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Feed Deprivation Affects Crop Environment and Modulates *Salmonella enteritidis* Colonization and Invasion of Leghorn Hens

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Leghorn hens over 50 weeks of age were assigned to two treatment groups designated as either unmolted controls or molted. A forced molt was induced by a 9-day feed withdrawal, and each hen was challenged orally with 10^5 *Salmonella enteritidis* organisms on day 4 of feed withdrawal. On days 4 and 9 of molt, the numbers of lactobacilli and the concentrations of lactate, acetate, propionate, and butyrate, and total volatile fatty acids in the crops decreased while crop pH increased significantly ($P < 0.05$) in the molted hens compared to the controls. *S. enteritidis* crop and cecal colonization, in addition to spleen and liver invasion, increased significantly ($P < 0.05$) in the molted hens compared to the controls. The invasive phenotype of *Salmonella* spp. is complex and requires several virulence genes which are regulated by the transcriptional activator HilA. Samples of the crop contents from the molted and unmolted birds were pooled separately, centrifuged, and filter sterilized. The sterile crop contents were then used to measure the expression of *hilA*. By using a *lacZY* transcriptional fusion to the *hilA* gene in *S. enteritidis*, we found that *hilA* expression was 1.6- to 2.1-fold higher in the crop contents from molted birds than in those from control birds in vitro. The results of the study suggest that the changes in the microenvironment of the crop caused by feed deprivation are important regulators of *S. enteritidis* survival and influence the susceptibility of molted hens to *S. enteritidis* infections. Furthermore, our in vitro results on the expression of *hilA* suggest that the change in crop environment during feed withdrawal has the potential to significantly affect virulence by increasing the expression of genes necessary for intestinal invasion.

During the past 10 to 15 years, the number of cases of gastroenteritis due to *Salmonella enteritidis* infections has increased markedly in the United States and Europe (20). Epidemiological studies have attributed episodes of *S. enteritidis* infection to the consumption of food containing contaminated Grade A eggs (33). *S. enteritidis* is invasive in poultry and therefore has the potential to contaminate eggs by transovarian transmission following colonization of the intestinal tract (35).

Practices, such as feed withdrawal, which increase the susceptibility of laying hens to *S. enteritidis* infection increase the risk of human salmonellosis from *S. enteritidis*-contaminated eggs. Feed withdrawal is used in the poultry industry to induce molting and stimulate multiple egg-laying cycles in laying hens (19). Unfortunately, the stress associated with feed withdrawal causes an increased susceptibility to *S. enteritidis* infection, marked by increased intestinal shedding and dissemination of *S. enteritidis* to internal organs such as the liver, spleen, and ovary (19, 20, 35). The incidence of *S. enteritidis*-positive eggs increases threefold within the first 10 weeks after hens are subjected to forced molting (38).

Virulence genes and phenotypes are coordinately regulated in response to environmental signals in vivo (26). Environmental conditions such as osmolarity, oxygen tension, pH, and the stimuli of short-chain fatty acids regulate *Salmonella* virulence by modulating the expression of invasion genes in *Salmonella* and in epithelial cell invasion (2, 11, 16, 22). Because the crop is the first host environment encountered by *S. enteritidis* after ingestion, it can influence the survival and virulence of *S. enteritidis*. Therefore, the purpose of the present study was to investigate the physiological changes in the crop associated with molt induced by feed withdrawal. HilA is a transcriptional activator that regulates the expression of invasion genes and the invasion phenotype in *Salmonella* (3). To improve our understanding of the potential role of the crop in *S. enteritidis* virulence, the expression of the *hilA* gene after growth of *S. enteritidis* in the crop contents of fed birds and birds deprived of feed was also evaluated in vitro.

