2 or 21 C) prior to incubation to ensure that the samples were held at the indicated temperature for the entire incubation time. Immediately following incubation, the samples were serially diluted and 100 μL of each dilution was spread plated on three BGA plates containing 25 μg / mL NO and 20 μg / mL NA.

TABLE 1. Rapid screening assay of candidate disinfectants for the removal of *Salmonella* in the presence of broiler feed

Test solution	Lowest effective concentrations	Level of inhibition[1]
	(%)	
Acetic acid	0.01, 0.1, 1	Ineffective
	10	Complete
Ascorbic acid	0.01, 0.1, 1, 10	Ineffective
Chlorhexidine	0.002, 0.02	Ineffective
	0.2	Moderate
Citric acid	0.01, 0.1, 1, 2	Ineffective
	10	Complete
Citric acid / *d*-Limonene	2 / 0.5	Complete
d-Limonene	0.01, 0.1, 0.5, 1, 10	Ineffective
Diocytlsulfosuccinate	0.01, 0.1, 1, 10	Ineffective
Ethanol	0.01, 0.1, 1	Ineffective
	10	Moderate
Formaldehyde	0.01, 0.1	Moderate
	1, 10	Complete
Formic acid	0.01	Ineffective
	0.1	Moderate
	1	Complete
Hipuric acid / ethanol	0.02 / 1	Ineffective
	0.2 / 10	Moderate
Iodine / ethanol	0.01 / 1	Ineffective
	0.1 / 10	Moderate
Potassium iodide	0.01, 0.1, 1	Ineffective
	10	Moderate
Quaternary ammonia	0.002, 0.02, 0.2	Ineffective
	2	Complete
Shikimic acid	0.04, 0.4, 4	Ineffective
Sodium hypochlorite	0.001, 0.01, 0.1	Ineffective
	1	Complete
2-imidazolidone	0.01, 0.1, 1, 10	Ineffective

[1]Evaluation of efficacy was done by categorizing colony-forming units streaked on brilliant green agar (BGA) plates following 1 h exposure to compounds at 37 C; 0 cfu (complete inhibition), 0 to 50 cfu (moderate inhibition), and >50 cfu (ineffective inhibition). The initial concentration of *Salmonella* was ~4 × 10^5 cfu.

[2]Sigma Chemical Co., St. Louis, MO 63178-9916.
[3]Difco Laboratories, Detroit, MI 48232.
[4]Becton-Dickinson, Cockeysville, MD 21030.

TABLE 2. Effect of selected concentrations of *d*-Limonene and citric acid on *Salmonella* growth in the presence of tryptic soy broth during 16 h incubation at 37 C

	Optical density[1]					
Treatment	0 h	2 h	6 h	10 h	12 h	16 h
Control	0	0.012	0.168	0.594	0.632	0.710
0.25% CA	0	0.015	0.101	0.208	0.401	0.460
0.0025% DL	0	0.013	0.232	0.604	0.594	0.599
0.25% CA / 0.0025% DL	0	0	0	0	0	0
Control	0	0.012	0.168	0.594	0.632	0.710
0.125% CA	0	0.104	0.152	0.502	0.501	0.505
0.005% DL	0	0.011	0.098	0.578	0.602	0.644
0.125% CA / 0.005% DL	0	0	0	0	0	0
Control	0	0.012	0.168	0.594	0.632	0.710
0.06125% CA	0	0.017	0.142	0.628	0.634	0.646
0.01% DL	0	0.010	0.112	0.390	0.411	0.495
0.06125% CA / 0.01% DL	0	0.006	0.010	0.110	0.210	0.323

[1]*Salmonella* growth indicated by increasing optical density measured by spectrophotometric quantification.

The plates were incubated at 37 C for 24 h, after which recoverable colony-forming units per milliliter were determined.

