In-Feed Supplementation of *trans*-Cinnamaldehyde Reduces Layer-Chicken Egg-Borne Transmission of *Salmonella enterica* Serovar Enteritidis

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Salmonella enterica serovar Enteritidis is a major foodborne pathogen in the United States, causing gastroenteritis in humans, primarily through consumption of contaminated eggs. Chickens are the reservoir host of *S. Enteritidis*. In layer hens, *S. Enteritidis* colonizes the intestine and migrates to various organs, including the oviduct, leading to egg contamination. This study investigated the efficacy of in-feed supplementation with *trans*-cinnamaldehyde (TC), a generally recognized as safe (GRAS) plant compound obtained from cinnamon, in reducing *S. Enteritidis* cecal colonization and systemic spread in layers. Additionally, the effect of TC on *S. Enteritidis* virulence factors critical for macrophage survival and oviduct colonization was investigated *in vitro*. The consumer acceptability of eggs was also determined by a triangle test. Supplementation of TC in feed for 66 days at 1 or 1.5% (vol/wt) for 40- or 25-week-old layer chickens decreased the amounts of *S. Enteritidis* on eggshell and in yolk ($P < 0.001$). Additionally, *S. Enteritidis* persistence in the cecum, liver, and oviduct in TC-supplemented birds was decreased compared to that in controls ($P < 0.001$). No significant differences in feed intake, body weight, or egg production in birds or in consumer acceptability of eggs were observed ($P > 0.05$). *In vitro* cell culture assays revealed that TC reduced *S. Enteritidis* adhesion to and invasion of primary chicken oviduct epithelial cells and reduced *S. Enteritidis* survival in chicken macrophages ($P < 0.001$). Follow-up gene expression analysis using real-time quantitative PCR (qPCR) showed that TC downregulated the expression of *S. Enteritidis* virulence genes critical for chicken oviduct colonization ($P < 0.001$). The results suggest that TC may potentially be used as a feed additive to reduce egg-borne transmission of *S. Enteritidis*.

Salmonella enterica serovar Enteritidis is one of the major foodborne pathogens in the United States responsible for causing enteric illnesses in humans (1). Eggs are the primary source of *S. Enteritidis* infection of humans (1, 2). Approximately 90 billion eggs are produced and 67.5 billion shell eggs consumed annually in the United States (3). Thus, the microbiological safety of eggs is a major concern to the government, the poultry industry, and consumers due to the potential impacts on public health and the economy. Chickens act as asymptomatic carriers of *S. Enteritidis*, resulting in its environmental dissemination and potential infection of humans. Humans contract *S. Enteritidis* infection via consumption of contaminated, raw, or undercooked eggs, and several epidemiological studies have confirmed this association between human salmonellosis and egg consumption (4, 5).

Despite the implementation of various pre- and postharvest control measures, *S. Enteritidis* remains a major cause of egg-borne disease outbreaks in the United States (1). Recently, the U.S. Centers for Disease Control and Prevention (CDC) reported that the incidence of foodborne salmonellosis did not decrease significantly in the last decade, highlighting the need for renewed efforts and alternative approaches for controlling *Salmonella* (6). Moreover, in light of increasing evidence linking human salmonellosis with consumption of eggs, the Food and Drug Administration (FDA) announced in 2009 that eggs constitute the primary source of *S. Enteritidis* infections of humans, and it issued a final rule that requires egg producers to implement measures to prevent the pathogen from contaminating eggs on the farm and growing further during storage and transportation (7).

The cecum is the primary site of *S. Enteritidis* colonization in

chickens (8, 9), with cecal carriage of the pathogen leading to contamination of the ovaries by a transovarian route (10). Additionally, the uptake of *Salmonella* by hen macrophages following bacterial invasion of intestinal cells aids in its dissemination within the host, including in the reproductive organs (11–14). Contamination of egg contents (yolk, albumen, and eggshell membranes) by *S. Enteritidis* can occur before oviposition (11, 12), where *Salmonella* colonizing reproductive organs invades and multiplies in the granulosa cells of the preovulatory follicles in the

Received 21 November 2014 Accepted 11 February 2015

Accepted manuscript posted online 20 February 2015

Citation Upadhyaya I, Upadhyay A, Kollanoor-Johny A, Mooyottu S, Baskaran SA, Yin H-B, Schreiber DT, Khan MI, Darre MJ, Curtis PA, Venkitanarayanan K. 2015. In-feed supplementation of *trans*-cinnamaldehyde reduces layer-chicken egg-borne transmission of *Salmonella enterica* serovar Enteritidis. *Appl Environ Microbiol* 81:2985–2994. doi:10.1128/AEM.03809-14.

Editor: C. A. Elkins

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AEM.03809-14>.

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reproductive tract (15, 16). Since *S. Enteritidis* colonization in the ceca of layers results in the transovarian spread of the pathogen to reproductive organs, a decreasing pathogen prevalence in flocks has been reported to result in a direct reduction in human health risk (17). Control measures implemented at the flock level may reduce human salmonellosis from egg consumption and have thus been suggested as a primary focus of control at the farm level (18). Therefore, innovative on-farm strategies for preventing *S. Enteritidis* colonization of birds are critical for preventing pathogen contamination of eggs. Besides reducing the colonization of *S. Enteritidis* in the chicken cecum, a viable approach may also be one that potentially reduces bacterial virulence, thereby preventing colonization in the reproductive tract and eventual transovarian transmission to eggs (10, 19, 20).

