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Caprylic acid reduces *Salmonella* Enteritidis populations in various segments of digestive tract and internal organs of 3- and 6-week-old broiler chickens, therapeutically[1,2]

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ABSTRACT We investigated the efficacy of feed supplemented with caprylic acid (CA), a natural, 8-carbon fatty acid for reducing *Salmonella enterica* serovar Enteritidis colonization in commercial broiler chickens. In separate 3- and 6-wk trials, 1-d-old straight-run broiler chicks (n = 70 birds/trial) were assigned to a control group (challenged with *Salmonella* Enteritidis, no CA) and 2 replicates of 0.7 and 1% CA (n = 14 birds/group). Water and feed were provided ad libitum. On d 1, birds were tested for any inherent *Salmonella* (n = 2 birds/group). For the 3-wk trial, on d 5, birds were challenged with 8 $\log_{10}$ cfu of *Salmonella* Enteritidis of a 4-strain mixture by crop gavage, and after 5 d postchallenge, birds (n = 2 birds/group) were euthanized to ensure *Salmonella* Enteritidis colonization. Caprylic acid was supplemented the last 5 d before tissue collection (n = 10 birds/group). For the 6-wk trial, on d 25, birds were challenged and confirmed for *Salmonella* Enteritidis colonization. The birds (n = 10 birds/group) were euthanized for tissue samples after CA supplementation for the last 5 d. Caprylic acid at 0.7 or 1% decreased *Salmonella* Enteritidis populations in cecum, small intestine, cloaca, liver, and spleen in both 3- and 6-wk trials. Body weight of birds did not differ between the groups ($P \geq 0.05$). Further, to elucidate a potential antibacterial mechanism of action of CA, we investigated if CA could reduce *Salmonella* Enteritidis invasion of an avian epithelial cell line and expression of invasion genes *hilA* and *hilD*. The cell invasion study revealed that CA reduced invasive abilities of all *Salmonella* Enteritidis strains by ~80% ($P < 0.05$). Gene expression studies indicated that CA downregulated ($P < 0.001$) *Salmonella* invasion genes *hilA* and *hilD*. These results suggest that supplementation of CA through feed could reduce *Salmonella* Enteritidis colonization in broiler chicken and potentially reduces the pathogen's ability to invade intestinal epithelial cells by downregulating key invasion genes, *hilA* and *hilD*.

Key words: caprylic acid, chicken, antibacterial, *Salmonella* Enteritidis, invasion

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INTRODUCTION

Salmonella enterica serovar Enteritidis is the most common serotype of *Salmonella* causing human salmonellosis in the United States (CDC, 2009). The total annual cost associated with *Salmonella* in the United States is estimated to be approximately $3 billion (USDA-ERS, 2009). The Centers for Disease Control and Prevention (Atlanta, GA) reported that despite extensive control efforts, the incidence of infections caused by *Salmonella* has not significantly changed in recent years (CDC, 2009).

Salmonella Enteritidis colonizes various parts of the chicken intestinal tract, with the cecum being the most common site (Stern, 2008; Gantois et al., 2009). Cecal colonization eventually leads to fecal shedding, contamination of eggshells with infected feces, carcass contamination during slaughter, and possible retro-contamination of reproductive organs (Keller et al., 1995; Gantois et al., 2009). Humans contract salmonellosis by the consumption of raw or undercooked chicken and eggs or contaminated products (Humphrey and Jorgensen, 2006; Marcus et al., 2007). Reducing the populations

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of *Salmonella* Enteritidis in the chicken intestinal tract would reduce contamination of poultry meat and eggs. Thus, *Salmonella* control strategies implemented at the farms could play a major role in delivering microbiologically safer poultry products to the consumer.

Various interventions have been investigated to control *Salmonella* Enteritidis at the farm level with varying degrees of success. Those include feeding chickens with competitive exclusion bacteria (Mead et al., 1996; Stern et al., 2001; Mead, 2002), bacteriophage (Loc Carrillo et al., 2005), enzymes such as xylanase (Fernandez et al., 2002), organic acids (Byrd et al., 2001; Heres et al., 2004), fructooligosaccharides (Schoeni and Wong, 1994), mannanoligosaccharides (Spring et al., 2000; Fernandez et al., 2002), chicory fructans (Yusrizal and Chen, 2003), vaccines (Dueger et al., 2001; Inoue et al., 2008), and antibacterial agents furazolidone and furaltadone (Chadfield and Hinton, 2003).

Fatty acids, especially medium-chain fatty acids (**MCFA**), are reported to possess antibacterial activity against various microorganisms (Bergsson et al., 1998; 1999). Caprylic acid (**CA**), an 8-carbon MCFA, naturally present in breast milk, caprine milk, and coconut oil, is a Generally Recognized as Safe (**GRAS**) molecule approved by the FDA. Previously, we reported that CA was effective in reducing cecal *Salmonella* Enteritidis populations in 18-d-old chicks, when supplemented in feed prophylactically (Johny et al., 2009). In addition, we found that CA reduced *Campylobacter jejuni* populations in the cecum of 10- and 42-d-old broiler chickens (Solís de los Santos et al., 2008, 2010).