Effect of Selected Concentrations of DL and CA on Salmonella *Growth*

The effect of selected concentrations of DL and CA on *Salmonella* growth in tryptic soy broth was evaluated by spectrophotometric quantification of optical density using a Spectronic 20D spectrophotometer[5] (Table 2). Sterile borosilicate tubes (13 × 100) containing 2.4 mL tryptic soy broth, 0.3 mL of saline containing the *Salmonella* inoculum (~1×10^5 cfu / mL final concentration) and 0.3 mL of saline containing DL or CA to achieve final concentrations of 0.0, 0.0025, 0.005, or 0.01% DL, 0.0, 0.06125, 0.125, or 0.25% CA, and combinations of 0.25% CA with 0.0025% DL, 0.125% CA with 0.005% DL, and 0.06125% CA with 0.01% DL. Tubes were incubated at 37 C and the optical density determined at 2, 6, 10, 12, and 16 h.

Evaluation of Bactericidal Action of CA and DL

To determine whether the combination of DL and CA was bacteriostatic or bactericidal, *Salmonella* (~1×10^5 cfu / mL) was incubated with or without DL (0.2%) and CA (1.0%) in tryptic soy broth for 1 h at 37 C in 15 mL conical centrifuge tubes prior to washing and spread plate determination of recoverable colony-forming units per milliliter as described above. Following incubation, the cell suspension was centrifuged (1,900 RCF) for 30 min and the cells resuspended in fresh tryptic soy broth. This process was repeated twice prior to dilution and spread plating. Total colony-forming units per milliliter were determined for both cells treated with DL / CA and cells untreated with DL / CA but subjected to the washing procedure to account for inefficiencies in recovery during centrifugal pelleting of bacteria.

Gelatin Encapsulation of CA and DL for Ingestion by Broilers

Because incorporation of DL and CA in drinking water or feed caused nearly absolute refusal of consumption by broilers in preliminary experiments (data not shown), the following method of encapsulation was employed. Num-

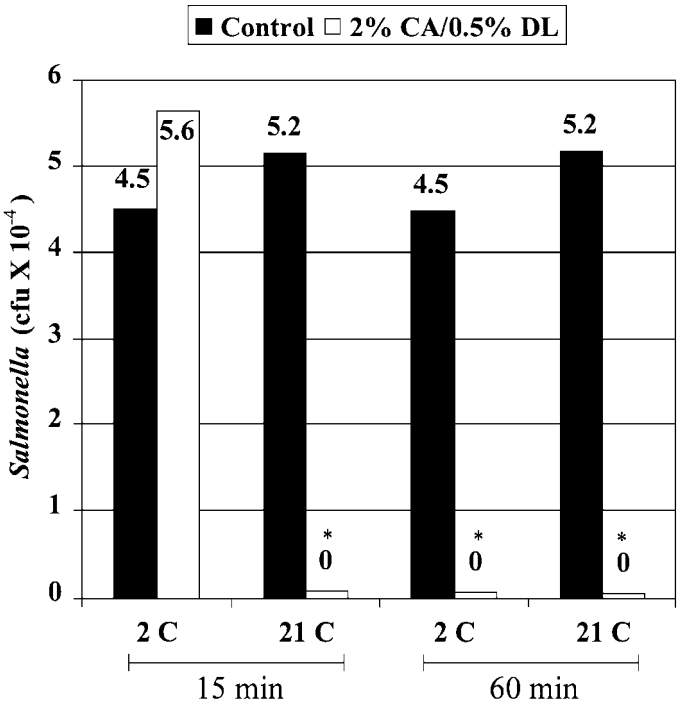

FIGURE 1. *d*-Limonene (DL) (0.5%) combined with citric acid (CA) (2%) were used in a feed slurry assay that contained approximately 1×10^6 cfu of *Salmonella* and incubated at 2 or 21 C for 15 or 60 min. *Asterisk indicates significantly ($P < 0.05$) lower recoverable colony-forming units of *Salmonella* than the control.

[5]Milton Roy Co., Rochester, NY 14625.

TABLE 3. Cumulative voluntary capsule[1] consumption per broiler during an 8 h feed withdrawal

	Time intervals[2]			
Pen[3]	2 h	4 h	6 h	8 h
1	6.6	12.6	17.0	19.6
2	9.0	19.6	21.8	22.4
3	8.8	16.8	22.2	23.4
4	8.8	14.2	19.4	20.4
$\bar{x}$	8.3	15.8	20.1	21.5

[1]Each gelatin capsule contained an average of 0.12 g citric acid and 0.1 mL *d*-Limonene.