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MATERIALS AND METHODS

Bacteria. A primary poultry isolate of *S. enteritidis* (phage type 13A), obtained from the National Veterinary Services Laboratory, Ames, Iowa, was selected for resistance to novobiocin and nalidixic acid (NO-NA) in the Agricultural Research Service Food Animal Protection Research Laboratory and maintained on nutrient agar. Media used to culture the resistant isolate in experimental studies contained 25 μg of NO and 20 μg of NA per ml. The challenge inocula were prepared from an overnight culture which had been previously transferred three times in Trypticase soy broth. The culture was serially diluted in sterile phosphate-buffered saline to a concentration of approximately 10^5 CFU per ml. The number of CFU in the challenge inoculum was confirmed by plating onto brilliant green agar (BGA) plates (Difco Laboratories, Detroit, Mich.).

Molt procedure. Hens were molted by a modification (19) of a previously described procedure (6). Seven days before feed removal, hens were exposed to an 8-h light–16-h dark photoperiod which was continued throughout the experiment. Beginning on day 0, feed was withdrawn for 9 days, after which the study was ended. Egg production by the molted hens ceased 4 to 7 days after feed withdrawal.

Experimental protocol. Single-comb White Leghorn hens (Hyline International) over 50 weeks of age were obtained from a commercial laying flock. Cloacal swab samples were collected from each hen and examined for salmonellae by successive culturing in tetrathionate (TT) broth and BGA (Difco) as described by Andrews et al. (1). *Salmonella* was detected in the swab samples from three hens. These hens were eliminated from the study. The remaining hens were placed in wire layer cages (2 hens per cage) and provided free access to

water and a balanced, unmedicated, corn-soya bean-meal-based mash layer feed ration that met National Research Council nutritional requirements (28). This diet provided 2,818 kcal of metabolizable energy per kg, 16.5% crude protein, 3.5% calcium, and 0.48% available phosphorus. Before use, three randomly selected 25-g samples of the feed were cultured successively in buffered peptone water, TT broth, and BGA as described by Andrews et al. (1) and examined for salmonellae. *Salmonella* was not detected in the feed samples. The hens were allowed to acclimate for 1 week and then were randomly assigned to two treatment groups of 24 hens each, designated as the unmolted control group and the molted group. The hens were housed in biohazard isolation facilities at the USDA Food Animal Protection Research Laboratory.

On day 4 of molt, before the *S. enteritidis* challenge, 12 hens were selected randomly from each group and euthanatized by cervical dislocation. The crop pH was determined by the insertion of a sterile glass pH electrode (model 05669-20; Cole Palmer, Niles, Ill.) through an incision in the crop wall, ensuring that the electrode remained in contact with the crop mucosal surface. 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 (Stomacher 80 lab blender; Stewart Medical, London, England). Samples of the blended crop were cultured for lactobacilli and were analyzed for concentrations of volatile fatty acids (VFAs) (acetic, propionic, butyric, isobutyric, valeric, and isovaleric acids) and lactic acid. On day 4 of molt, all of the remaining hens in both groups were challenged by crop gavage with 1 ml of inoculum containing approximately 10^5 CFU of NO-NA-resistant *S. enteritidis*. The challenge dose was approximately equal to the 5.6×10^4 CFU dose reported previously to be the mean infectious dose for *S. enteritidis* in unmolted hens (19).

On day 9 of molt, the 12 hens in each group challenged with *S. enteritidis* were euthanatized, and the crop, cecum, liver, and spleen were excised aseptically. Crop pH, numbers of lactobacilli, and VFA and lactic acid concentrations were determined as described previously. The crop and cecum were cultured separately for *S. enteritidis*. The liver and spleen from each hen were combined as a single sample and cultured for *S. enteritidis*. The experimental protocol was repeated in two separate trials with hens obtained from the same commercial flock.

Crop VFA and lactic acid concentrations. The concentrations of VFAs in the crop contents were determined by gas-liquid chromatography as described by Corrier et al. (10). Briefly, the analyses were conducted with a gas chromatograph equipped with a flame ionization detector and peak profiles integration-quantification integrator (Shimadzu Corp., Columbia, Md.). Each sample peak profile was integrated and quantified relative to an internal standard of methylbutyric acid placed in the same sample. Analyses were conducted at an oven temperature of 200°C and a flow rate of 85 ml/min. The concentration of each acid was expressed in micromoles per milliliter. Lactic acid concentrations were determined by an enzymatic method (17).

