Evaluation of *Salmonella enteritidis* in Molting Hens After Administration of an Experimental Chlorate Product (for Nine Days) in the Drinking Water and Feeding an Alfalfa Molt Diet

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ABSTRACT The method most commonly used to induce molting and stimulate multiple egg-laying cycles in laying hens for commercial egg production is to fast the hens. Unfortunately, increased risk of *Salmonella enteritidis* (SE) infection may result from the use of this method. Methods to stimulate multiple egg-laying cycles without increasing the risk of SE infection are needed. Hens over 50 wk of age were divided into 12 groups of 11 hens each and placed in individual laying cages. One week prior to dietary changes, hens were placed on an 8-h light and 16-h dark photoperiod that continued for the 9-d molt. All hens were challenged orally with 10^6 cfu of SE on the fourth day of the molt. Treatments were nonfed hens with distilled water (NFD), nonfed hens with the experimental chlorate product (ECP, which provided 15 mM chlorate ion) water (NFECP), alfalfa diets with distilled water (ALD), and alfalfa diets with ECP water (ALECP). In the NFD hens, 67% (log_{10} 2.74) of the crops and 94% (log_{10} 5.62) of the ceca were colonized, whereas for the NFECP hens significant reductions to 22% (log_{10} 1.05) of the crops and 61% (log_{10} 2.44) of the ceca were observed. In the ALD hens, 61% (log_{10} 2.52) of the crops and 94% (log_{10} 4.06) of the ceca were colonized. In the ALECP hens, highly significant reductions to 11% (log_{10} 1.26) of the crops and 39% (log_{10} 1.12) of the ceca were observed. When compared with the NFD hens, significant reductions in SE invasion of the ovary, liver, and spleen occurred in all other treatments, except the ovary in the ALD hens. The low alfalfa intake is probably a factor in our lowered protection against SE when compared with previous results. For several parameters, these results suggest that ECP or the combination of ECP and alfalfa may be a useful tool to reduce the risk of SE during an induced molt.

(Key words: *Salmonella*, molt, alfalfa diet, chlorate, drinking water)

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INTRODUCTION

Salmonellosis is one of the most common foodborne diseases with an estimated 800,000 to 4 million human infections reported each year in the United States alone. From 1996 to 1999 *Salmonella enteritidis* (SE) illness rates declined 48% and from 1996 to 2000 the incidence per 100,000 population decreased from 2.5 to 1.8 (USDHHS-CDC, 2000). Unfortunately based on the most recent epidemiological reports, this previously reported decline in SE incidence has now been eliminated by a renewed upsurge in SE infections (USDHHS-CDC, 2003). Some of these increases could be partially attributed to increased technology and surveillance procedures incorporated by the industry over the last several years. Contamination of egg products by SE is the most commonly linked source of human foodborne illness (Patrick et al., 2004). Therefore, developing preharvest intervention strategies to reduce this source of contamination is warranted.

It has been suggested that the high incidence of SE infection may be linked to the specific stressful management practice of inducing a molt to stimulate multiple egg-laying cycles in hens (Holt, 2003; Ricke, 2003; Park et al., 2004). According to a National Animal Health Monitoring Service report (2000), over 94% of commercial laying facilities in the western United States use induced molting as a means of increasing productivity in flocks. Feed withdrawal is the primary method used in the layer industry to induce the molt but has been shown experimentally to increase SE recovery from crops, increase invasion of organs in chickens, and increase horizontal