[2]Capsules ingested were calculated every 2 h during an 8 h feed withdrawal.

[3]Five 59-d-old broilers were used in each pen.

ber 4 pharmaceutical grade gelatin capsules[6] were manually filled by hand with a paste containing CA, DL, DSS[2], and all purpose wheat flour (local grocery), blended at a ratio of 12 g CA:1 mL DL:1 g DSS:2 g flour, with a mortar and pestle. Prior to blending of these substances, DL was solubilized with DSS at a 1:1 vol/wt ratio. Each capsule contained an average 0.16 g of prepared paste. The capsules were then briefly wetted with distilled water and immediately rolled in white flour. Flour-coated capsules were then lightly sprayed by hand using an adjustable spray/wash bottle[7] and diluted yellow food coloring[8] (1 mL food coloring to 100 mL water) to achieve a uniform color distribution of the approximate color density of yellow corn. Capsules were allowed to dry for 5 to 10 h at 22 C prior to use.

Experimental Birds

Broilers used in these experiments were obtained from a local poultry producer and placed within a biological hazard isolation building. The isolation building temperature was held at approximately 22 C with 24 h fluorescent lighting. Feed and water were provided for *ad libitum* consumption, the ration consisted of a standard unmedicated diet based on corn and soybean meal (Texas A&M University Poultry Center) that contained or exceeded level of nutrients recommended by the National Research Council (NRC, 1984). Water was continuously available during the feed withdrawal period.

Efficacy of Encapsulated DL and CA

Initially, voluntary consumption of DL/CA filled capsules by market age broilers was determined. Eight-wk-old broilers were divided into four pens and maintained on used pine shaving litter for 1 d. Following 24 h of acclimation to the pen setting, the broilers were subjected to an 8 h feed withdrawal and provided unlimited access to water and DL/CA filled capsules. Capsules were provided in feed pans with feed removed at the initiation of feed withdrawal and the number of cumulative capsules ingested per broiler was determined at 2, 4, 6, and 8 h (Table 3).

To control for possible variation in capsule consumption and to determine the efficacy of ingested capsules on *Salmonella* recovery from crops, the effects of forced administration of DL/CA filled capsules on incidence of *Salmonella* recovery in broiler crops was evaluated. Twenty-five 8-wk-old broilers were divided into five groups and challenged with ~1×10^5 cfu of *Salmonella* by oral gavage 1 h prior to an abbreviated 4 h feed withdrawal. Prior to challenge, the broilers were maintained on used pine shaving litter. Two hours following initiation of feed withdrawal, the groups were treated with either 0, 1, 2, 4, or 8 capsules. Water was provided for *ad libitum* intake during the withdrawal period. Immediately following feed withdrawal, the broilers were killed and the crops were removed and processed for culture as described below (Table 4).

Finally, the effects of *ad libitum* access to DL/CA capsules on the incidence of *Salmonella* recovery from the crop during an 8 h feed withdrawal was evaluated (Table 5). Thirty-four 8-wk-old broilers were divided into two groups and challenged with ~1×10^8 cfu of *Salmonella* by oral gavage 5 d prior to feed withdrawal. On the 5th d postchallenge, one group was provided with unlimited access to capsules during the entire 8 h feed withdrawal for comparison to feed withdrawal alone. In a subsequent experiment, 35 8-wk-old broilers were similarly challenged and divided into two groups and subjected to either feed withdrawal alone or unlimited access to capsules during the final 45 min of the feed withdrawal period. Immediately following the withdrawal period, broilers were killed and crops were removed and subjected to culture for *Salmonella* as described below. Additionally, the number of capsules ingested were enumerated and presented as the average number of capsules consumed per broiler (Table 5).

TABLE 4. Forced oral administration[1] of capsules[2] during an abbreviated 4 h feed withdrawal

Treatment[3]	*Salmonella* positive crops/total
Control	5/5
8 capsules	0/5*
4 capsules	5/5
2 capsules	4/5
1 capsule	4/5

[1]Capsules administered 2 h into a 4 h feed withdrawal.