Various approaches to reducing *S. Enteritidis* colonization in poultry have been investigated, with various degrees of success. These include feeding chickens competitive exclusion bacteria (21, 22), bacteriophages (23), organic acids (24, 25), oligosaccharides (26, 27), or antibiotics (28) and vaccinating birds (29). Due to the limited efficacy of the aforementioned approaches, along with concerns over the toxicity of synthetic chemicals and the development of multidrug resistance in bacteria, there is a growing interest in exploring the potential of natural antimicrobials for controlling pathogens (30, 31).

Since ancient times, plants have played a critical role in human health and well-being. Plant extracts have been used widely in herbal medicine, both prophylactically to prevent infections and therapeutically for the treatment of various ailments and diseases (32). The antimicrobial activity of several plant-derived compounds has been reported previously (33, 34), and a wide array of active components have been identified (35). A majority of these compounds are secondary metabolites and are produced by plants in response to microbial infection or animal predation (36, 37). Among various plant compounds, *trans*-cinnamaldehyde (TC), a major ingredient in cinnamon (*Cinnamomum zeylandicum*), has been reported to exhibit antibacterial properties against both Gram-negative and Gram-positive bacteria (33). It is a GRAS (generally regarded as safe) chemical approved for addition to foods by the U.S. FDA (approval TC-21CFR182.60). Previously, our laboratory observed that TC was effective at reducing *S. Enteritidis* in chicken cecal contents *in vitro* and in various internal organs in broilers (38). In addition, TC was found to inhibit biofilm formation by *Cronobacter sakazakii* (39) and uropathogenic *Escherichia coli* (40), by downregulating critical genes involved in biofilm synthesis.

The objective of this study was to investigate the efficacy of feed-supplemented TC in reducing *S. Enteritidis* colonization, systemic spread, and contamination of eggs in layer chickens. In addition, the potential effect of TC on *S. Enteritidis* virulence factors critical for egg-borne transmission in layers was determined using cell culture and gene expression studies. Moreover, the effect of TC supplementation in birds on consumer acceptability of eggs was studied.

MATERIALS AND METHODS

Bacterial strains and dosing. A four-strain mixture of *S. Enteritidis* isolated from chickens (obtained from the Connecticut Veterinary Diagnostic Medical Laboratory, University of Connecticut) was used to inoculate the birds. The isolates were SE-12 (chicken liver, phage type 14b), SE-21 (chicken intestine, phage type 8), SE-28 (chicken ovary, phage type 13a),

and SE-31 (chicken gut, phage type 13a). Each strain was preinduced for resistance to 50 µg/ml of nalidixic acid (NA; Sigma-Aldrich, St. Louis, MO) for selective enumeration (38). One hundred microliters of each NA-resistant strain was cultured separately in 10 ml tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) overnight, transferred to flasks containing 100 ml TSB supplemented with 50 µg/ml of NA, and incubated overnight at 37°C with shaking (100 rpm). Equal volumes of the *S. Enteritidis* cultures were combined and centrifuged at $3,600 \times g$ for 15 min at 4°C. The pellet was washed and resuspended in 100 ml of phosphate-buffered saline (PBS; pH 7.0) and then used as the inoculum ($\sim 10^{10}$ CFU/ml). The bacterial counts in the individual cultures and the four-strain cocktail were confirmed by plating 0.1-ml portions of appropriate dilutions on xylose lysine desoxycholate agar (XLD; Difco) plates containing NA (XLD-NA) and incubating the plates at 37°C for 24 h.

Experimental birds and housing. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Connecticut. Twenty-five- and 40-week-old *Salmonella*-free layer hens (single comb, White Leghorn) were procured from the University of Connecticut poultry farm and allocated to floor pens, with non-medicated feed *ad libitum*, *Salmonella*-free water, age-appropriate ambient temperatures, and bedding, at the Isolation Facility of the University of Connecticut.

Two separate experiments with TC were conducted, wherein 40-week-old (experiment 1) and 25-week-old (experiment 2) layers were randomly allocated to 6 treatments (20 birds/treatment group). The treatments included a negative control (no *S. Enteritidis* challenge and no supplemental TC), a low-dose compound control (no *S. Enteritidis* challenge but 1.0% supplemental TC [vol/wt]), a high-dose compound control (no *S. Enteritidis* challenge but 1.5% supplemental TC [vol/wt]), a positive control (*S. Enteritidis* challenge but no supplemental TC), a low-dose treatment (*S. Enteritidis* challenge and 1% supplemental TC), and a high-dose treatment (*S. Enteritidis* challenge and 1.5% supplemental TC). On day 0, two birds from each experimental group were randomly selected and sacrificed to confirm that the birds were initially devoid of any *Salmonella*. TC was supplemented in the feed for 66 days, starting on day 0. Appropriate amounts of TC were added to feed and mixed thoroughly to obtain concentrations of 1 and 1.5% in the feed. On day 10, birds in the positive-control, low-dose, and high-dose treatment groups were challenged with *S. Enteritidis* ($10 \log_{10}$ CFU/bird) by crop gavage. After 3 days of *S. Enteritidis* challenge (day 13), three birds from each treatment group were sacrificed to determine pathogen colonization in the ceca, liver, and oviduct. After 7 days of challenge (day 17), eggs were collected daily from each treatment group and tested for the presence or absence of *S. Enteritidis* until day 66. In addition, cloacal swabs from all birds were analyzed weekly until day 66 for the presence or absence of *Salmonella*. At the end of 66 days, the birds from all treatment groups were euthanized by CO₂ asphyxiation. Cecum, oviduct, and liver samples from birds were collected for *S. Enteritidis* detection.