Undissociated-VFA concentrations. The concentrations of undissociated lactic and acetic acids were calculated by using the Henderson-Hasselbalch equation ($pH = pK_a + \log_{10} [A-]/[HA]$), where A− is dissociated acids and HA is undissociated acids, with crop pH values, the pK_a of each acid, and the total acid concentration as determined by gas chromatography. The respective pK_as (under standard conditions) of lactic and acetic acids used in calculating the concentration of undissociated acid present were 3.08 and 4.75 (39).

Lactobacilli CFU per milliliter of crop contents. One milliliter of crop contents was serially diluted in sterile buffered peptone water to a final dilution of 10^{-10}. Each dilution (0.1 ml) was plated onto Rogosa SL (Difco) agar in duplicate. The plates were transferred immediately to an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) and incubated for 96 h at 37°C.

Crop colonization by *S. enteritidis*. One milliliter of the blended crop sample was transferred into 10 ml of TT broth and incubated for 24 h at 37°C. After incubation, the broth was streaked onto NO-NA-BGA plates, incubated for an additional 24 h at 37°C, and examined for the presence of suspect *S. enteritidis* colonies. Suspect colonies were confirmed by biochemical tests on triple sugar iron agar and lysine-iron agar (Oxoid, Unipath Ltd., Basingstoke, Hampshire, England) and further identified serologically as *S. enteritidis* by using *Salmonella* type O antiserum, group D, factors 1, 9, and 12 (Difco). Identification of the NO-NA-resistant *S. enteritidis* by the culture on the NO-NA-BGA plates and the biochemical and serological procedures described above was considered confirmatory without further serotyping.

Cecal colonization by *S. enteritidis*. One cecum from each hen was cut into several pieces, placed in 30 ml of TT broth, shaken vigorously, and incubated for 24 h at 37°C. After incubation, the broth was streaked on NO-NA-BGA plates, incubated for an additional 24 h at 37°C, and examined for the presence of suspect *S. enteritidis* colonies. Suspect colonies were confirmed biochemically and serologically as described above.

***S. enteritidis* CFU per gram of cecal contents.** The contents of a cecum from each hen were serially diluted and spread plated on NO-NA-BGA plates at dilutions of 10^{-1} through 10^{-4}. The plates were incubated for 24 h at 37°C, and the number of CFU of *S. enteritidis* per gram of cecal contents was determined on an automatic colony counter (Biotran III; New Brunswick Scientific Co., Edison, N.J.). *S. enteritidis* colonies were confirmed biochemically and serologically as described above. Cecal contents in which *S. enteritidis* was not detected at the 10^{-1} dilution on BGA plates and after TT broth enrichment and BGA plating were scored as 0 CFU. Cecal contents that were negative at a 10^{-1} dilution on BGA plates but were positive after TT enrichment and BGA plating were arbitrarily assigned scores of log 0.95 CFU of *S. enteritidis* per g of cecal contents.

Liver and spleen colonization by *S. enteritidis*. Liver and spleen specimens were combined, minced with scissors, and cultured as a single combined sample according to National Poultry Improvement Plan guidelines (37). The organ samples were incubated for 24 h at 37°C in TT broth. After incubation, the broth was streaked onto NO-NA-BGA plates, incubated for an additional 24 h at 37°C, and examined for the presence of *S. enteritidis* colonies. Suspect colonies were confirmed biochemically and serologically as described previously.

Preparation of the *hilA-lacZY* fusion strain of *S. enteritidis*. Bacteriophage P22 HT105/1 *int*-201 was used to transduce a *hilA-lacZY* transcriptional operon fusion from *Salmonella typhimurium* (EE658) (2) into the poultry isolate of *S. enteritidis* used in this experiment. Generalized transduction was conducted as described by Maloy et al. (24). Transductants selected on Luria-Bertani (LB) agar plates containing relevant antibiotics were purified on Evans blue-uranin (24) plates and subsequently cross-streaked with P22 H5 for identification and subsequent elimination of pseudolysogens and lysogens. The fusion strain was grown in LB broth and stored in glycerol at −70°C. Prior to the experiment, a sample of culture was taken from the frozen stock and streaked onto LB agar containing a 40-μg/ml concentration of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in *N-N*-dimethylformamide. The plates were incubated overnight at 37°C. After incubation, a single colony was used to inoculate 2 ml of LB broth in a 13- by 100-mm borosilicate tube. The tube was placed on a roller drum at 37°C for 18 h. This culture was used for the *S. enteritidis* inoculum in the β-galactosidase assay.