In this study, we investigated the therapeutic efficacy of CA supplemented through feed in reducing *Salmonella* Enteritidis populations in the various segments of digestive tract and internal organs such as liver and spleen in 3- and 6-wk-old broiler chickens. In addition, we used a model avian intestinal epithelial cell line to elucidate the possible antibacterial mechanism by which caprylic acid might reduce *Salmonella* Enteritidis colonization in chickens.

MATERIALS AND METHODS

All the experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Connecticut (Protocol no. A09–028).

Experimental Birds and Management

For experiments with both 3- and 6-wk-old birds, we procured 1-d-old commercial, nonvaccinated, straight-run broiler chicks (Cornish cross) from Burr Farms (CT) and were distributed into floor pens in the University of Connecticut avian isolation facility. The facility is equipped with provisions for age-appropriate temperature, floor space, light, and bedding. The birds were given *Salmonella*-free feed (Blue Seal Feeds Inc., Londonderry, NH) and water ad libitum.

Bacterial Strains and Culture Conditions

Four isolates of *Salmonella* Enteritidis, namely SE 12 (chicken liver, phage type 14b), SE 22 (chicken intestine, phage type 8), SE 28 (chicken ovary, phage type 13a), and SE 31 (chicken gut, phage type 13a), were used for inoculating birds. Each strain was pre-induced for resistance to 50 μg/mL of nalidixic acid (**NA**; catalog no. N4382, Sigma-Aldrich, St. Louis, MO) to facilitate selective enumeration of the pathogen (Johny et al., 2009). Briefly, each strain was cultured separately in 10 mL of tryptic soy broth (**TSB**; catalog no. DF 0370173, Difco, Sparks, MD) supplemented with 50 μg/mL of NA and incubated at 37°C for 24 h with agitation (100 rpm). After 3 successive transfers, 1 mL of each strain was transferred separately to 100 mL of TSB and incubated overnight. The cultures were combined and sedimented by centrifugation (3,600 × g for 15 min at 4°C), and the pellet was resuspended in 100 mL of phosphate buffered saline (**PBS**, pH 7.0) and used as the inoculum. The bacterial counts in the individual cultures and 4-strain mixture was confirmed by plating 0.1-mL portions of appropriate dilutions on xylose lysine desoxycholate agar (**XLD**; catalog no. DF 078817–9, Difco) plates containing NA (XLD-NA) and incubating the plates at 37°C for 24 h (Johny et al., 2009).

Experimental Design

Experiment 1 (3-wk Trial). Seventy straight-run 1-d-old broiler chicks were weighed at the beginning of the trial and randomly assigned to a control group [challenged with *Salmonella* Enteritidis, no supplemental CA (octanoic acid; catalog no. 129390025, Sigma-Aldrich, St. Louis, MO)] and 2 replicates each for low dose CA (challenged with *Salmonella* Enteritidis, 0.7% CA) and high dose CA (challenged with *Salmonella* Enteritidis, 1% CA) treatments (n = 14 birds/group). On d 1, birds were tested for the presence of any inherent *Salmonella* (n = 2 birds/group). On d 5, the birds were challenged by crop gavage with 1 mL of the inoculums, containing approximately 8.0 $\log_{10}$ cfu of the 4-strain *Salmonella* Enteritidis mixture. After 5 d postchallenge (**PC**), 2 birds from each treatment were euthanized to ensure *Salmonella* Enteritidis colonization (n = 2 birds/group). Appropriate volumes of CA were measured using a graduated cylinder, added to the feed, and mixed thoroughly to obtain a final concentration of 0.7 or 1% CA. The feed was given to the birds for the last 5 d before killing on d 21 to collect organ samples for bacteriological analysis (n = 10 birds/group). Autopsy and tissue collection were performed at the Connecticut Veterinary Diagnostic Laboratory (CVDL), University of Connecticut. The samples were collected in 50-mL sterile tubes containing ice-cold PBS and were transported to the laboratory on ice for bacteriological analysis performed on the same day.

Experiment 2 (6-wk Trial). Similar to experiment 1, 1-d-old chicks (n = 70) were assigned to groups as described before. On d 25, the birds were challenged with ~8.0 $\log_{10}$ cfu of the *Salmonella* Enteritidis mix by crop gavage and confirmed colonization with the bacterium after 5 d. The CA was supplemented through feed from d 37 for 5 d until the birds were killed by CO_2 asphyxiation on d 42 (n = 10 birds/group). Autopsy, tissue collection, and bacteriological analysis were done on the same day as before.

Determination of Salmonella Enteritidis in Digestive Tract and Internal Organs

Various segments of the digestive tract (crop, small intestine, cloaca, and cecum) and liver and spleen from each bird were weighed, processed using a tissue homogenizer (TM125, Omni Tissue Master; Omni International, Marietta, GA), and diluted 10-fold in sterile PBS. A volume of 0.1-mL portion of appropriate dilutions was surface-plated on duplicate XLD-NA plates. The colonies were enumerated after incubation at 37°C for 48 h. Representative colonies from XLD-NA plates were confirmed as *Salmonella* using *Salmonella* rapid detection kit (catalogue no. 24-C008, Microgen Bioproducts Ltd., Camberley, UK). When the colonies were not detected by direct plating, the samples were tested for surviving cells by enrichment in 100 mL of selenite cysteine broth (**SCB**; catalog no. OXCM0699B, Oxoid, supplied by Fisher Scientific) at 37°C for 48 h (Fernandez et al., 2002), followed by streaking on XLD-NA plates. Representative colonies from the plates were confirmed as *Salmonella* by *Salmonella* rapid detection kit.

