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ABSTRACT The objectives of this study were to enumerate Salmonella enterica serovar Enteritidis colonization in fecal, cecal, and internal organs, and to compare the level of virulence gene expression (hilA) of experimentally challenged laying hens fed different dietary molt-induction regimens. Twelve Salmonella-free Single Comb Leghorn hens (>50 wk old) hens were randomly assigned to each of 6 treatment groups designated based on diet in 2 trials: 1) feed withdrawal Salmonella Enteritidis-positive (FW+), 2) fully fed Salmonella Enteritidis-positive (FF+), 3) 100% alfalfa crumble Salmonella Enteritidis-positive (ALC+), 4) feed withdrawal Salmonella Enteritidis-negative, 5) fully fed Salmonella Enteritidis-negative, and 6) 100% alfalfa crumble Salmonella Enteritidis-negative. A forced molt was induced by a 12-d alfalfa diet and a feed-withdrawal regimen. On d 4 of the molt, all hens in groups 1, 2, and 3 were challenged by crop gavage with 1 mL of inocula containing approximately 10⁶ cfu of nalidixic acid- and novobiocin-resistant Salmonella Enteritidis (phage type 13A). At the conclusion of both trials, all hens were euthanized and Salmonella Enteritidis colonization was enumerated in the cecal contents, liver, spleen, and ovaries. In addition, fecal (d 4 and 8) and cecal samples (necropsy at d 12) were collected postchallenge from treatment groups 1, 2, and 3 (Salmonella Enteritidis-positive) to quantify hilA expression by PCR. In both trials, all nonchallenged birds were Salmonella Enteritidis-negative; therefore, no further analysis was done. In trial 1, a 2-fold reduction in Salmonella Enteritidis colonization was observed in the ALC+ hens (log₁₀ Salmonella Enteritidis of 1.99) compared with the FW+ hens (log₁₀ Salmonella Enteritidis of 3.89). In trial 2, a 4-fold reduction in Salmonella Enteritidis colonization was observed in the ALC+ hens (log₁₀ Salmonella Enteritidis of 1.27) compared with the FW+ hens (log₁₀ Salmonella Enteritidis of 5.12). In trial 2, Salmonella Enteritidis colonization in spleens was higher (P ≤ 0.05) in FW+ hens compared with ALC+ and FF+ hens. Relative expression of hilA was higher (P ≤ 0.05) in FW+ compared with FF+ hens, whereas the FF+ and ALC+ groups were not different (P > 0.05). In trial 2, hilA expression in FW+ hens was higher (P ≤ 0.05) for d 6, 11, and 12, respectively, when compared with ALC+ and FF+ hens. The results of these studies support the concept that changes in the gastrointestinal tract microenvironment, such as those created during feed deprivation, encourage Salmonella Enteritidis virulence and susceptibility in molted hens.

Key words: Salmonella, hilA, alfalfa, molting, virulence

INTRODUCTION

To induce molting, feed withdrawal has been the primary method of choice to achieve a new egg-laying cycle rapidly and economically (Brake, 1993; Holt, 1995). However, feed withdrawal compromises the birds’ immune system, making them susceptible to infection by a number of microorganisms, including Salmonella enterica serovar Enteritidis (Holt, 2003). Feed withdrawal-associated stress causes increased susceptibility to Salmonella Enteritidis infection in the gastrointestinal (GI) tract (Holt, 1993, 2003; Corrier et al., 1997; Durant et al., 1999; Ricke, 2003), usually marked by increased intestinal shedding and colonization in the internal organs, such as the liver, spleen, and ovaries (Holt, 1993; Thiagarajan et al., 1994; Holt et al., 1995). The genes required for Salmonella pathogenesis and subsequent infection are located on Salmonella pathogenicity island 1, at centisome 63 on the chromosome (Galán and Sansonetti, 1996). The transcriptional activator HiiA, encoded by Salmonella pathogenicity island 1, coordinate regulates the expression of invasion genes in

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Salmonella in response to environmental conditions, including stress and low nutrient concentrations (Bajaj et al., 1996). In vitro experiments have indicated a positive relationship between increased hilA expression and potential for increased Salmonella Enteritidis infection in hens during feed withdrawal (Durant et al., 1999, 2000). Therefore, evaluation of hilA expression could be an important consideration for estimating the potential susceptibility of hens to Salmonella Enteritidis colonization and infection.