The β-galactosidase assay. For trials 1 and 2, samples of the blended crops of the molted and unmolted birds on days 4 and 9 were pooled separately. The pooled samples were centrifuged for 10 min at $1{,}000 \times g$. The supernatant was recovered and filter sterilized. A 1:50 ratio of the transduced *S. enteritidis* inoculum was added to the filtered crop samples. Three milliliters of the inoculated crop contents was dispensed into sterile 10- by 100-mm borosilicate tubes, and the tubes were incubated at 37°C. After 1, 3, and 5 h of growth, the β-galactosidase activity was determined as described by Miller (27). Three tubes were used for each time point. The β-galactosidase activity is expressed as Miller units (units of optical density at 420 nm per min per unit of optical density at 600 nm).

Statistical analysis. Chi-square analysis was used to determine significant differences between treatment groups for incidences in *S. enteritidis* colonization of the crop, cecum, liver, and spleen (23). Differences in the cecal pH, VFA and lactic acid concentrations, $\log_{10}$ *Lactobacillus* counts, and log CFU of *S. enteritidis* counts among treatment groups were determined by analysis of variance using the general linear-models procedures. Significant differences were further separated by using Duncan's multiple-range test and commercial statistical analysis software (SAS Institute, Cary, N.C.) (23). The concentrations of crop VFAs within each group of hens did not differ significantly between each of the trials as determined by statistical analysis and comparison of the individual trial data. The VFA data from each trial were therefore combined and analyzed statistically. All other data were analyzed by individual trial. All statistical analyses were considered significant ($P < 0.05$).

RESULTS AND DISCUSSION

Molting induced by feed withdrawal is commonly used by the United States layer industry to stimulate egg laying in aging flocks. However, this method of inducing molt reduces the resistance of hens to *S. enteritidis* colonization (8, 18–20). The results of the present study indicate that changes which occur in the crop during feed withdrawal may contribute to the survival of *S. enteritidis* in the crop and also stimulate expression of virulence factors in *S. enteritidis*. The stimulation of *S. enteritidis* virulence factors in the crop may result in increased colonization of the intestine and translocation of *S. enteritidis* to the liver and spleen in the molted birds.

The crop is a nonsecretory organ which functions to store food before passage into the gizzard (13). Lactobacilli are the predominant colonizers of the stratified squamous epithelium of the crop and maintain the crop pH at approximately 5. Previously, the lactobacilli from the crop were shown to be important in maintaining a low pH that prevented coliform establishment in the crop (13). Lactobacilli from the crop contents were found to inhibit the growth of *Escherichia coli* in vitro, and at least 10^7 lactobacilli per g were required to prevent multiplication of *E. coli* (12). Scrapings of the crop epithelium from birds deprived of feed for 12 h were previously reported to be free from bacteria, as determined microscopically (14). Our results indicated that feed withdrawal caused a

TABLE 1. Effect of 9-day feed withdrawal (induced molt) on number of lactobacilli and lactic acid concentration in leghorn hen crops

Sampling time[a]	Concn in group[b]			
	Lactobacilli ($\log_{10}$ CFU/ml)		Lactic acid (μmol/ml)	
	Control	Molted	Control	Molted
Trial 1				
Day 4	8.73 ± 0.45 A	6.82 ± 0.47 B	51.4 ± 46.4 A	14.6 ± 4.8 B
Day 9	8.91 ± 0.68 A	6.53 ± 1.23 B	33.5 ± 27.9 A	10.4 ± 2.9 B
Trial 2				
Day 4	8.33 ± 0.89 A	6.28 ± 0.67 B	24.4 ± 16.8 A	9.1 ± 2.6 B
Day 9	9.01 ± 0.49 A	6.42 ± 0.72 B	35.4 ± 26.1	14.4 ± 2.9