Detection of *S. Enteritidis* on egg surfaces and in egg contents. After 7 days of *S. Enteritidis* challenge, eggs from each treatment group were collected daily and checked for the presence or absence of the pathogen until day 66 of the experiment. The presence of *S. Enteritidis* on eggshell surfaces and in egg contents was determined according to the method of Miyamoto et al. (11). Each egg was rinsed separately in a sterile stomacher bag containing 50 ml of selenite cysteine broth supplemented with NA (50 µg/ml) for 2 min. After washing, the egg was removed and the broth was incubated at 37°C for 48 h, followed by streaking on XLD-NA plates to detect the presence of *S. Enteritidis* on the eggshell. The bacterial colonies were confirmed as *Salmonella* by use of a *Salmonella* rapid detection kit (Microgen Bioproducts Ltd., Camberley, United Kingdom).

The eggs that were washed in selenite cysteine broth as described above were disinfected by wiping with 70% ethanol, dried, and cracked open aseptically, and the shell and egg contents were collected into separate stomacher bags containing 50 ml of selenite cysteine broth containing NA. The bags with the egg contents or shells were homogenized for 1 min in a

stomacher and then incubated at 37°C for 24 to 48 h to detect *Salmonella* present inside the egg. The bacterial colonies were confirmed as *S. Enteritidis* as described previously.

Detection of *S. Enteritidis* in internal organs. *S. Enteritidis* populations in the oviduct, liver, and cecum were determined as described previously (38). The organ samples and their contents from each bird were weighed and homogenized. Each homogenate was serially diluted (1:10) in PBS, and appropriate dilutions were plated on XLD-NA plates for bacterial enumeration. Representative colonies from XLD-NA plates were confirmed as *Salmonella* by use of a *Salmonella* rapid detection kit (Mikrogen Bioproducts Ltd.). When colonies were not detected by direct plating, samples were tested for surviving *Salmonella* by enrichment in 100 ml selenite cysteine broth (Oxoid) for 48 h at 37°C (38), followed by streaking on XLD-NA plates. In addition, endogenous cecal bacteria were enumerated by plating appropriate dilutions of the cecum samples on duplicate thioglycolate agar (TGA) plates (Difco), followed by incubation at 37°C under 5% CO₂ for 24 h.

Determination of SICs of TC. Subinhibitory concentrations (SICs) of TC against *S. Enteritidis* were determined as previously described (38). Sterile 24-well polystyrene tissue culture plates (Costar; Corning Incorporated, Corning, NY) containing TSB (1 ml/well) were inoculated separately with ~6.0 log CFU of *S. Enteritidis*, followed by the addition of 1 to 10 µl of TC (Sigma-Aldrich), in increments of 0.5 µl. The plates were incubated at 37°C for 24 h, and bacterial growth was determined by culturing on duplicate tryptic soy agar (TSA) and XLD plates. The two highest concentrations of TC below the MIC that did not inhibit bacterial growth after 24 h of incubation compared to the control were selected as the SICs for the study. Duplicate samples were included, and the experiment was repeated three times.

Cell culture. Primary chicken oviduct epithelial cells (COEC) were isolated as described previously (41, 42). The oviduct tissues of 25- to 28-week-old *Salmonella*-free layer hens (single comb, White Leghorn) were obtained from the University of Connecticut poultry farm. The isthmus portion of the oviduct was collected and flushed with Hanks' balanced salt solution (HBSS) (Sigma-Aldrich) containing 200 U/ml penicillin (Sigma-Aldrich) and 200 mg/ml streptomycin (Sigma-Aldrich). The epithelial cells were gently scraped off the oviduct and treated with 20 ml of HBSS containing 1 mg/ml collagenase (Sigma-Aldrich) for 30 min at 37°C. After collagenase treatment, the supernatant was discarded, and trypsinization of tissue fragments was done using 0.25% trypsin and 3 mM EDTA (Sigma-Aldrich) in 20 ml of HBSS for 10 min at 37°C. Heat-inactivated fetal bovine serum (10% HI-FBS; Gibco, Invitrogen, Grand Island, NY) was added to the cell suspension to inactivate trypsin. The cell suspension was then passed through a sterile cell strainer (100 µm; Fisher Scientific) to remove undigested tissue debris. The cell suspension was centrifuged at 50 × g for 5 min to separate epithelial cells from erythrocytes and platelets. The supernatant obtained after centrifugation was discarded, and the pellet containing epithelial cells was resuspended in minimal essential medium (MEM; Invitrogen) supplemented with 10% HI-FBS, 2% heat-inactivated chicken serum (HICS; Gibco, Invitrogen), insulin (0.12 U/ml; Sigma-Aldrich), and estradiol (50 nM; Sigma-Aldrich). The COEC were incubated for 2 h at 39°C under 5% CO₂ to allow fibroblast attachment. Following incubation, the unattached epithelial cells were collected by gentle pipetting, followed by centrifugation at 125 × g for 10 min. The pelleted epithelial cells were resuspended in whole medium and allowed to grow until a confluent monolayer was formed. After four successive passages, the cells were seeded onto 24-well cell culture plates (~2 × 10⁵ cells per well) and grown at 39°C under 5% CO₂ for 24 to 36 h. The identity of COEC was confirmed by determining the constitutive expression of the avian beta defensin (AvβD) genes by real-time quantitative PCR (RT-qPCR) as described previously (42).