Alternative molting methods are known to reduce Salmonella Enteritidis invasion in the GI tract of experimental hens (Seo et al., 2001; Holt, 2003; Ricke, 2003; Moore et al., 2004; Park et al., 2004a, b; Ricke et al. 2004; McReynolds et al., 2005, 2006; Woodward et al., 2005). Fermentable high-fiber diets such as alfalfa meal have been examined as potential molting approaches that retain normal microbial flora and reduce proliferation of Salmonella Enteritidis (Woodward et al., 2005). Alfalfa meal and pellets, alone or in combination with a layer ration, have been shown to be capable of causing ovarian regression during molting and restoration of optimal postmolt egg production comparable to feed withdrawal-induced molt (Donalson et al., 2005; Landers et al., 2005a, b). The use of alfalfa meal as a single dietary source appears to support microbial fermentation in the chicken ceca sufficient to restrict Salmonella Enteritidis colonization, but inconsistent intake can be a problem (Woodward et al., 2005). The use of crumble and pelleted physical forms of feeds has been examined as a means to increase the feed-conversion ratios of birds (Kilburn and Edwards, 2001). Furthermore, Nir et al. (1994) noted that crumble feed is more suitable in the development of the chicken digestive tract compared with mash, which is uniform in particle size. The objectives of this study were to enumerate Salmonella Enteritidis colonization in fecal, cecal, and internal organs and compare the level of virulence gene expression (hilA) of experimentally challenged laying hens fed an alfalfa crumble diet, with hens either undergoing feed withdrawal or fed a layer ration.

**MATERIALS AND METHODS**

**Experimental Design**

Experiments 1 and 2 were conducted with Single Comb White Leghorn hens, >50 wk of age, obtained from a local commercial laying flock. The hens were placed in wire layer cages and given free access to water and an unmedicated corn-soybean meal-based mashed layer ration that met NRC (1994) recommendations for nutrients. The alfalfa crumble diet used in the study was considered high in crude fiber (24 to 25%), moderate in CP (17 to 18%), and low in ME (1,200 kcal/kg; NRC, 1994). Feed samples and fecal samples (1 g) were collected and examined for Salmonellae. Samples were cultured in tetrathionate broth (Becton, Dickinson and Company, Sparks, MD) and on brilliant green agar (BGA) plates (Becton, Dickinson and Company) as previously described by Andrews et al. (1995). All hens and feed used in both trials tested negative for Salmonella.

**Trials 1 and 2**

The hens were allowed to acclimate to the cages for 2 wk, then were exposed to an 8L:16D photoperiod for 1 wk before changing the diets or, for hens in the feed-withdrawal group (FW), removing feed. This light schedule continued for a 12-d period, after which the experiment was terminated. Twelve hens were randomly assigned to 6 treatment groups, designated as follows: 1) feed withdrawal Salmonella Enteritidis-positive (FW+), 2) fully fed Salmonella Enteritidis-positive (FF+), 3) 100% alfalfa crumble Salmonella Enteritidis-positive (ALC+), 4) feed withdrawal Salmonella Enteritidis-negative (FW−), 5) fully fed Salmonella Enteritidis-negative (FF−), and 6) 100% alfalfa crumble Salmonella Enteritidis-negative (ALC−). Treatment diets were applied to each treatment on d 1 of the molt at the same time feed was removed from hens in the FW group. Treatment diets were administered for 12 d to coincide with the time period that hens in the FW group were deprived of feed. Hens in all the treatment groups were provided water ad libitum. On d 4 of the molt, all hens in groups 1, 2, and 3 were challenged by crop gavage with 1 mL of inocula containing approximately 106 cfu of nalidixic acid (NA)- and novobiocin (NO)-resistant Salmonella Enteritidis (Sigma Aldrich Co., St. Louis, MO). Groups, 4, 5, and 6 were not challenged with Salmonella Enteritidis. The Salmonella Enteritidis-positive and Salmonella Enteritidis-negative hens were placed in separate rooms. Footbaths were located at the doors of all the rooms in the facilities and noninfected hens were cared for before the infected hens each day. At the conclusion of both trials, all hens were euthanized and Salmonella Enteritidis colonization was enumerated in the cecal contents, liver, spleen, and ovaries. In addition, fecal (d 4 and 8) and cecal samples (necropsy at d 12) were collected from 5 postchallenged hens per treatment group 1, 2, and 3 (Salmonella Enteritidis-positive) for determination of hilA expression by PCR.