[a] Times are days of feed withdrawal.
[b] Data are presented as means ± standard deviations, $n = 16$. Values in a row followed by different capital letters differ significantly ($P < 0.05$).

significant and consistent decrease in the numbers of lactobacilli in the crop during trials 1 and 2 (Table 1). The numbers of lactobacilli ranged from 8.33 ± 0.89 to 9.01 ± 0.49 $\log_{10}$ CFU/ml in the control birds and from 6.28 ± 0.67 to 6.82 ± 0.47 $\log_{10}$ CFU/ml in the molted birds. Previously, starvation has been shown to reduce the total viable numbers of organisms in the crops of broiler chickens (21) and in the intestines of mice (34).

Lactate is the primary fermentation product of the lactobacillus species that are present in the crop (12). The concentration of lactate in the crops of the molted hens was significantly lower than that in the crops of the control hens (Table 1). Lactate concentrations ranged from 24.4 ± 16.8 to 51.4 ± 46.4 μmol/ml in control birds versus 9.1 ± 2.6 to 14.6 ± 4.8 μmol/ml in molted birds. The significant decrease in lactate in the molted birds was expected, since there was a significant decrease in the numbers of lactobacilli in these birds. In our experiments, feed withdrawal also resulted in significant changes in the concentrations of total VFAs and acetic, propionic, and butyric acids in the crop contents of molted hens compared to the control birds (Table 2). On day 4, the total VFA and acetate concentrations were significantly decreased in molted birds compared to controls. On day 9, the concentrations of total VFAs along with acetic, propionic, and butyric acids were significantly lower in the molted hens than in the control birds. The decrease in total VFAs may be the result of decreased *Lactobacillus* numbers in addition to other VFA-producing microorganisms in the crop. Humphrey et al. (21) found that the total viable counts of organisms in the crop contents of birds starved for 24 h were lower than in fed birds. Acetate was the predominant VFA present in the crop contents of both the control and molted hens. Our results are in agreement with those of Fuller and Brooker (13), who found lactate to be the predominant organic acid in the crop contents, followed by acetate and traces of propionate and butyrate.

TABLE 2. Effect of 9-day feed withdrawal (induced molt) on concentration of volatile fatty acids (VFA) in the crops of leghorn hens[a]

Day and group	Concn (μmol/ml) of VFA			
	Total[b]	Acetic	Propionic	Butyric
Day 4				
Control	21.09 ± 13.21 A	14.94 ± 11.61 A	3.58 ± 3.93 A	1.19 ± 0.87 A
Molted	11.83 ± 3.63 B	5.22 ± 2.03 B	2.75 ± 1.17 A	1.09 ± 0.27 A
Day 9				
Control	27.48 ± 11.92 A	20.22 ± 13.26 A	5.70 ± 2.14 A	1.62 ± 0.38 A
Molted	13.99 ± 3.31 B	5.40 ± 2.62 B	4.22 ± 1.02 B	1.16 ± 0.41 B

[a] The experiment was repeated in two replicate trials, and the data were combined. Data are presented as means ± standard deviations, $n = 24$. Values in a column followed by different capital letters differ significantly ($P < 0.05$).
[b] Total VFAs include acetic, propionic, butyric, isobutyric, valeric, and isovaleric acids.

TABLE 3. Effect of 9-day feed withdrawal (induced molt) on the pH of the crops and proventriculi of leghorn hens[a]

Group	Crop pH			
	Trial 1		Trial 2	
	Day 4	Day 9	Day 4	Day 9
Control	4.98 ± 0.31 A	4.96 ± 0.53 A	5.33 ± 0.60 A	4.98 ± 0.44 A
Molted	6.25 ± 0.25 B	6.10 ± 0.64 B	6.25 ± 0.25 B	6.21 ± 0.37 B

[a] Data are presented as means ± standard deviations, $n = 12$. Values in a column followed by different capital letters are significantly different ($P < 0.05$).