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**Abbreviation Key:** ALD = alfalfa diets with distilled water; ALECP = alfalfa diets with ECP water; BGA = brilliant green agar; ECP = experimental chlorate product; NFD = nonfed with distilled water; NFECP = nonfed with ECP water; NA = nalidixic acid; NO = novobiocin; NREP = nitrate reductase enzymatic pathway; ST = *Salmonella* Typhimurium.
The poultry layer industry needs to assess new intervention strategies that reduce enteric pathogens. These products should be a part of an alternative molting procedure that does not require feed withdrawal but allows layer house managers to retain the economic advantages of obtaining a second laying cycle with a high production rate of quality eggs from laying hens via molting without increasing the risk of a SE problem. One preharvest intervention strategy currently being evaluated for pathogen reduction is the use of a chlorate ion based product that uses the respiratory nitrate reductase enzymatic pathway (NREP). The NREP is part of a nitrate respiration process in some Enteric and sulfate-reducing bacteria; this pathway uses nitrate as a terminal electron acceptor during anaerobic metabolism (Richardson, 2001). The NREP also co-metabolically reduces chlorate to a cytotoxic chlorite ion; increased levels of this ion become lethal to bacteria (Stewart, 1988). This NREP utilizing the chlorate ion has been shown to reduce enteric pathogens in chickens, sheep, beef cattle, and pigs (Anderson et al., 2001; Byrd et al., 2003; Edrington et al., 2003).

Use of molting diets that retain protective microflora during induced molting would provide poultry producers with dietary approaches that could more easily be incorporated into current management practices and avoid the more drastic measure of feed withdrawal that is compounded by increases in SE contamination (Park et al., 2004). One dietary supplement that has been used is alfalfa. Alfalfa has been shown to reduce growth and egg production in layers when fed in high concentrations (Heywang, 1950). Alfalfa has also been shown to effectively cause ovarian regression as well as retaining initial post molt egg production responses when compared with feed withdrawal (Landers et al., 2005 a,b). Woodward et al. (2005) demonstrated that 100% alfalfa diets reduced SE infection during molt. However, they observed that elimination of SE was incomplete in some trials, suggesting that additional amendments might be needed to increase effectiveness of this alternative molting regimen. The objective of the present investigation was to determine if ECP in the presence or absence of an alfalfa diet would further reduce intestinal colonization by SE in laying hens and to determine if key characteristic changes in the chicken intestinal microenvironment could be linked to diets, feed consumption, or ECP in the drinking water and SE colonization. This report will provide the poultry industry with a scientifically based rationale for possible management alternatives that reduce molting as a major risk for SE contamination and will be more acceptable to the populace.

**Experimental Design**

Single Comb White Leghorn hens (W-36) over 50 wk of age were obtained from a local commercial laying flock. Laying hens were placed in wire layer cages and provided free access to water and a balanced unmedicated corn-soybean meal based mash layer diet that met or exceeded the National Research Council recommendations for nutrients (1994). The diet was calculated to provide 2,818 kcal of metabolizable energy per kilogram, 16.5% crude protein, 3.5% calcium, and 0.48% available phosphorus. Feed samples (25 g) and fecal samples (1 g) were collected and examined for salmonellae by successive culturing in tetrathionate broth and brilliant green agar2 (BGA) as described by Andrews et al. (1995). Salmonella-positive hens were eliminated from the study. Salmonella-positive feed was not found. Hens were allowed to acclimate for 2 wk, followed by random assignment to 3 replicates of 11 hens in each of 4 treatment groups, designated as follows: (1) nonfed hens with distilled water (NFD), (2) nonfed hens with ECP (provided 15 mM chloride ion) water (NFECW), (3) alfalfa diets with distilled water (ALD), and (4) alfalfa diets with ECP water (ALECP).

On fourth day of the molt induction, all hens in each treatment group were challenged by crop gavage with 1 mL of inoculum containing approximately 10^6 cfu of naladixic acid (NA)-novobiocin (NO)-resistant SE. The challenge dosage was slightly higher than the 5.6 × 10^4 cfu dosages reported previously to be the mean infectious dosage (ID50) for SE in nonmolted hens (Holt, 1993).