BW and Feed Consumption

The feed consumption and BW of birds were determined for each trial. Birds were weighed individually at the start and end of each experiment and averaged. The average feed consumption per bird was calculated by dividing the total amount of feed consumed per group by the number of birds in the respective group.

Cell Culture

Avian Epithelial Cell Line. Budgerigar abdominal tumor cells (**BATC**), a permanent avian abdominal epithelial cell line, a gift from Margie Lee, College of Veterinary Medicine, University of Georgia, Athens were used. The cell line is a published model for studying *Salmonella* invasion and pathogenesis in avian species (Dodson et al., 1999; Henderson et al., 1999; Hudson et al., 2000). The cell culture assay was used to determine if CA reduced *Salmonella* Enteritidis invasion of avian intestinal epithelial cells. The cells were cultured in Dulbecco's modified eagle medium (**DMEM**; catalog no. 21063029, Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (**FCS**; catalog no. 10099141, Invitrogen). After 3 successful propagations, the cells were seeded into wells of 24-well tissue culture plates containing 1 mL of whole medium (DMEM + 10% FBS) at 1×10^5 cells/well and incubated at 37°C with 5% CO_2 to reach a confluency of >95% within 48 h. The viability of cells was confirmed using trypan blue vital dye exclusion assay (Pazos et al., 2002). Briefly, 50 µL of the diluted BATC suspension before each experiment was mixed with 10 µL of the dye, and 10 µL of the mixture was loaded in the counting chambers of the hemocytometer. After a minute, the number of non-stained cells was counted under the low-power objective of the light microscope.

Salmonella Invasion Assay. Subinhibitory concentration (**SIC**) of CA against *Salmonella* Enteritidis was determined in whole medium and Luria Bertani (**LB**; catalog no. DF0446–17–3, BD Diagnostic Systems, supplied by Fisher Scientific) broth, separately. The SIC (or sub-MIC) of an antimicrobial is the concentration below the minimum inhibitory concentration (**MIC**) that does not kill the bacteria but can modify their physicochemical characteristics and may interfere with bacterial functions (Fonseca et al., 2004). The SIC of CA (0.075%) was used to determine if the molecule exerted any effect on the virulence in *Salmonella* that help them invade intestinal epithelial cells. After 3 successful propagations, the BATC were seeded into wells of 24-well tissue culture plates containing 1 mL of whole medium (DMEM + 10% FBS) and incubated at 37°C with 5% CO_2 to reach a confluency of >95% within 48 h. The *Salmonella* Enteritidis strains were grown separately in LB broth with (0.075%) or without the SIC of CA (0%) with agitation (100 rpm) at 37°C, to mid-log phase. The cultures were sedimented by centrifugation (3,600 × g for 10 min at 4°C) and the pellet resuspended in DMEM and used as the inoculum. The bacterial counts in the individual cultures were confirmed by plating 0.1-mL portions of appropriate dilutions on TSA plates and incubating the plates at 37°C for 24 h. The BATC were inoculated with each *Salmonella* Enteritidis strain separately (multiplicity of infection of ~50). The tissue culture plates were then centrifuged at 1,000 × g for 3 min at 23°C and incubated for 45 min at 37°C with 5% CO_2. Thereafter, the medium was removed from the wells and replaced with fresh medium supplemented with 100 µg/mL of gentamicin (catalogue no. 15750078; Gibco, Invitrogen). The samples were incubated for 1 h to kill all the extracellular bacteria. The wells were then washed with PBS 3 times, and then 1 mL of PBS containing 0.1% Triton X (catalogue no. HFH10; Invitrogen) was added, followed by incubation at 37°C with 5% CO_2 for 15 min to lyse the BATC and release the intracellular *Salmonella*. The cell lysates were serially diluted, plated on TSA plates, and incubated at 37°C for 24 h before counting. Triplicate samples were included and the experiment was repeated 2 times.