[2]Each gelatin capsule contained an average of 0.12 g citric acid and 0.1 mL *d*-Limonene.

[3]Broilers orally challenged with 1×10^5 *Salmonella* 1 h prior to 4 h feed withdrawal.

*Asterisk indicates significantly different ($P < 0.05$) from the control group.

[6]Professional Compounding Centers of America, Houston, TX 77099.
[7]VWR Scientific Products, McGaw Park, IL 60085.
[8]McCormick and Co., Hunt Valley, MD 21030.

TABLE 5. *Ad libitum* feeding of *d*-Limonene (DL) and citric acid (CA) capsules[1] to *Salmonella* infected broilers during an 8 h feed withdrawal

Treatment	*Salmonella*-positive crops/total	Percentage positive	Average number of capsules consumed/broiler
Control	12/17	70.6	N/A
DL/CA capsules[2]	13/17	76.5	24.79
Control	16/17	94.1	N/A
DL/CA capsules[3]	7/18	38.9*	22.22

[1]Each gelatin capsule contained an average of 0.12 g CA and 0.1 mL DL.

[2]Capsules were provided during the entire 8 h feed withdrawal.

[3]Capsules were provided during the final 45 min of feed withdrawal.

*Asterisk indicates significantly ($P < 0.05$) lower recoverable colony-forming units of *Salmonella* than the control.

Sample Collection and Culture

All broilers were killed by cervical dislocation, and the crops and crop contents of each broiler were removed aseptically by clamping across the pre- and post-crop esophagi using a surgical Carmalt forcep and immersed in boiling water for 1 s to reduce external contamination of the crop. As previously reported, immersion of crops or ceca in boiling water for 1 s effectively removed all detectable *Salmonella* from the surface of intentionally contaminated crops while not affecting recovery of *Salmonella* injected into the lumen of the tissue (Ramirez *et al.*, 1997). The crop was sectioned aseptically below the clamp and the body of the crop, with the lumen and contents exposed, was collected aseptically in individual Whirlpak™[9] bags. The crops were enriched in 20 mL of tetrathionate broth,[4] stomached for 30 s, incubated for 24 h at 37 C, and streaked on BGA plates containing 25 μg/mL NO and 20 μg/mL NA. The plates were incubated for 24 h at 37 C and examined for the presence of lactose-negative, NA-resistant *Salmonella* colonies. Those plates supporting non-*Salmonella* bacterial growth with some potential *Salmonella* colonies were restreaked on NO-NA plates using one isolated suspect *Salmonella* colony. These plates were incubated for 24 h at 37 C, and colonies consistent with *Salmonella* were further confirmed serologically.

Statistical Analysis

The chi-square test of independence was used to determine significant differences ($P < 0.05$) in *Salmonella* recovery between treatments within experiments as described in the tables and figure (Zar, 1984).

[9]Nasco, Fort Atkinson, WI 53583.

RESULTS AND DISCUSSION

Several candidate disinfectants were initially compared for anti-*Salmonella* activity under simulated crop conditions *in vitro*. Solutions of ascorbic acid, DL, DSS, or 2-imidazolidone did not reduce *Salmonella* at concentrations ≤ 10% final concentration. Potassium iodide, ethanol, chlorhexidine, acetic acid, or CA caused apparent reduction in recovered *Salmonella* at concentrations of 10% but did not apparently affect *Salmonella* recovery at lower concentrations (1, 0.1, or 0.01%). A final concentration of 0.1% iodine or 0.2% hippuric acid combined with 10% ethanol caused an apparent reduction, but not elimination, of recovered *Salmonella*; and lower concentrations were not effective. Ethanol alone at 10% had limited inhibitory activity, and shikimic acid at 4% or less caused no inhibitory activity. Concentrations of 1% formic acid, 1% formaldehyde, 1% sodium hypochlorite, or 2% quaternary ammonia or higher resulted in elimination of recoverable *Salmonella*. Concentrations of DL (e.g., ≤ 10%) or CA (e.g., ≤ 2%) that did not reduce *Salmonella* recovery when tested alone completely eliminated our ability to recover *Salmonella* when used in combination (0.5% DL/2% CA). Because DL and CA are relatively inexpensive and nontoxic, combinations of these compounds were selected for further evaluation.