**Bacterial Strain**

A primary poultry isolate of Salmonella Enteritidis (phage type 13A) from the National Veterinary Services Laboratory (Ames, IA), selected for resistance to NO and NA in the USDA-ARS facility (College Station, TX), was used. The media used to culture the resistant isolate contained 25 μg of NO and 20 μg of NA/mL (Sigma Aldrich Co.). The culture was prepared from an overnight culture previously transferred 3 times in trypticase soy broth (Becton, Dickinson and Company). The challenge inoculum was prepared by serially diluting the culture in sterile PBS to a concentration of approximately 106 cfu/mL. The colony-forming units of the challenge inoculum were confirmed by plating on BGA plates (Becton, Dickinson and Company).
**Necropsy**

At the conclusion of both studies, hens were euthanized and the ceca, liver, spleen, and ovaries were excised aseptically. Serial dilutions were performed using 0.25 g of the cecal contents. One hundred microliters from each dilution tube was subsequently placed onto a BGA plate (Becton, Dickinson and Company) containing NA and NO and spread-plated using a bacterial cell spreader. Plates were incubated for 24 h at 37°C, and colony-forming units enumerated and expressed as \( \log_{10} \) Salmonella Enteritidis/g of cecal contents. Samples of the ceca, liver, spleen, and ovaries of each hen were cultured for Salmonella Enteritidis. The organ samples were incubated for 24 h at 41°C in Rappaport-Vassiliadis R10 broth (Difco Laboratories, Detroit, MI). After incubation, the broth was streaked onto NA/NO BGA plates (Becton, Dickinson and Company) and incubated for an additional 24 h at 37°C. On the following day, plates were examined for Salmonella Enteritidis colonies and were recorded as either negative or positive for Salmonella Enteritidis.

**Immunomagnetic Separation of Salmonella Enteritidis with Dynabeads**

Anti-Salmonella Dynabeads (Dynal Biotech ASA, Oslo, Norway) were used in an immunomagnetic separation technique to remove Salmonella Enteritidis from feces (Olsvik et al., 1994) for hilA detection by real-time PCR. Fecal and cecal samples were suspended in a 1:2 (wt/vol) ratio of RNAlater (Sigma Aldrich Co.) in Whirl-Pak filter bags (Nasco, Fort Atkinson, WI). Triplicate 1-mL portions were placed into 1.5-mL sterile microcentrifuge tubes with 20 
\( \mu \)L of RNAlater (Sigma Aldrich Co.) and frozen until RNA was extracted.

**RNA Extraction and Primer Design**

Ribonucleic acid was extracted from the Dynabead-Salmonella Enteritidis complexes according to the manufacturer’s instructions (RNase Mini Kit, Qiagen, Valencia, CA). Salmonella RNA was subjected to reverse transcription (RT)-PCR to obtain cDNA. Primers were designed for hilA and 16S rRNA genes using sequence data obtained from the GenBank Web site and optimized using Primer Express 1.0 software (Perkin-Elmer Applied Biosystems, Foster City, CA). Optimized sequences were processed on the National Center for Biotechnology Information Web site to determine their cross-reactivity with other species of bacteria. The primers used in this study are listed in Table 1 (McClelland et al., 2001).