Real-Time Quantitative PCR

RNA Isolation and cDNA Synthesis. Each strain of *Salmonella* Enteritidis was grown in LB without (0%)

and with the SIC of CA (0.075%) to mid-log phase at 37°C. Three milliliters of bacterial culture was centrifuged at 14,000 × *g* for 2 min at 4°C and the resultant pellet incubated with 1 mL of RNAprotect reagent (catalogue no. 76506; Qiagen, Valencia, CA) for 5 min at room temperature. Total RNA was extracted from the control and treated *Salmonella* Enteritidis cells using the RNeasy minikit (catalogue no. 74104; Qiagen) according to manufacturer's instructions. Quantitation of RNA was done by measuring the absorbance at 260 and 280 nm. Complimentary DNA was synthesized using the Superscript II Reverse transcriptase kit (catalogue no. 18080–044; Superscript, Invitrogen). The cDNA was used as the template for the amplification of *Salmonella* genes, *hilA* and *hilD*. Specific primers for *Salmonella* invasion genes, *hilA* (forward primer: 5′-TTACTGTGCGCTGGCAGAAT-3′; reverse primer: 5′-TCGCCTTAATCGCATGTTCTT-3′) and *hilD* (forward primer: 5′-GGCGGTACCCACAGAGAAAG-3′; reverse primer: 5′-TCGTACAGGAGAACGCCGTT-3′) and 16S rRNA (endogenous control; forward primer: 5′-GTATGCGCCATTGTAGCACG-3′; reverse primer: 5′-TCATCATGGCCCTTACGACC-3′) were designed using Primer Express software (Applied Biosystems) based on *Salmonella* Enteritidis strain P125109 genome (NCBI Reference Sequence: NC_011294.1) and were custom synthesized by Integrated DNA Technologies (Foster City, CA). Real-time quantitative PCR (RT-qPCR) was performed with the ABI PRISM 7900 sequence detection system (Applied Biosystems) using SYBR Green assay (Applied Biosystems) under custom thermal cycling conditions. The biological replicates were analyzed in triplicate and normalized against 16S rRNA gene expression. The comparative Ct method ($2^{-\Delta\Delta Ct}$) was used to assess the relative changes in *hilA* and *hilD* mRNA expression levels between the control and CA-treated *Salmonella* Enteritidis and expressed as relative quantitation of *hilA* or *hilD* expression (RQ; Bookout and Mangelsdorf, 2003; Schmittgen and Livak, 2008).

Statistical Analysis

A completely randomized design was used to analyze the effect of CA on *Salmonella* Enteritidis in either 3- or 6-wk trials. The treatment structure included 3 concentrations of CA (0, 0.7, or 1% CA) and 6 organ samples (crop, small intestine, cecum, cloaca, liver, and spleen), and the experimental unit was pen. The number of *Salmonella* colonies was logarithmically transformed ($\log_{10}$ cfu/g) before analysis to achieve homogeneity of variance (Byrd et al., 2003). The samples from which no bacteria were recovered after spread plating, but positive after enrichment, were assumed a value of 0.95 for analysis (9 cfu/mL; Seo et al., 2000; Young et al., 2007). The data were analyzed using the PROC-MIXED procedure of the statistical analysis software (version 9.3, SAS Institute Inc., Cary, NC), with pen within treatment as the random statement. Differences among the least squares means were detected using Fisher's least significance difference test. A *P*-value <0.05 was considered statistically significant. The data were also analyzed with a binary approach using the PROC-GENMOD procedure of SAS to determine the effect of CA treatments on the presence (positive after either direct plating or enrichment) or absence (negative after both direct plating and enrichment) of *Salmonella* in different organ samples. Pen was the experimental unit, and the analysis was done for each organ separately. For cell invasion assays, differences between groups were analyzed using one-way ANOVA, and $P < 0.05$ was considered statistically significant (n = 6). The RT-qPCR data were subjected to nonparametric (PROC-NPAR1WAY) Wilcoxon tests (SAS), and a $P < 0.001$ was considered significant.

RESULTS

3-wk Trial

The results from the 3-wk trials are presented in Figures 1 through 6. Supplementation of CA reduced ($P < 0.05$) *Salmonella* Enteritidis counts in the cecum of birds (Figure 1A). Compared with controls, *Salmonella* Enteritidis populations in the cecum were reduced by ~3.0 $\log_{10}$ cfu/g in birds supplemented with 0.7 or 1% CA. Similarly, both the groups of CA-treated birds consistently yielded lower counts of the pathogen in the cloacal samples compared with control (Figure 2A). The mean *Salmonella* Enteritidis populations recovered from the cloaca of control and CA-treated birds were 5.0 $\log_{10}$ cfu/g and 3.0 $\log_{10}$ cfu/g, respectively. Consistent with the results from cecal and cloacal samples, 0.7 or 1% CA also decreased ($P < 0.05$) *Salmonella* counts in the small intestine of birds at the end of 3 wk (Figure 3A). A significant effect of CA supplementation on *Salmonella* Enteritidis populations was also observed in liver (Figure 4A), spleen (Figure 5A), and crop (Figure 6A), where the pathogen was reduced by 1.2 to 2.5 $\log_{10}$ cfu/g ($P < 0.05$) in comparison to control birds. Although both concentrations of CA consistently decreased the pathogen populations in all the organ samples, there was no difference ($P > 0.05$) in *Salmonella* Enteritidis counts between 0.7 and 1% treatment groups.

6-wk Trial

Similar to the 3-wk trial, supplementation of CA at 0.7 or 1% levels reduced ($P < 0.05$) *Salmonella* Enteritidis counts in the cecum, cloaca, intestine, liver, and spleen of 6-wk-old birds (Figures 1B–5B). For the crop, only 1% CA brought about a reduction in pathogen counts compared with control birds (Figure 6B).