Decreases in the concentrations of lactate and total VFAs as a result of feed withdrawal were accompanied by a significant increase in crop pH in the molted hens (Table 3). The pH of the crop in molted birds ranged from 6.1 ± 0.64 to 6.25 ± 0.25, compared to 4.96 ± 0.53 to 5.33 ± 0.60 in control birds. An increase in crop pH was previously noted in hens deprived of feed for 24 h (21). The introduction of *S. enteritidis* into the crop environment with high pH and lowered concentrations of lactate and total VFA was accompanied by increased crop colonization (Table 4). Fifty percent of the crops of molted birds were *Salmonella* positive, compared to 8.3% of control birds (Table 4). Previously, Humphrey et al. (21) reported an increase in the recovery of *S. enteritidis* from the crops of broilers deprived of feed for 24 h. The present study also demonstrates that the changes in the crop environment after feed withdrawal were more favorable for the survival of and colonization by *S. enteritidis*.

The native intestinal bacteria are known to have a protective effect against *Salmonella* colonization of the cecum (4, 9, 29, 30) and the crop (4) in poultry. The importance of VFAs and pH in preventing *Salmonella* colonization of the cecum has been positively correlated with increased concentrations of VFAs and decreased pHs (5, 9, 29). A lowered pH is thought to promote the bacteriostatic action of VFA by increasing the concentration of the undissociated state, which can permeate the cell membrane (7). During the present study, the concen-

TABLE 4. Effect of feed withdrawal (induced molt) on *S. enteritidis* colonization of the crops, spleens, livers, and ceca of leghorn hens[a]

Group	No. of positive hens/total (%)			$\log_{10}$ SE per g of cecum
	Crop	Spleen-liver	Cecum	
Trial 1				
Control	1/12 (8.3)	1/12 (8.3)	2/12 (16.7)	0.16 ± 0.38
Molted	6/12 (50.0)*	5/12 (41.7)†	11/12 (91.7)*	3.78 ± 2.29*
Trial 2				
Control	1/12 (8.3)	1/12 (8.3)	2/12 (16.7)	0.46 ± 1.33
Molted	6/12 (50.0)*	6/12 (50.0)*	7/12 (58.3)*	2.99 ± 3.26*

[a] Hens were challenged by crop gavage with 10^5 CFU of *S. enteritidis* on day 4 of molt and cultured for *Salmonella* on day 9 of molt. Data are presented as means ± standard deviations, $n = 12$. *, $P < 0.05$, and †, $P < 0.10$, compared with control values.

TABLE 5. Expression of *hilA* after growth of *S. enteritidis* in the crop contents of fed and molted birds

Time (h)	β-Galactosidase activity[a] on:			
	Day 4		Day 9	
	Control	Molted	Control	Molted
Trial 1				
1	341 ± 42	695 ± 83 (2)	396 ± 34	740 ± 58 (1.9)
3	343 ± 62	712 ± 49 (2.1)	417 ± 23	847 ± 63 (2)
5	362 ± 71	686 ± 58 (1.9)	406 ± 42	690 ± 49 (1.7)
Trial 2				
1	414 ± 43	745 ± 63 (1.8)	326 ± 63	684 ± 54 (2.1)
3	436 ± 59	828 ± 98 (1.9)	356 ± 41	694 ± 43 (1.9)
5	427 ± 64	790 ± 42 (1.9)	387 ± 38	619 ± 41 (1.6)

[a] Data are presented as means ± standard deviations, $n = 6$. Activity was measured in Miller units (see the text). The fold increase over the control is given in parentheses.

trations of the two predominant organic acids (lactate and acetate) in the undissociated form decreased markedly in the molted hens compared to the controls. On day 4 of molt, the concentrations of undissociated lactate were 3.62 μmol/ml in the crops of the control hens and 0.059 μmol/ml in the molted hens. On day 9, the concentration of undissociated lactic acid was 2.47 μmol/ml in the controls, compared to 0.06 μmol/ml in the molted hens. Similarly, the concentrations of undissociated acetic acid in the crops of the control and molted hens were 4.92 versus 0.16 μmol/ml on day 4 and 6.65 versus 0.18 μmol/ml on day 9, respectively.