***Salmonella* adhesion and invasion assays.** The adhesive and invasive abilities of three *S. Enteritidis* isolates on COEC were investigated as previously described (42). *S. Enteritidis* was cultured to mid-log phase in either the absence (control) or presence of TC at the SICs before inocula-

tion onto COEC. The COEC were seeded into 24-well tissue culture plates at ~10⁵ cells per well and inoculated with ~6.0 log CFU of each *S. Enteritidis* isolate separately (multiplicity of infection [MOI] of 10). The inoculated COEC were incubated at 39°C in a humidified 5% CO₂ incubator for 1 h to facilitate *S. Enteritidis* attachment, followed by gentle washing with PBS to remove unattached bacteria. The infected COEC were lysed with 0.1% Triton X-100 (Invitrogen), and the number of viable adherent *S. Enteritidis* organisms was determined by serial dilution and plating on TSA and XLD plates. For the invasion assay, a gentamicin protection assay was performed as described previously (38, 42). The *S. Enteritidis*-inoculated monolayer was incubated for 1 h, followed by three washings in specific minimal medium (MEM). The infected cells were incubated for an additional 2 h in whole medium-10% FBS containing gentamicin (100 µg/ml; Sigma-Aldrich) to kill the extracellular *S. Enteritidis*. Subsequently, the wells were washed three times with PBS, followed by addition of 1 ml 0.1% Triton X-100 solution and incubation at 39°C for 15 min to lyse the cells and release the invaded *S. Enteritidis*. The cell lysates were serially diluted, plated on TSA/XLD plates, and incubated at 37°C for 24 h.

Macrophage cultivation and *S. Enteritidis* survival assay. Chicken macrophages (HTC; chicken monocyte cell line) were cultivated in RPMI 1640 with 10% FBS. The cells were activated and plated as described previously (42, 43). Twenty-four hours prior to infection, the cells were seeded in 24-well tissue culture plates and incubated at 39°C under 5% CO₂ to form a monolayer. Each *S. Enteritidis* isolate, grown to mid-log phase in the presence or absence of TC at the SICs, was centrifuged (3,600 × g) and resuspended in RPMI medium with 10% FBS. Macrophages were infected with 6.0 log CFU of each *S. Enteritidis* isolate at an MOI of 10 and incubated at 37°C for 45 min under 5% CO₂. After incubation, the macrophages were treated with a whole medium containing 100 µg of gentamicin/ml for 2 h at 37°C to kill extracellular bacteria. The macrophages were then washed twice and maintained in whole medium supplemented with 10 µg of gentamicin/ml for 24, 48, and 72 h. The medium was replaced every 24 h. The macrophages were washed twice, lysed with 0.5% Triton X-100, serially diluted, and plated on TSA and XLD agar plates to determine the surviving population of *S. Enteritidis* at the aforementioned time intervals. All assays were performed in duplicate at least three times.

RNA isolation and RT-qPCR. To determine the basal expression of the AvβD genes in COEC, RT-qPCR was performed using total RNA extracted from COEC and primers specific for the AvβD genes (42). The amplification of AvβD genes, including the AvβD-4, AvβD-5, AvβD-9, AvβD-10, AvβD-11, and AvβD-12 genes, was achieved with primers specific for each gene, and the β-actin gene served as an endogenous control. Data for this experiment are presented in Fig. S1 in the supplemental material.

In addition, the effect of TC on the expression of *S. Enteritidis* virulence genes was investigated using RT-qPCR. Each *S. Enteritidis* strain was grown separately to mid-log phase, with and without TC at the SICs, in TSB at 37°C. Total RNA was extracted using an RNeasy RNA isolation kit (Qiagen, Valencia, CA). cDNA was synthesized using a Superscript II reverse transcriptase kit (Invitrogen) and was used as the template for RT-qPCR. The primers (Table 1) for each gene were designed from published *S. Enteritidis* sequences by using Primer Express software (Applied Biosystems, Foster City, CA). Relative gene expression was determined according to the comparative critical threshold (*C_T*) method, using a model 7500 Fast Step One Plus real-time PCR machine (Applied Biosystems). Data were normalized to the endogenous control (16S rRNA), and the levels of candidate gene expression in treated and control samples were determined.

Sensory evaluation of eggs. The sensory evaluation of eggs collected from TC-treated and control birds was conducted at the Sensory Laboratory, Department of Poultry Science, Auburn University, AL. Eggs were collected from control and TC-treated groups of birds once a week for 3 weeks and were tested using the triangle test (44) to assess whether consumers could detect a difference between the eggs from TC-supplemented