BW and Feed Consumption

The body weights of birds did not differ between the groups ($P > 0.05$) for both 3- or 6-wk trials. The mean

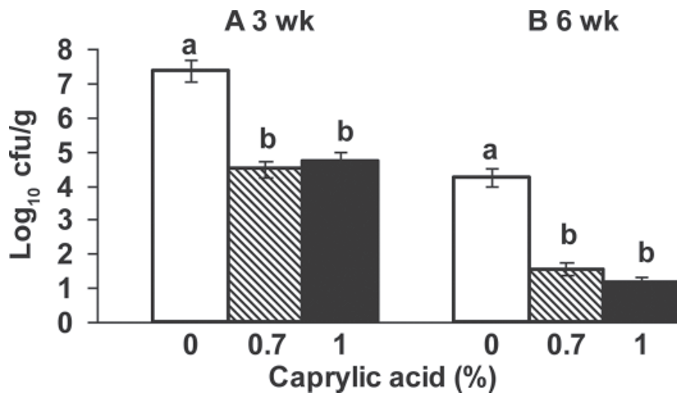

Figure 1. Effect of caprylic acid (catalog no. 129390025, Sigma-Aldrich, St. Louis, MO) on *Salmonella* Enteritidis counts in cecum of (A) 3-wk-old chickens (n = 50 birds/trial) and (B) 6-wk-old chickens (n = 50 birds/trial) fed caprylic acid for 5 d before necropsy. The error bars indicate SE. Columns with no common letters differ at $P < 0.05$.

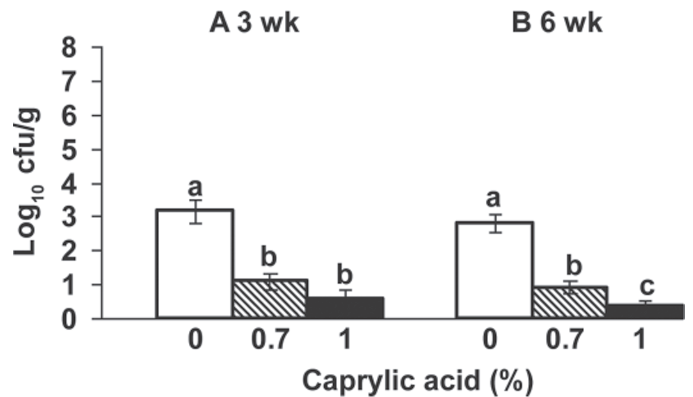

Figure 3. Effect of caprylic acid on *Salmonella* Enteritidis counts in the small intestine of (A) 3-wk-old chickens (n = 50 birds/trial) and (B) 6-wk-old chickens (n = 50 birds/trial) fed caprylic acid for 5 d before necropsy. The error bars indicate SE. Columns with no common letters differ at $P < 0.05$.

BW of control, 0.7, and 1% CA groups (in kg) were 0.62 ± 0.21, 0.58 ± 0.17, 0.61 ± 0.17 and 2.86 ± 0.06, 2.80 ± 0.05, 2.90 ± 0.05, respectively, at the end of 3- and 6-wk trials. The average feed intake was similar among the groups in both trials (data not shown).

Invasion Assay

Because 0.075% CA did not inhibit the growth of *Salmonella* Enteritidis, it was selected as the SIC. Caprylic acid at its SIC reduced the invasion ($P < 0.05$) of all 4 *Salmonella* Enteritidis isolates in BATC (Figure 7). Caprylic acid was found to reduce ($P < 0.05$) the number of invaded *Salmonella* Enteritidis cells in BACT by ~80%, relative to control.

hilA and hilD Expression

It was observed that the SIC of CA reduced *hilA* and *hilD* expression in all strains of *Salmonella* Enteritidis ($P < 0.05$), compared with that in untreated controls. Caprylic acid reduced *hilA* expression by approximately 2.5-, 4.0-, 2.0-, and 1.5-fold (Figure 8A) and *hilD* expression by approximately 2.5-, 5.0-, 2.0-, and 1.5-fold (Figure 8B), respectively in SE12, SE21, SE28, and SE31.

DISCUSSION

It is widely accepted that *Salmonella* Enteritidis colonizes the intestinal tract of chickens, with large numbers colonizing specifically in the cecum (Cerquetti and Gherardi, 2000; Van Immerseel et al., 2004). Besides the ceca, other parts of the digestive tract, including the crop, small intestine, and cloaca are locations where the pathogen survive and get disseminated into the external environment (Khan et al., 2003; Li et al., 2003; Van Immerseel et al., 2004). In addition, organs such as liver and spleen are implicated during the infection episodes, because *Salmonella* Enteritidis reaches the organs by lymphatic or circulatory routes (Cerquetti and Gherardi, 2000; Van Immerseel et al., 2004). Therefore, in the current study, we investigated the efficacy of caprylic acid for reducing *Salmonella* Enteritidis popula-