Most of the genes required for the penetration of *Salmonella* into intestinal epithelial cells are located on *Salmonella* pathogenicity island 1 (SPI1) at centisome 63 on the chromosome (15). This virulence locus encodes a complex type III secretion system (*spa*, *inv*, and *org*), secreted effector proteins (*sip* and *spt*), and regulatory components (*hilA* and *invF*). Genes on SPI1 are also important for virulence in poultry, since *S. enteritidis* strains with mutations in genes of the *inv* operon (32) and *sipC* (36) were attenuated for virulence and deficient in intestinal colonization in 1-day-old chicks. HilA regulates invasion gene expression in response to environmental signals (3). *Salmonella* strains with a *hilA* mutation are noninvasive for cultured epithelial cells (2) and in mice (31). In order to understand the role of the crop environment in *S. enteritidis* virulence, the expression of a *hilA-lacZY* gene fusion transduced into the *S. enteritidis* poultry isolate was measured.

In the present study, a 1.6- to 2.1-fold increase in the expression of the *hilA* gene was observed when *S. enteritidis* was grown in the crop contents of molted birds, compared to the crop contents of fed birds (Table 5). These in vitro results suggest that the changes in the crop environment as a result of feed withdrawal may cause *S. enteritidis* to become more virulent. An increase in virulence could have a significant impact on the invasive capability of *S. enteritidis* as it travels along the intestinal tract. *S. enteritidis* may become more efficient at invasion of the epithelial cells lining the intestines. This may partly explain the increase in spleen and liver invasion and the higher number of ceca which were culture positive for *S. enteritidis* in this study (Table 4). Spleen and liver invasion was significantly increased from 8.3% in control birds to 41.7 and 50% in molted birds during trials 1 and 2, respectively (Table 4). Cecal colonization by *S. enteritidis* occurred in 16.7% of the control birds, compared to 58.3 and 91.7% of molted birds (Table 4). Additionally, a significantly higher number of *S. enteritidis* were isolated per gram of cecal contents in control hens versus molted hens. Our results suggest that the conditions of the crop during feed withdrawal may cause *S. enteritidis* to become more efficient at invasion of the intestinal epithelium. In fed birds, *S. enteritidis* colonization is localized in the cecum (20). However, Holt et al. also observed that during feed withdrawal, the dynamics of *S. enteritidis* infection were changed and the organism was more evenly distributed along the intestinal tract, including the ileum.

The association of molting with an increased severity of early intestinal lesions may be related to an impaired immune system (18, 25). The cell-mediated immune response plays an important role in protection against the invasion of intracellular pathogens. Conditions that disrupt the functioning of the immune system dramatically alter the ability of the host to reduce infection (18). Holt (18) showed that in laying hens, the total numbers of lymphocytes and $CT4^+$ (helper) T-cells significantly decreased in the peripheral blood during feed withdrawal and molting. An increase in the expression of *S. enteritidis* virulence genes, combined with a depressed cellular immune response, may be partly responsible for the higher frequency of *S. enteritidis* organ invasion observed in the present study.

The results of the present study suggest that the changes in the microenvironment of the crop contents, which were influenced by feed deprivation, are important regulators of *S. enteritidis* survival and influence the susceptibility of molted hens to *S. enteritidis* infections. Our results show that feed withdrawal alters the microenvironment of the crop by causing significant reductions in the *Lactobacillus* population and in lactate and total VFA concentrations and an increase in pH. This alteration of the crop environment was accompanied by increased *S. enteritidis* colonization of the crop and cecum with invasion of the spleen and liver. Furthermore, our in vitro results on the expression of *hilA* suggest that the change in crop environment during feed withdrawal has the potential to significantly affect virulence by increasing the expression of genes necessary for intestinal invasion.

Our observation that crop composition may affect the virulence of *S. enteritidis* may have important implications for understanding the factors necessary for protection against *S. enteritidis* infection. Therefore, further in vitro research is necessary to identify the components of the crop which modulate the expression of invasion genes.

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