**RT Reaction**

To obtain Salmonella cDNA for real-time PCR, RT reactions were performed using the reagents from the TaqMan OneStep RT-PCR Kit (Perkin-Elmer Applied Biosystems). Each reaction contained 10× RT-PCR buffer, 500 
\( \mu \)M dNTPs Mix (Perkin-Elmer Applied Biosystems), 20 U/reaction of RNase inhibitor (Perkin-Elmer Applied Biosystems), 25 mM MgCl\(_2\), 200 ng of RNA, 2.0 
\( \mu \)L of each primer (2.5 
\( \mu \)M), 1.25 U/\( \mu \)L of MultiScribe RT (Perkin-Elmer Applied Biosystems), and RNase-free water to a final volume of 20 
\( \mu \)L. A positive RT reaction was run to ensure the proper procedure. The positive reaction contained all the same components, except that in place of the RNA sample template, a DNA sample supplied with the kit was used as the template. To determine whether RNA samples were contaminated with DNA, 2 negative RT reactions were run on each RNA sample. One negative RT reaction contained the same components as the positive RT reactions, except that it lacked the RNA sample template and contained more water to ensure that the final concentrations of the remaining components were the same. The other negative RT reaction did not contain the RT enzyme to ensure that there was no DNA contamination. All 1-step RT reactions were performed on a Gene Amp PCR System (Perkin-Elmer GeneAmp PCR Systems, Wellesley, MA) under the following conditions: incubation for 10 min at 25°C, RT for 30 min at 45°C, RT inactivation for 5 min at 95°C, and 3-step cycling for 1 min at 94°C, 1 min at 53°C, and 1 min at 72°C for 40 cycles. The samples were held at 4°C until they could be removed.

<table>
<thead>
<tr>
<th>Table 1. Primer sequences used in real-time PCR reactions</th>
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<tr>
<td>Gene</td>
<td>Primer sequence</td>
</tr>
<tr>
<td>hilA FWD</td>
<td>5′ TATCCGACTATCCGCCCTT 3′</td>
</tr>
<tr>
<td>hilA REV</td>
<td>3′ TGTAATGTCACCGCAG 5′</td>
</tr>
<tr>
<td>16S rRNA FWD</td>
<td>5′ TGGCCGACCGCTAAA 3′</td>
</tr>
<tr>
<td>16S rRNA REV</td>
<td>3′ TTACACACTGCGAGTATTAAGC 5′</td>
</tr>
</tbody>
</table>

1From McClelland et al. (2001).
Real-Time PCR Reaction

Real-time PCR reactions were performed on an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems). Each 20 μL of SYBR Green PCR reaction contained 1 μL of cDNA, 0.2 μL of each primer, 10 μL of 1× SYBR Green PCR Master Mix (Perkin-Elmer Applied Biosystems), and PCR water. Thermal cycling conditions were as follows: 48°C for 30 min, 95°C for 10 min, and 40 repeats of 95°C for 15 s and 60°C for 1 min. The same positive and negative RT reactions were run for the real-time PCR as for the RT reactions.

Gene Analysis and Expression

Samples for real-time PCR reactions (Orlando et al., 1998; Bustin, 2000; Livak and Schmittgen, 2001; Pierson et al., 2003) were run in triplicate to determine Salmonella Enteritidis expression. Data were analyzed using the relative quantification method ($2^{-\Delta\Delta CT}$; Livak and Schmittgen, 2001), which describes the change in expression of the target gene (hilA) relative to the 16S rRNA reference gene (rsmC) from an untreated Salmonella Enteritidis control sample (Tschirner et al., 1999; Kundinger et al., 2007). Data were analyzed by averaging the C_{T} values (cycle at which
Salmonella Enteritidis Colonization and Hila Response