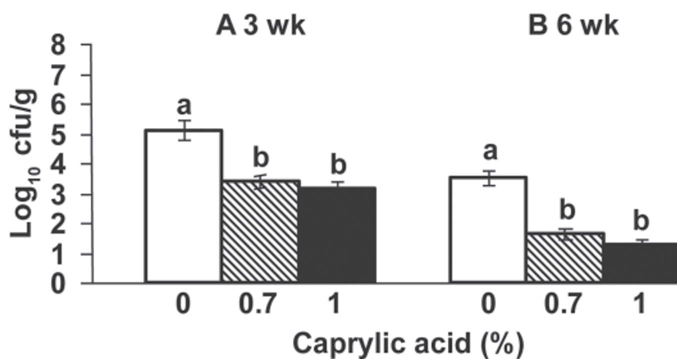

Figure 2. Effect of caprylic acid on *Salmonella* Enteritidis counts in cloaca of (A) 3-wk-old chickens (n = 50 birds/trial) and (B) 6-wk-old chickens (n = 50 birds/trial) fed caprylic acid for 5 d before necropsy. The error bars indicate SE. Columns with no common letters differ at $P < 0.05$.

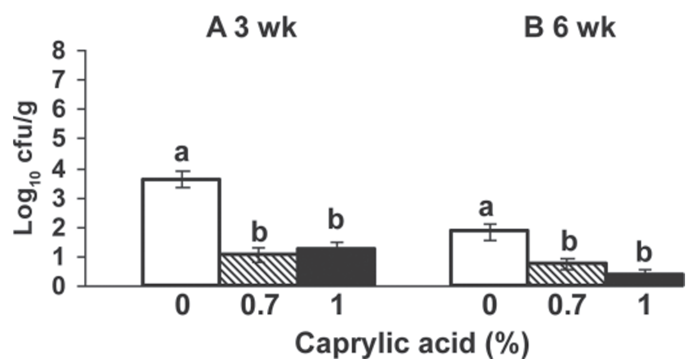

Figure 4. Effect of caprylic acid on *Salmonella* Enteritidis counts in liver of (A) 3-wk-old chickens (n = 50 birds/trial) and (B) 6-wk-old chickens (n = 50 birds/trial) fed caprylic acid for 5 d before necropsy. The error bars indicate SE. Columns with no common letters differ at $P < 0.\ 05$.

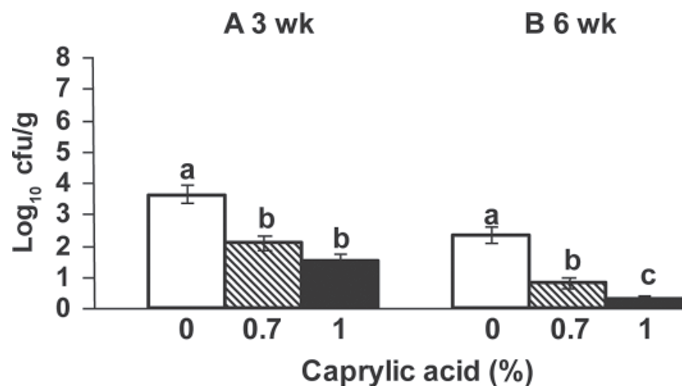

Figure 5. Effect of caprylic acid on *Salmonella* Enteritidis counts in spleen of (A) 3-wk-old chickens (n = 50 birds/trial) and (B) 6-wk-old chickens (n = 50 birds/trial) fed caprylic acid for 5 d before necropsy. The error bars indicate SE. Columns with no common letters differ at $P < 0.05$.

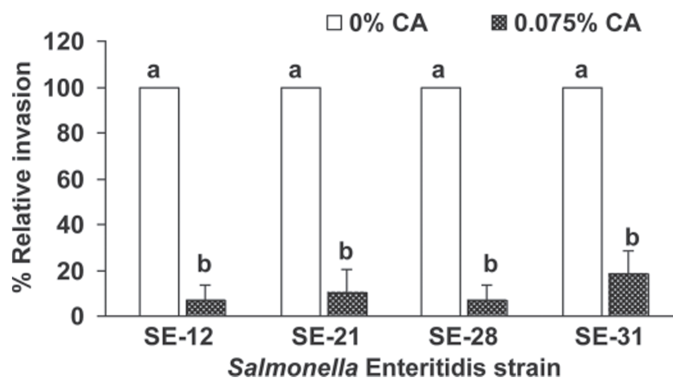

Figure 7. Effect of subinhibitory concentration of caprylic acid (CA; 0.075%) on *Salmonella* Enteritidis invasion of avian abdominal epithelial cell line, Budgerigar abdominal tumor cells. Four strains of *Salmonella* (SE-12, SE-21, SE-28, and SE-31) were grown in the presence of subinhibitory concentration of caprylic acid to mid-log phase and were inoculated separately (n = 6) on to cell lines at an multiplicity of infection (MOI) of ~50 and incubated for 45 min. Afterward, gentamicin (catalogue no. 15750078; Invitrogen, Carlsbad, CA) protection assay was performed for 1 h. Total invaded bacteria were determined after treatment with 0.1% triton X (catalogue no. HFH10; Invitrogen) and expressed as percentage invaded bacteria. The error bars indicate SE. Columns with no common letters differ at $P < 0.05$.

tions in different parts of the chicken gastrointestinal tract, including liver and spleen.