TABLE 1 Primers used for RT-qPCR analysis of *S. Enteritidis* genes

Accession no.	Primer	Gene product	Primer sequence (5'–3')
NC_011294.1	<i>fimDF</i> <i>fimDR</i>	Outer membrane usher protein FimD	CGCGGCGAAAGTTATTTCAA CCACGGACGCGGTATCC
NC_011294.1	<i>flgGF</i> <i>flgGR</i>	Flagellar basal body rod protein	GCGCCGGACGATTGC CCGGGCTGGAAGCATT
NC_011294.1	<i>hflKF</i> <i>hflKR</i>	FtsH protease regulator	AGCGCGGCGTTGTGA TCAGACCTGGCTCTACCAGATG
NC_011294.1	<i>invHF</i> <i>invHR</i>	Cell adherence/invasion protein	CCCTTCTCCGTGAGCAAA TGGCCAGTTGCTCTTTCTGA
NC_011294.1	<i>lrpF</i> <i>lrpR</i>	Leucine-responsive transcriptional regulator	TTAATGCCGCGGTGCAA GCCGGAACCAAATGACACT
NC_011294.1	<i>mrr1F</i> <i>mrr1R</i>	Pseudo/restriction endonuclease gene	CCATCGCTTCCAGCAACTG TCTCTACCATGAACCCGTACAAATT
NC_011294.1	<i>ompRF</i> <i>ompRR</i>	Osmolarity response regulator	TGTGCCGGATCTTCTTCCA CTCCATCGACGTCCAGATCTC
NC_011294.1	<i>orf245F</i> <i>orf245R</i>	Pathogenicity island protein	CAGGGTAATATCGATGTGGACTACA GCGGTATGTGGAAAACGAGTTT
NC_011294.1	<i>pipBF</i> <i>pipBR</i>	Pathogenicity island protein	GCTCCTGTTAATGATTTGCTAAAG GCTCAGACTTAACGACACCAAACTAA
NC_011294.1	<i>prot6EF</i> <i>prot6ER</i>	Fimbrial biosynthesis	GAACGTTTGGCTGCCTATGG CGCAGTGACTGGCATCAAGA
NC_011294.1	<i>rfbHF</i> <i>rfbHR</i>	RfbH dehydratase	ACGGTCGGTATTTGTCAACTCA TCGCCAACCGTATTTTGCTAA
NC_011294.1	<i>rpoSF</i> <i>rpoSR</i>	RNA polymerase sigma factor RpoS	TTTTTCATCGGCCAGGATGT CGCTGGGCGGTGATTC
NC_011294.1	<i>sipAF</i> <i>sipAR</i>	Pathogenicity island 1 effector protein	CAGGGAACGGTGTGGAGGTA AGACGTTTTTGGGTGTGATACGT
NC_011294.1	<i>sipBF</i> <i>sipBR</i>	Pathogenicity island 1 effector protein	GCCACTGCTGAATCTGATCCA CGAGGCGCTTGCTGATTT
NC_011294.1	<i>sodCF</i> <i>sodCR</i>	Superoxide dismutase	CACATGGATCATGAGCGCTTT CTGCGCCGCTCTGA
NC_011294.1	<i>sopBF</i> <i>sopBR</i>	Cell invasion protein	GCGTCAATTTTCATGGGCTAAC GGCGGCGAACCTATAAACT
NC_011294.1	<i>ssaVF</i> <i>ssaVR</i>	Secretion system apparatus protein SsaV	GCGCGATACGGACATATTCTG TGGGCGCCACGTGAA
NC_011294.1	<i>ssrAF</i> <i>ssrAR</i>	Sensor kinase	CGAGTATGGCTGGATCAAAACA TGTACGTATTTTTGCGGGATGT
NC_011294.1	<i>tatAF</i> <i>tatAR</i>	Twin-arginine translocase protein A	AGTATTTGGCAGTTGTTGATTGTTG ACCGATGGAACCGAGTTTTTT
NC_011294.1	<i>xthAF</i> <i>xthAR</i>	Exonuclease III	CGCCCGTCCCCATCA CACATCGGGCTGGTGTTTT
NC_011294.1	<i>16S f</i> <i>16S r</i>	SENr010, 16S rRNA	CCAGGGCTACACACGTGCTA TCTCGCGAGGTGCTTCT
NC_011294.1	<i>mgtCF</i> <i>mgtCR</i>	Mg ²⁺ transport ATPase protein C	CGAACCTCGCTTTCATCTTCTT CCGCCGAGGGAGAAAAAC
NC_019120.1	<i>spvBF</i> <i>spvBR</i>	Actin ADP ribosyltransferase 2C toxin SpvB	TGGGTGGGCAACAGCAA GCAGGATGCCGTTACTGTCA

FIG 1 Effect of TC on *S. Enteritidis* (SE) contamination of eggs in 40-week-old birds at 7 weeks postinoculation ($n = 2,195$; $P < 0.001$). (a) Eggshell. (b) Egg yolk. (c) Cumulative effect of TC treatment for 7 weeks on eggshell. (d) Cumulative effect of TC treatment for 7 weeks on yolk. Negative and compound controls were not included in the statistical analysis because *S. Enteritidis* was not recovered from those treatment groups.

and control birds. Briefly, the test controls included a partitioned test area in which each subject worked independently. Equal numbers of six possible combinations for control (A) and TC (B) were presented at random (ABB, BAA, AAB, BBA, ABA, and BAB) to the subjects. The panelists were presented with three coded samples and instructed that two samples were identical and one was different. The panelists were randomly served three coded scrambled egg samples for tasting and detection of organoleptic differences. The subjects were asked to taste (feel, examine) each cooked product in a sensory booth under white light from left to right and to select the odd sample. The effect of residual taste in the mouth was minimized by using a water-based mouth rinse between each sampling. The sensory testing was done with 36 panelists (students, staff, faculty, and local townspeople) per experiment, and the experiment was repeated thrice over a period of 3 weeks. The number of correct replies was recorded per the method of Roessler et al. (44). Significance was calculated at the 5% level, using a table of “minimum numbers of correct judgments” based on published series of tables and using the binomial formula to calculate the number of correct judgments and their probability of occurrence.

Statistical analysis. The numbers of *S. Enteritidis* colonies in the organs were logarithmically transformed ($\log_{10}$ CFU per gram) before analysis to achieve homogeneity of variance. These data were analyzed using the PROC-GLM procedure of the statistical analysis software (SAS, version 9.2; SAS Institute Inc., Cary, NC). Differences among the means were detected with a P value cutoff of <0.001 , using Fisher’s least significance difference (LSD) test. For cell culture and RT-qPCR assays, the results are provided as mean values with standard errors. Differences between two independent treatments were analyzed using the two-tailed t test, and P

values of <0.001 were considered statistically significant. For the sensory study, analysis of results was done for a probability level of 5%, using a table of “minimum numbers of correct judgments” (44).