At the conclusion of the study, all hens were euthanized and the crop, ceca, liver, spleen, and ovary were excised aseptically. Crop pH was determined as described previously (Durant et al., 1999). Briefly, crop pH was determined insertion of a sterile glass pH electrode through an incision in the crop wall, ensuring that the electrode remained in contact with the crop mucosal surface (Durant et al., 1999). Each crop was excised and cut open aseptically, and the entire crop and contents together with 10 mL of sterile distilled water were blended for 1 min and serial dilutions were preformed. Samples of the crop, ceca, liver, spleen, and ovary of each hen were cultured for SE.

**Molt Procedure**

Feed deprivation by a modification (Holt, 1993) of a previously described procedure (Brake et al., 1982) was used to induce molt. Seven days before feed removal or feeding the alfalfa diet and adding ECP to the drinking water, hens were exposed to a photoperiod of 8 h light: 16 h dark, which was continued throughout the experiment. Beginning on d 0, feed was withdrawn for 9 d, or hens received the alfalfa diet, after which the study was terminated.
Bacterial Strain

A primary poultry isolate of SE (phage type 13A), selected for resistance to NO and NA at USDA-ARS, College Station, TX, was used. Media to culture the resistant isolate in experimental studies contained 25 µg of NO and 20 µg of NA per milliliter. The challenge inoculum was prepared from an overnight culture, which had been previously transferred 3 times in trypticase soy broth. The culture was serially diluted in sterile phosphate-buffered saline to approximately 10⁶ cfu/mL. The colony-forming units of the challenge inoculum was confirmed by plating onto BGA plates.²

Recovery of Salmonella

All samples tested for Salmonella (+/−) including ceca, crop, liver, spleen, and ovary were minced with sterile scissors and cultured. The organ samples were incubated for 24 h at 41°C in Rappaport-vassiliadis R10 broth.² After incubation, the broth was streaked onto a BGA² plate containing 25 µg/mL NO and 20 µg/mL NA, incubated for an additional 24 h at 37°C, and examined for the presence of SE colonies. Samples that were direct plated for enumeration of total colony-forming units were stomached, and 0.25 g of cecal or crop contents were placed into a 6-mL snap cap polypropylene tube containing 2.25 mL of Butterfield’s solution. Serial dilutions of each sample were performed using 0.5 mL of the sample and placed into 4.5 mL of Butterfield’s solution for final concentrations of 10, 100, and 1,000 cfu/mL. One hundred microliters from each dilution tube was placed onto a BGA plate and spread plated with a bacterial cell spreader. All of the plates were incubated for 24 h at 37°C, and the number of Salmonella colony-forming units were determined and expressed as log₁₀ Salmonella per gram of cecal or crop content. Cecal and crop contents that were negative at a 100-fold dilution on BGA plates but were positive at a 10-fold dilution on BGA plating were assigned 1.00 log₁₀ Salmonella per gram of cecal contents (Corrier et al., 1993; 1995). Suspect colonies were confirmed by biochemical tests on triple sugar-iron agar and lysine-iron agar and further identified as SE serologically using Salmonella O antiserum group D, factors 1, 9, 12.²

Statistical Analysis

Chi-squared analysis was used to determine significant differences between treatment groups for incidences of SE colonization of the crop, ceca, liver, spleen, and ovary (Luginbuke and Schlotzhauer, 1987). Differences in the log₁₀ colony-forming units of SE counts among treatment groups were determined by analysis of variance using the general linear models procedures. Significant differences were further separated using Duncan’s multiple range tests and commercial statistical analysis software (SAS Institute, Cary, NC; Luginbuke and Schlotzhauer, 1987). All data analyzed by statistical analyses were considered significant at P < 0.05.