RESULTS AND DISCUSSION

Salmonella Enteritidis Colonization in the Organs

The results of Salmonella Enteritidis colonization in the liver, spleen, and ovaries are shown in Table 2. In both trials, all nonchallenged birds (FF−, FW−, and ALC− treatments) were Salmonella Enteritidis-negative (data not shown). In trial 1 of this study, the ALC+ treatment group showed a trend of numerically lower Salmonella Enteritidis colonization in the ovaries 8% (1/12), spleen 17% (2/12), and liver 25% (3/12), compared with FW+ hens [ovaries 33% (4/12), spleen 33% (4/12), and liver 50% (6/12)], but these differences were not statistically significant (P > 0.05). In the same trial, FF+ hens were Salmonella Enteritidis-negative for the liver, spleen, and ovaries; however, only Salmonella Enteritidis colonization in the liver was less (P ≤ 0.05) compared with FW+ and ALC+ hens. In general, trial 2 demonstrated significantly (P ≤ 0.001) higher Salmonella Enteritidis infection compared with trial 1. In trial 2, the FW+ hens exhibited 42% Salmonella Enteritidis infection in the liver, and 63% of the hens were positive in the spleen. Salmonella Enteritidis colonization in the spleen (trial 2) was not statistically different for both FF+ (5%) and ALC+ (10%) but was significantly (P ≤ 0.001) less than in FW+ hens. This indicated that the alfalfa crumble diet effectively limited the incidence of colonization in these organs. The ALC+ treatment group exhibited a 25% infection of Salmonella Enteritidis in the ovaries, compared with 47% in the ovaries in FW+ hens; only FF+ ovaries were less (P < 0.05). Woodward et al. (2005) reported that Salmonella Enteritidis colonization generally increased in the liver, spleen, and ovaries in feed-withdrawal hens, compared with alfalfa meal molt-induced hens. In a molt induction study of hens fed alfalfa meal and alfalfa combined with a standard commercial layer diet, McReynolds et al. (2006) observed substantial reductions in Salmonella Enteritidis colonization in the liver of hens fed the 100% alfalfa meal diet and more than a 3-fold reduction in alfalfa meal-layer ration combinations when compared with feed-withdrawal hens.

Salmonella Enteritidis Colonization of the Cecal Contents

In both trials, based on a chi-squared analysis, there were no differences between the ALC+ diet and the FF+ control in cecal Salmonella Enteritidis enrichment positives; however, hens in the FW+ treatment yielded significantly (P ≤ 0.05) higher Salmonella Enteritidis infectivity colony-forming units per gram of cecal contents (Figure 1). In trial 1, ALC+ hens exhibited a 2-fold (log_{10} 1.99) Salmonella Enteritidis reduction in colonization when compared with FW+ (log_{10} 3.89). In trial 2, a 4-fold (log_{10} 1.29) Salmonella Enteritidis reduction in colonization occurred for ALC+ hens compared with FW+ hens (log_{10} 5.12). Woodward et al. (2005) saw a 2- to 7-fold reduction in Salmonella Enteritidis colonization in the ceca of alfalfa meal-fed hens compared with feed-withdrawal hens. McReynolds et al. (2006) reported similar reductions of Salmonella Enteritidis ceca colonization in birds fed a 100% alfalfa meal diet and a 70% alfalfa meal:30% standard

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**Table 2. Colonization of Salmonella Enteritidis in the organs of molted laying hens (trials 1 and 2)**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Liver</th>
<th>Spleen</th>
<th>Ovaries</th>
<th>Liver</th>
<th>Spleen</th>
<th>Ovaries</th>
</tr>
</thead>
<tbody>
<tr>
<td>FW+</td>
<td>6/12</td>
<td>4/12</td>
<td>4/12</td>
<td>8/19</td>
<td>12/19</td>
<td>9/19</td>
</tr>
<tr>
<td>FF+</td>
<td>0/12*</td>
<td>0/12</td>
<td>0/12</td>
<td>4/20</td>
<td>1/20*</td>
<td>0/20**</td>
</tr>
<tr>
<td>ALC+</td>
<td>3/12</td>
<td>2/12</td>
<td>1/12</td>
<td>3/20</td>
<td>2/20*</td>
<td>5/20</td>
</tr>
</tbody>
</table>