Results revealed that 0.7 or 1% CA supplemented through the feed were effective in reducing *Salmonella* Enteritidis populations in various parts of the chicken intestinal tract and internal organs such as liver and spleen. Although CA reduced *Salmonella* counts in all the tested sites ($P < 0.05$), its effect was more pronounced in the cecum (Figure 1A,B), with a maximum reduction of ~3.0 $\log_{10}$ cfu/g in *Salmonella* Enteritidis counts. Additionally, CA decreased the pathogen population by ~2 $\log_{10}$ cfu/g in the cloaca (Figure 2A,B). These reductions in *Salmonella* counts in the cecum and cloaca are important for the microbiological safety of poultry products, because these 2 sites represent 2 common locations in the birds where the bacteria are present in high numbers (Cerquetti and Gherardi, 2000; Li et al., 2003; Van Immerseel et al., 2004). *Salmonella* recovery from the crop, small intestine, liver, and spleen samples were comparatively lower than that recovered from cecum and cloaca; however, the bacterial counts from these samples were reduced in CA-treated birds ($P < 0.05$).

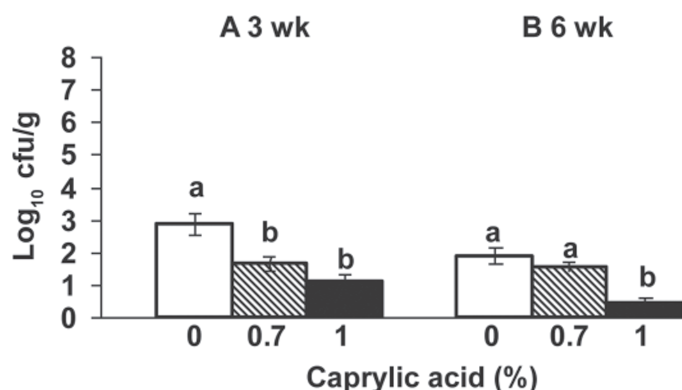

Figure 6. Effect of caprylic acid on *Salmonella* Enteritidis counts in crop of (A) 3-wk-old chickens (n = 50 birds/trial) and (B) 6-wk-old chickens (n = 50 birds/trial) fed caprylic acid for 5 d before necropsy. The error bars indicate SE. Columns with no common letters differ at $P < 0.05$.

Although birds from both trials (3-wk and 6-wk) were challenged with ~8.0 $\log_{10}$ cfu of *Salmonella* Enteritidis, the younger birds were colonized with more *Salmonella* than their older counterparts. For example, approximately 7.0 $\log_{10}$ cfu/g of *Salmonella* was recovered from the ceca of 3-wk-old birds (Figure 1A) compared with 4.0 $\log_{10}$ cfu/g recovered in the ceca of the 6-wk-old birds (Figure 1B). The reduced recovery of the pathogen was also observed in other organs from the 6-wk-old chickens, except small intestine ($P > 0.05$). It was previously reported that chickens become more resistant to *Salmonella* with increasing age due to the developing immune system (Holt et al., 1999; Bjerrum et al., 2003; Beal et al., 2004a,b).

Several mechanisms are attributed to the antibacterial action of MCFA, including CA. It is reported that CA can directly penetrate and be incorporated into the bacterial plasma membrane, changing the membrane permeability (Bergsson et al., 1999). It can also diffuse into the bacterial protoplasm and dissociate, leading to intracellular acidification affecting the enzymes and amino acid transport adversely (Freese et al., 1973; Viegas and Sa-Correia, 1991; Sun et al., 1998). It may also result in alterations in the cecal microflora populations and change in the physical characteristics of the intestine (Solís de los Santos et al., 2008). However, from our recent investigations, we found that CA did not alter cecal pH or normal cecal endogenous flora in chickens (Johny et al., 2009; Solís de los Santos et al., 2010).

Under nutrient-rich conditions, 2 transcriptional regulators, HilC and HilD, activate *hilA* expression in *Salmonella*, which in turn activates another transcriptional regulator, *invF* (Bajaj et al., 1995; Fahlen et al.,