RESULTS

TC reduces *S. Enteritidis* on eggshells and in egg yolks and internal organs. The dietary supplementation of TC at 1 or 1.5% did not significantly alter ($P > 0.05$) the body weight or egg production of birds compared to that of controls in experiment 1 and experiment 2. In both experiments, TC supplementation (1 and 1.5%) decreased the amounts of *S. Enteritidis* on shells and in yolks ($P < 0.001$). In experiment 1, a total of 2,195 eggs from the inoculated birds were evaluated over a period of 7 weeks for the presence of *Salmonella* on the shell and in yolk. As observed in Fig. 1, TC at 1% and 1.5% consistently decreased the amounts of *Salmonella* both on the shell (Fig. 1a) and in yolk (Fig. 1b) from week 1 to week 7 of supplementation ($P < 0.001$). The cumulative data on the prevalence of *Salmonella* from 2,195 eggs over the 7-week period revealed that dietary supplementation of 1.5% TC decreased the presence of *S. Enteritidis* to 16% on the shell and 4% in yolk compared to the levels for control birds, which yielded a 60% presence of *S. Enteritidis* on the shell (Fig. 1c) and a 40% presence in yolk (Fig. 1d).

In the experiment with 25-week-old birds, a total of 2,350 eggs from inoculated birds were assayed for the presence of *Salmonella*

FIG 2 Effect of TC on *S. Enteritidis* contamination of eggs in 25-week-old birds at 7 weeks postinoculation ($n = 2,350$; $P < 0.001$). (a) Eggshell. (b) Egg yolk. (c) Cumulative effect of TC treatment for 7 weeks on eggshell. (d) Cumulative effect of TC treatment for 7 weeks on yolk. Negative and compound controls were not included in the statistical analysis because *S. Enteritidis* was not recovered from those treatment groups.

in eggs. As observed in experiment 1, TC supplementation at both tested concentrations decreased *S. Enteritidis* contamination of eggshell and yolk ($P < 0.001$) compared to the case for untreated control birds (Fig. 2a and b). In-feed supplementation of TC at 1.5 and 1% reduced *S. Enteritidis* contamination of eggshell and yolk to 15% and 2% and to 28% and 4%, respectively, compared to that for control birds, which produced 63% positive eggs based on shell and ~39% positive eggs based on yolk (Fig. 2c and d).

Similar to the results observed in eggs, TC supplementation reduced *S. Enteritidis* colonization of the cecum, liver, and oviduct in both 40- and 25-week-old birds ($P < 0.001$). For 40-week-old layers, 60% of cecal samples, 20% of liver samples, and 30% of oviduct samples from control birds tested positive for *S. Enteritidis* (Fig. 3a). However, TC supplementation decreased *S. Enteritidis* in all the aforementioned organs, with the pathogen recovered from only 35% of cecal samples and 10% of liver and oviduct samples from birds. Similar results were also observed in the experiment with 25-week-old birds (Fig. 3b). In addition, the cecal endogenous bacterial counts did not differ ($P > 0.05$) among birds from the various treatment groups (see Table S1.1 in the supplemental material).

When the eggs were subjected to sensory analysis by the triangle test, only 43 of the 108 panelists were able to detect the eggs from TC-treated birds, and the remaining 65 panelists failed to identify the treatments from controls, thus resulting in a 0.005 confidence that the panelists were not able to detect a

difference between the eggs from TC-supplemented and untreated birds.

TC reduces *S. Enteritidis* adhesion to and invasion of COEC *in vitro*. The ability of TC to inhibit *S. Enteritidis* colonization of chicken oviduct epithelium was assessed by standard adhesion and invasion assays. The two highest SICs of TC against *S. Enteritidis* were 0.0075% (0.565 mM) and 0.01% (0.750 mM) (data not shown). Cell culture assays indicated that TC at both SICs significantly reduced *S. Enteritidis* adhesion to and invasion of COEC ($P < 0.001$) (Fig. 4), by ~3.0 log CFU/ml and 2.0 log CFU/ml, respectively. Since no significant differences were observed between the strains, only the data for strain 28 are presented here.

TC reduces *S. Enteritidis* survival in chicken macrophages. The results from the macrophage survival assay revealed that TC at its SICs significantly decreased *S. Enteritidis* survival in chicken macrophages, although at different levels (Fig. 5). For example, TC decreased *S. Enteritidis* survival in macrophages by ~1.5 to 2.0 log CFU/ml at 24 and 48 h and by ~2.5 log CFU/ml by 72 h of incubation for strains 21 and 28 compared to controls ($P < 0.001$). Since *S. Enteritidis* 457 failed to survive in macrophages even in the absence of TC (control), no data are available for inclusion here. These findings are in alignment with RT-qPCR results, where TC significantly downregulated *sodC*, a critical gene for *Salmonella* survival in macrophages (45), and *mgfC*, which is required for *Salmonella* growth at low Mg^{2+} concentrations and for intramacrophage survival (46).

FIG 3 Effect of TC on *S. Enteritidis* in internal organs (liver, cecum, and oviduct) of 40-week-old layer hens ($P < 0.001$) (a) and 25-week-old layer hens ($P < 0.001$) (b). For the 40-week-old layer hens, values with different letters (a, b, c, a', b', c', a'', b'', and c'') differ significantly within the organ between treatments ($P < 0.001$).

TC downregulates the expression of *S. Enteritidis* genes that are critical for virulence and oviduct colonization. The RT-qPCR results indicated that TC significantly downregulated ($P < 0.001$) several oviduct-specific colonization genes in *S. Enteritidis* (Table 1). The downregulated genes (Table 2) included genes critical for regulating *Salmonella* functions, such as (i) motility, namely, *flgG*, *fimD*, and *prot6E*; (ii) adherence and invasion, namely, *sopB* and *invH*; (iii) type three secretion systems (TTSS), namely, *sipA*, *sipB*, *pipB*, *ssaV*, and *orf245*; (iv) cell membrane and cell wall integrity, namely, *hflK*, *lrpF*, *ompR*, and *tatA*; (v) exo/endonuclease activity, namely, *xthA* and *mrr1/SEN4287*; (vi) metabolism, namely, *rfbH*, *rpoS*, and *ssrA*; and (vii) survival in macrophages, namely, *sodC*, *spvB*, and *mgtC*.