1FW+ = feed withdrawal, challenged with Salmonella Enteritidis; FF+ = fully fed, challenged with Salmonella Enteritidis; ALC+ = alfalfa crumble, challenged with Salmonella Enteritidis.

2Values represent the mean of 12 or 20 hens per treatment. A significant difference was found between the number of positive controls and positive, treated internal organs (*P ≤ 0.05). Significant differences were seen between the number of positive controls and positive, treated internal organs (**P ≤ 0.001).
commercial layer diet, respectively, compared with molted feed-withdrawal hens.

**Hila Response**

The relative expression of *Salmonella* hilA genes was examined from fecal (d 6 and 11) and cecal contents (d 12 necropsy) of post-*Salmonella* Enteritidis-challenged molted hens (Figure 2). Relative expression of hilA was higher (P ≤ 0.05) on all days examined in trial 2 compared with trial 1. There were no significant (P > 0.05) differences in hilA expression between fecal and cecal contents. In trial 1, fecal and cecal *Salmonella* hilA expression in FW+ hens was higher (P ≤ 0.05) than in FF+ hens (Figure 2, panels A, C, and E). However, there were no significant differences (P > 0.05) between hilA expression in the ALC+ group compared with the FF+ group. In trial 2, hilA expression was higher (P ≤ 0.05) in FW+ hens for d 6, 11, and 12, respectively (Figure 2, panels B, D, and F), when compared with ALC+ and FF+ hens. This corresponds to previous in vitro studies by Durant et al. (1999), in which sterile crop contents were used to incubate *Salmonella* Enteritidis; they observed that hilA expression was nearly doubled in crop contents from hens undergoing feed withdrawal compared with crops from layer ration-fed hens, corresponding to a 5- to 6-fold increase in *Salmonella* Enteritidis colonization in the spleen and liver on molted hens compared with those of layer ration-fed hens. In addition, they reported a 3.5- to 5.5-fold increase of *Salmonella* colonization in the ceca of feed-withdrawal hens compared with nonmolted hens.

It has previously been demonstrated that molted hens shed significantly more *Salmonella* Enteritidis in their feces (Holt and Porter, 1992; Holt, 1993; Holt et al., 1995) and generally exhibit much higher levels of *Salmonella* Enteritidis survival and invasion in their internal organs (Holt et al., 1995). The quantification of hilA expression has been proposed as an accurate indicator of the level of *Salmonella* virulence in the GI tract and potential susceptibility of hens (Durant et al., 1999, 2000; Ricke, 2003). In the current study, the lower hilA expression in the ALC+ and FF+ diets generally paralleled organ infection levels and suggests that both diets provided available substrates that minimized *Salmonella* Enteritidis virulence. In addition to limiting *Salmonella* Enteritidis infection, the effectiveness of FF+ and ALC+ diets in reducing cecal *Salmonella* colonization may rely on their ability to support normal microflora fermentation in the intestines (Nurmi and Rantala, 1973; Barnes et al., 1980; Nisbet et al., 1994; Corrier et al., 1995; Van der Wielen et al., 2000, 2001, 2002; Ricke, 2003; Woodward et al., 2005). The results of the current study suggest that alfalfa would be capable of retaining protective microflora and providing the desired fermentative capacity needed during molt to establish resistance to enteropathogens such as *Salmonella* Enteritidis. However, determining this will require in vitro and in vivo profiling of both the molecular ecology and fermentation capacity of the cecal microorganisms in hens fed these diets.

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