RESULTS AND DISCUSSION

When evaluating the effects of the ECP on Salmonella in the crops, in the drinking water alone or in combination with an alfalfa diet, there were significant reductions in the number of SE recovered and in the overall incidence as compared with the controls (Table 1). In the NFD controls the hens had a 69.4% (log₁₀ 2.74) infectivity rate. Interestingly the hens in the NFEC and the ALECP had significant reductions (P ≤ 0.005) in Salmonella with 33.3% (log₁₀ 1.22) and 11.1% (log₁₀ 1.27), respectively. When evaluating the ceca of hens provided ECP in the drinking water alone or in combination with an alfalfa diet there were significant reductions in the number of SE recovered from the ceca and the overall incidence as compared with the controls (Table 2). In these trials the approximate alfalfa feed intake was 4.6 g / d per hen, which was considerably lower than the intake of 17.5 g observed by Woodward et al. (2005). This lower intake of alfalfa was probably a factor in our lowered protection against SE when compared with previous results. Woodward et al. (2005) suggested the incomplete elimination of SE in alfalfa molted hens observed in their trials was possibly due to decreases in feed intake and general decreases in volatile fatty acid production. In the NFD controls, the hens had a 97.2% (log₁₀ 5.35) infectivity rate; however, hens on the NFEC and ALECP treatments had significant reductions (P ≤ 0.001) in Salmonella of 58.3% (log₁₀ 3.10) and 41.6% (log₁₀ 1.73), respectively.

These results show similar trends that have been previously reported by our laboratory. McReynolds and coworkers showed that ECP, when administered in the drinking water, significantly reduced Salmonella Typhimurium (ST) colonization in the ceca of challenged seed-

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²National Veterinary Services Laboratory, Ames, IA.
³Oxoid, Unipath Ltd., Hampshire, UK.
ers and nonchallenged contacts. The data revealed that the ECP and a CE culture provided the best protection against *Salmonella* when administered together, indicating that the ECP did not adversely affect many of the populations of bacteria in the gastrointestinal system and that bacteria using the NREP were affected (McReynolds et al., 2004). Byrd and coworkers showed that the ECP, when administered in the drinking water, significantly reduced ST. The incidence of ST was reduced in the crop from 36.7% in the controls to 2% in the treated groups (Byrd et al., 2003). In the present investigation, it has been shown that the ALECP treatment group did have the most significant reduction in *Salmonella* among the treatment groups.

There was a significant increase in the lactic acid concentration in the ceca; the NFD controls had 16.11 µM/g of cecal contents compared with 30.83 µM/g of cecal contents in the ALECP treatment group. There was a significant increase in the production of lactic acid in hens in the ALECP treatment group when compared with the NFD controls with levels of 14.03 µM/g of crop contents compared with 10.53 µM/g of crop for the respective groups. Woodward et al. (2005) also observed increased cecal lactic acid concentrations in alfalfa molted birds when compared with feed withdrawal birds.

Understanding the dynamics of the ecology in the crop and gastrointestinal system in poultry has been an extremely difficult subject, and only limited studies have examined the relationship between foodborne microbial pathogenesis and fermentation characteristics of the indigenous population (Ricke, 2003). However, Durant and coworkers have shown in vitro that the expression of virulence genes in *Salmonella* can be up-regulated during a feed deprived state; this research has illustrated the nutrient requirements for the up-regulation or down-regulation of the *HilA* gene that is required for epithelial cell invasion. In their work, they showed that increased levels of lactate in a culture medium with adjusted pH significantly inhibits expression of the *HilA* gene (Durant et al., 1999). Lactate (lactic acid) is the fermentation product of lactobacillus species that are located in the crop (Fuller, 1977). These bacteria regulate the pH of the crop and maintain the homeostatic environment; fluctuations in commensal bacteria can give rise to opportunistic bacteria. This is one possible explanation as to why the ALECP treatment group was so effective in reducing SE.