DISCUSSION

Despite substantial progress in food safety achieved through pathogen reduction programs, *S. Enteritidis* remains one of the most common foodborne pathogens transmitted to humans through the consumption of contaminated eggs. Since chickens serve as the reservoir of *S. Enteritidis*, innovative on-farm strategies for reducing pathogen colonization in birds are critical for controlling human infections. An antimicrobial treatment that can be applied through feed represents the most practical and economically viable method for controlling *S. Enteritidis* in chickens. In addition, a natural and safe feed additive will be better accepted by producers, including organic farmers, without concerns for toxicity.

S. Enteritidis primarily colonizes the chicken cecum (47, 48),

FIG 4 Effect of TC on SE-28 adhesion to and invasion of primary COEC. The COEC were seeded into 24-well tissue culture plates at $\sim 10^5$ cells per well and inoculated with ~ 6.0 log CFU of each *S. Enteritidis* strain (MOI = 10). The infected monolayer was incubated for 1 h at 39°C. The cells were washed thrice with PBS, followed by Triton-mediated cell lysis, and the number of viable adherent *S. Enteritidis* cells was enumerated. For the invasion assay, monolayers incubated for 1 h following *S. Enteritidis* infection were rinsed with specific minimal medium (MEM) and incubated for an additional 2 h in whole medium-10% FBS containing gentamicin (100 μ g/ml). Following incubation, the cells were lysed, and invading *S. Enteritidis* organisms were enumerated. Since there was no significant difference between the three strains studied, results are shown for SE-28. Treatments for TC differed significantly from the control ($P < 0.001$). The letters indicate that adhesion of control SE-28 (a) was significantly different from that with treatments "b" and "c" ($P < 0.001$). Similarly, invasion of control SE-28 (d) was significantly different from that with both treatments (e) ($P < 0.001$).

and it spreads to the spleen and liver by lymphatic or circulatory routes, creating a repertoire for subsequent colonization and spread (47, 48). In addition, *S. Enteritidis* colonizes the reproductive organs in layers, thereby contaminating the yolk. The results from the chicken trials indicated that in-feed administration of TC significantly reduced *S. Enteritidis* colonization in layer chickens, as well as egg-borne transmission of the bacterium. TC supplementation to birds not only decreased *S. Enteritidis* levels on eggshell and in the yolk but also reduced pathogen populations in the cecum, liver, and oviduct compared to those in control birds ($P < 0.001$). However, we did not observe any difference in the total endogenous bacterial microbiota of birds supplemented with TC compared to controls, as depicted in Table S1.1 in the supplemental material. This is in accordance with a previous study where TC supplementation in 20-day-old broilers did not affect the endogenous microbiota of birds but was effective at reducing cecal colonization of *Salmonella* (38). Similarly, Tihihonen and coworkers observed that oral supplementation of cinnamaldehyde against *Salmonella* revealed no effect on the gut microbiota in chickens (49). In yet another study, Jamroz et al. (50) reported that a combination of plant molecules (capsaicin, cinnamaldehyde, and carvacrol) decreased *E. coli* and *Clostridium perfringens* levels but resulted in increased populations of beneficial lactobacilli in 41-day-old commercial broiler chickens.

Our results from the cell culture assay revealed that TC significantly reduced *S. Enteritidis* attachment to and invasion of chicken oviduct epithelium, which are critical for transovarian transmission of the bacterium. In addition, we determined the persistence of *S. Enteritidis* in macrophages following exposure to TC, since *S. Enteritidis* can persist in chicken macrophages and spread via the circulatory system to the reproductive system (48). The results revealed that TC reduced *S. Enteritidis* survival in chicken macrophages compared to controls.

FIG 5 Effects of TC on SE-21 (a) and SE-28 (b) survival in chicken macrophages (HTC) for 24, 48, and 72 h. About 10^5 macrophages were infected with $6.0 \log$ CFU *S. Enteritidis* and incubated at 39°C for 45 min under 5% CO_2 . The macrophages were then washed twice and maintained in whole medium supplemented with $10 \mu\text{g}$ of gentamicin/ml for 72 h. At 24, 48, and 72 h, the cells were lysed, and the surviving *S. Enteritidis* organisms were enumerated on XLD and TSA. Both treatments differed significantly from the control ($P < 0.001$).

In order to determine the potential mechanism(s) behind TC's anti-*Salmonella* effect on egg-borne transmission of layer chickens, we investigated the effect of TC at SICs on *S. Enteritidis* virulence and colonization factors in chickens. Since SICs of antimicrobials, including antibiotics, modulate bacterial physiochemical functions through gene modulations, we investigated the effect of SICs of TC on *Salmonella* virulence *in vitro* (51, 52). Since the SICs of TC were neither bacteriostatic nor bactericidal, any inhibitory effect on *S. Enteritidis* colonization of COEC or survival in macrophages could be attributed to the downregulation of *Salmonella* virulence mechanisms. To ascertain this, we determined the effect of TC on the transcription of 22 published *S. Enteritidis* genes critical for colonization of the chicken reproductive tract and for macrophage survival, using RT-qPCR. The results indicated that TC significantly decreased the expression of several of the tested genes, although at different magnitudes. The downregulated genes included those critical for regulating *Salmonella* motility, namely, *flgG* (14), *fimD* (53, 54), and *prot6E* (20); adherence and invasion, namely, *sopB* (55) and *invH* (56); TTSS, namely, *sipA*, *sipB*, *pipB*, *ssaV*, and *orf245* (55); cell membrane and cell wall integrity, namely, *hflK*, *lrp*, *ompR*, and *tatA* (14); exo/endonuclease activity, namely, *xthA* (57) and *mrr1/SEN4287* (20); and metabolism, namely, *rfbH* (58), *rpoS* (59), and *ssrA* (60). Among these genes, *ssaV* and *pipB*, in addition to being important for the *Salmonella* TTSS, also play a major role in macrophage survival of *S. Enteritidis* in host cells (55). Additionally, *ssrA* has been ob-