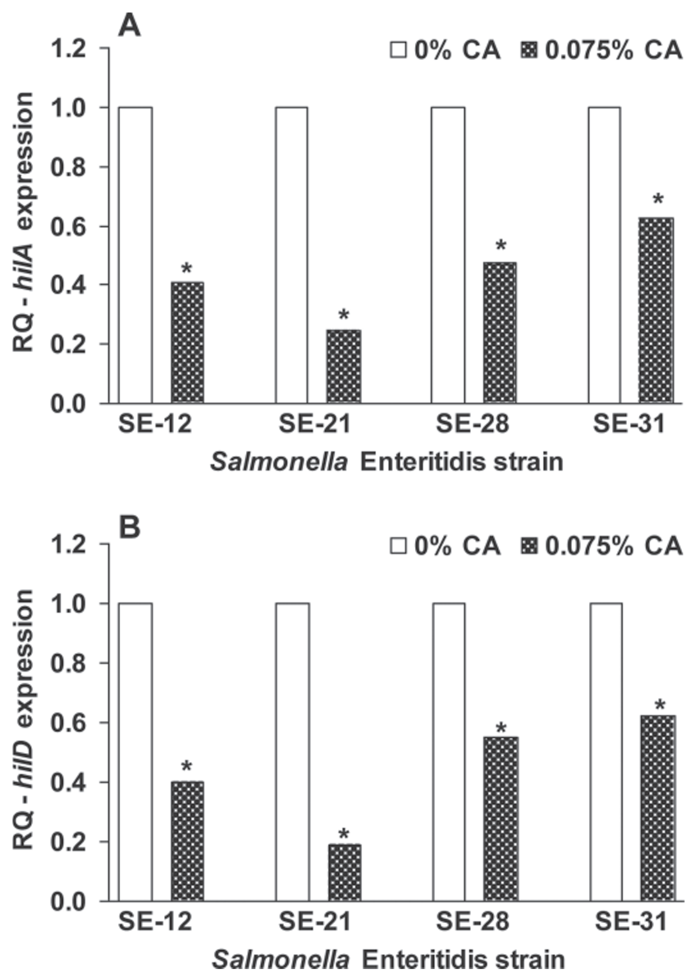

Figure 8. A) Effect of subinhibitory concentration of caprylic acid (CA; 0.075%) on *hilA* expression in *Salmonella* Enteritidis. Four strains of *Salmonella* Enteritidis were grown in the presence of subinhibitory concentration of caprylic acid to mid-log phase and the total RNA was extracted. Complementary DNA was synthesized and real-time quantitative PCR was done for *hilA* gene. Two biological replicates were analyzed in triplicate. *CA significantly downregulated *hilA* in all strains ($P < 0.001$). B) Effect of subinhibitory concentration of CA (0.075%) on *hilD* expression in *Salmonella* Enteritidis. Four strains of *Salmonella* Enteritidis were grown in the presence of subinhibitory concentration of caprylic acid to mid-log phase and the total RNA was extracted. Complementary DNA was synthesized and real-time quantitative PCR was done for *hilD* gene. Two biological replicates were analyzed in triplicate. *CA significantly downregulated *hilD* in all strains ($P < 0.001$).

2000; Ellermeier et al., 2005). This *invF* activates genes encoding effector proteins for invasion process (Darwin and Miller, 1999). These genes are critically affected by several environmental factors, including osmolarity, oxygen concentration, and pH (Ellermeier and Slauch, 2007). In vitro investigations have reported that low oxygen tension, high osmolarity, and slightly alkaline pH induce invasion of *Salmonella* Typhimurium into cultured epithelial cells (Lee and Falkow, 1990; Francis et al., 1992; Bajaj et al., 1995, 1996). We investigated if CA exerted any inhibitory effect on *Salmonella* Enteritidis invasion of host tissue, using an avian epithelial cell line model, namely BATC. For the invasion assay, SIC of CA was used because it is neither bacteriostatic nor bactericidal, and any inhibitory effect on pathogen invasion of intestinal cells could be attributed to the downregulation of *Salmonella* invasion mechanisms. The results from the cell culture assay revealed that CA reduced the invasion of the pathogen in BACT. Moreover, RT-qPCR results indicated CA substantially decreased the expression of *hilA* and *hilD*, the key regulators of *Salmonella* invasion of host tissue. Similarly, we observed that CA downexpressed *invF* by more than 2-fold in all 4 *Salmonella* Enteritidis strains ($P < 0.05$; data not shown). Van Immerseel et al. (2004) reported that caproic acid (3 g/kg of feed) supplemented to chicks resulted in significant reductions in the colonization of *Salmonella* Enteritidis in the ceca. These investigators suggested that MCFA could potentially reduce invasive abilities of *Salmonella* in T-84 epithelial cell lines and reduce *hilA* expression. It has been previously reported that short-chain fatty acids, such as butyric acid and propionic acid, decreased invasion of *Salmonella* Enteritidis in avian epithelial cells, although acetic acid and formate resulted in increased invasion (Van Immerseel et al., 2003). Gantois et al. (2006) reported that butyrate downregulated the expression of *Salmonella* Pathogenicity Island 1 (SPI 1) regulators *hilD* and *invF* in *Salmonella*.

Our previous study reported that prophylactic supplementation of CA before challenging birds with *Salmonella* Enteritidis reduced the pathogen colonization in 18-d-old chicks, with no deleterious effects (Johny et al., 2009). The results from this current study underscore that CA was not only effective in reducing *Salmonella* Enteritidis colonization in young and market-age birds but could also be used as a potential preslaughter treatment to reduce the pathogen carriage in birds. This is critical because enteric contents containing the pathogen could potentially contaminate the broiler carcasses during evisceration process and chilling (Morris and Wells, 1970), and substantial reductions in *Salmonella* Enteritidis on chicken carcasses can be achieved by delivering birds to the processing plants that are minimally contaminated with the pathogen (Bailey, 1993). It is also important to note that 0.7 or 1% CA did not reduce feed consumption and BW in birds. Therefore, CA could potentially be used as a safe antimicrobial feed additive to reduce *Salmonella* Enteritidis colonization in chickens and improve the safety of poultry meat.

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