One of the characteristics of SE is being able to infect key organ tissues, including livers, spleens, and ovaries of molting hens. In the present investigation, the ECP alone or in combination with the alfalfa diet significantly reduced SE invasion in all these tissues (Table 3). When comparing the colonization of the ovaries, the NFD (control) hens had a 75.0% infectivity rate, which was significantly reduced (P ≤ 0.005) to 41.6% in the NFECP and to 25.0% in the ALECP treatment groups. The reductions of SE were similar for the spleens; the NFD control hens had a 86.1% infectivity rate, which was significantly reduced (P ≤ 0.005) to 50.0% in the NFECP and to 25.0% in the ALECP treatment groups.

In the present investigation, ECP added to the drinking water of laying hens being induced to molt by fasting was effective in reducing the incidence and severity of colonization in the crop and ceca and organ invasion by SE. The ECP was also effective when included in the drinking water of laying hens being induced to molt by fasting or feed withdrawal. Furthermore, concentrations of lactic acid were also significantly increased in the crop and cecum of birds administered ECP combined with the alfalfa diet. Use of these combinations of alfalfa and ECP would allow the industry an opportunity to reduce stress factors and bacterial pathogen loads from commercial poultry. Use of these new innovative technologies could potentially reduce the number of foodborne pathogens entering the food chain.

**REFERENCES**


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**TABLE 2. Evaluation of *Salmonella enteritidis* (SE) colonization in the ceca, in hens that were administered an experimental chlorate compound (ECP) with and without alfalfa (2 trials)**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SE in ceca contents (log10/g)</th>
<th>SE (+) ceca /total hens (%)</th>
<th>LA in ceca contents (µM/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFD²</td>
<td>5.35 ± 0.34⁴</td>
<td>35/36 (97.2)</td>
<td>16.11 ± 2.89⁵</td>
</tr>
<tr>
<td>NFECP</td>
<td>3.10 ± 0.43⁶</td>
<td>21/36 (58.3)</td>
<td>10.53 ± 1.43⁵</td>
</tr>
<tr>
<td>ALD</td>
<td>4.76 ± 0.32⁵</td>
<td>33/36 (91.6)</td>
<td>16.99 ± 2.96⁶</td>
</tr>
<tr>
<td>ALECP</td>
<td>1.73 ± 0.30⁰</td>
<td>15/36 (41.6)</td>
<td>30.83 ± 3.96⁶</td>
</tr>
</tbody>
</table>

²ECP concentration equivalent to a 15 mM chlorate ion concentration.
³Mean ± standard deviation.
⁴A significant difference was found between the number of positive controls and positive, treated crops or ceca (*P ≤ 0.05*).
⁵A significant difference was found between the number of positive controls and positive, treated crops or ceca (*P ≤ 0.05*).

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**TABLE 3. Evaluation of *Salmonella enteritidis* (SE) invasion of organ tissue, in hens that were administered an experimental chlorate compound (ECP) with and without alfalfa (2 trials)**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SE (+) ovaries /total hens (%)</th>
<th>SE (+) spleens /total hens (%)</th>
<th>SE (+) livers /total hens (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFD²</td>
<td>27/36 (75.0)</td>
<td>31/36 (86.1)</td>
<td>34/36 (94.4)</td>
</tr>
<tr>
<td>NFECP</td>
<td>15/36 (41.6)</td>
<td>18/36 (50.0)</td>
<td>19/36 (52.7)</td>
</tr>
<tr>
<td>ALD</td>
<td>28/36 (77.7)</td>
<td>24/36 (66.6)</td>
<td>29/36 (80.5)</td>
</tr>
<tr>
<td>ALECP</td>
<td>9/36 (25.0)</td>
<td>10/36 (27.7)</td>
<td>7/36 (19.4)</td>
</tr>
</tbody>
</table>

¹ECP concentration equivalent to a 15 mM chlorate ion concentration.
²NFD = nonfed; NFECP = nonfed + ECP; ALD = alfalfa diet; ALECP = alfalfa + ECP.
³A significant difference was found between the number of positive controls and positive, treated crops or ceca (*P ≤ 0.05, **P ≤ 0.001*).