When the effect of selected concentrations of DL or CA on *Salmonella* growth in broth culture was evaluated, clear additive or synergistic inhibitory effects of combination of these compounds were observed (Table 2). Furthermore, when a selected combination of DL (0.2%) and CA (1%) were incubated with *Salmonella*, prior to repeated washing of cells by centrifugation and resuspension in fresh medium, *Salmonella* could not be recovered on nutrient agar plates (data not shown). This observation suggests that the unknown anti-*Salmonella* action of DL/CA in combination may be bactericidal, rather than bacteriostatic, in nature.

Further evaluation of the combination of DL and CA indicated that 2% CA combined with 0.5% DL completely eliminated detectable *Salmonella* in the presence of autoclaved broiler feed and saline at 21 C for 15 or 60 min (Figure 1). At an incubation temperature of 2 C, this combination was not effective at the 15 min incubation but did cause a marked and significant ($P < 0.05$) decrease in the number of *Salmonella* recovered following 60 min of incubation (Figure 1).

Because preliminary experiments indicated that feed and water refusal were near absolute levels when adulterated with effective concentrations of DL and CA (data not shown), these compounds were encapsulated for voluntary consumption experiments. Calculating the number of DL/CA treated capsules that were voluntarily ingested by 8-wk-old broilers during an 8 h feed withdraw indicated that they readily consumed the capsules during the first 4 h (~15 capsules). During the final 4 h of feed withdrawal, voluntary consumption

substantially subsided with only 1.4 capsules consumed per broiler during the final 2 h of feed withdrawal (Table 2).

To evaluate the effect of consumption of known numbers of capsules, selected numbers of capsules were administered to *Salmonella*-infected broilers after 2 h of a simulated 4 h feed withdrawal. Nearly simultaneous force-administration of eight capsules in this experiment eliminated our ability to recover *Salmonella* from crops (Table 3). However, forced ingestion of four or fewer capsules was not effective for *Salmonella* removal. Residual feed in the crop at necropsy varied but was not associated with treatment efficacy.

When *ad libitum* access to DL/CA filled capsules was provided during feed withdrawal, 24.8 capsules per broiler were consumed during the 8 h period. Interestingly, no effect of DL/CA consumption on *Salmonella* recovery from crops was noted when capsules were continuously available during withdrawal (Table 5). As previous studies have indicated that ingested material may immediately bypass the crop in fasted birds (Duke, 1986), we hypothesized that the capsules were not retained by the crop when access was provided over this protracted 8 h period. Alternatively, as voluntary ingestion tended to be reduced during the latter part of an 8 h feed withdrawal (Table 3), this failure might have been due to recontamination of the crops during the late stages of feed withdrawal. For these reasons, broiler access to capsules was restricted to the last 45 min of an 8 h feed withdrawal in a subsequent experiment, resulting in similar total consumption of capsules during this abbreviated time and improved efficacy for *Salmonella* removal from crops (Table 5).

While the combination of DL and CA has remarkable anti-*Salmonella* properties, via an unknown mechanism in the presence of large amounts of organic matter, the practical administration of these compounds to poultry during feed withdrawal is complicated by broiler refusal to consume these compounds when solubilized in water or dispersed in feed at effective concentrations (data not shown). The present data suggests that methods for disguising the odor or flavor of potential disinfectants for voluntary consumption can be accomplished and that effective disinfectants can reduce or eliminate the ability to recover *Salmonella* from contaminated crops ante-mortem. A number of practical considerations must be addressed prior to commercial utilization of ante-mortem crop disinfection, including development of easy and cost-effective delivery systems, human and animal toxicity evaluations, and retention of the disinfectant material in the crop for sufficient times to reduce or eliminate foodborne pathogens. Additionally, the possibility of recontamination of crops during preslaughter transport and holding remains to be evaluated.

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