served to be associated with *Salmonella* survival in macrophages (60). Other genes reported to play a role in *Salmonella* survival in macrophages are *sodC* (61), *spvB* (62, 63), and *mgtC* (46). The *spvB* gene ribosylates macrophage actin and destabilizes the cytoskeleton (62, 63). Yet another virulence gene studied, *invH*, encodes an outer membrane lipoprotein responsible for *Salmonella* adhesion to and invasion of the host cell (56), which in turn is facilitated by *sopB*, which allows uptake of the pathogen into the host system (55). On the other hand, *orf245* (55) and *prot6E* (20) are specific to oviduct colonization of *S. Enteritidis*, and *pipB*, *sipA*, and *sipB* aid in *Salmonella* invasion and translocation of proteins through the TTSS (55). The genes downregulated >5-fold included genes critical for the *Salmonella* pathogenicity island effector protein (*sipA*), cell membrane and cell wall integrity (*ompR*), exonuclease activity (*xthA*), and metabolism (*rfbH*). Similar results were reported by Kollanoor-Johny (64), who analyzed a DNA microarray of TC-treated *S. Enteritidis* and found that several critical *S. Enteritidis* genes, associated with *Salmonella* pathogenicity island 1, the type three secretion system, motility, chemotaxis, adherence, replication, cell division, transcription, translation, and metabolic and biosynthetic pathways, were downregulated. In addition, cinnamaldehyde and its derivatives were found to interfere with quorum sensing (QS)-regulated activities in *Pseudomonas aeruginosa* (65) and with autoinducer 2 (AI-2)-mediated QS in different *Vibrio* spp. (66), where the target protein of TC was found to be LuxR (66).

In summary, TC supplementation in chickens reduced *S. Enteritidis* contamination of egg yolk and shell without adversely affecting egg production or consumer acceptability of eggs from treated birds. Follow-up mechanistic studies using cell culture and gene expression analysis revealed that TC decreased *S. Enteritidis*

TABLE 2 Expression of SE-28 genes critical for virulence and oviduct colonization in the presence of TC, using real-time PCR

Gene	Fold change ^a	
	0.0075% TC	0.01% TC
<i>fimD</i>	-1.1 ± 0.2	-1.9 ± 0.4
<i>flgG</i>	-1.1 ± 0.2	-1.3 ± 0.4
<i>hflK</i>	$-0.6 \pm 0.2^{\text{NS}}$	$-0.7 \pm 0.3^{\text{NS}}$
<i>invH</i>	-3.3 ± 0.4	-3.7 ± 0.5
<i>lrpF</i>	-4.0 ± 0.2	-4.5 ± 0.6
<i>mrr1</i>	-3.0 ± 0.2	-3.6 ± 0.5
<i>ompR</i>	-10.2 ± 0.4	-11.8 ± 1.0
<i>orf245</i>	-4.8 ± 0.3	-5.7 ± 0.5
<i>pipB</i>	-3.7 ± 0.3	-4.0 ± 0.5
<i>prot6E</i>	-3.1 ± 0.3	-3.3 ± 0.4
<i>rfbH</i>	-6.6 ± 0.1	-8.0 ± 1.0
<i>rpoS</i>	-3.4 ± 0.1	-3.3 ± 0.5
<i>sipA</i>	-6.5 ± 0.3	-6.5 ± 0.5
<i>sipB</i>	-3.5 ± 0.2	-5.9 ± 1.0
<i>sodC</i>	-1.7 ± 0.2	-3.8 ± 0.5
<i>spvB</i>	$-0.4 \pm 0.2^{\text{NS}}$	$-0.5 \pm 0.5^{\text{NS}}$
<i>mgtC</i>	-4.2 ± 0.2	-5.9 ± 0.5
<i>sopB</i>	-4.2 ± 0.2	-4.6 ± 0.5
<i>ssaV</i>	-2.2 ± 0.1	-2.3 ± 0.4
<i>ssrA</i>	-0.7 ± 0.1	-1.9 ± 0.2
<i>tatA</i>	-1.4 ± 0.1	-1.4 ± 0.2
<i>xthA</i>	-9.8 ± 0.5	-12.8 ± 0.2

^a The data show fold changes in gene expression with treatments relative to control gene expression. Data are means $\pm$ standard errors. NS, nonsignificant ($P > 0.05$).

colonization of the oviduct epithelium and survival in chicken macrophages by downregulating critical virulence genes in the bacterium. We concluded that TC may potentially be used as an antimicrobial feed additive to reduce egg-borne transmission of *S. Enteritidis*, in combination with standard hygienic practices used on the farm. This study demonstrates the effectiveness of a feed-supplemented natural antimicrobial compound in reducing the transovarian route of transmission of *S. Enteritidis* in layer chickens.

ACKNOWLEDGMENTS

This study was supported by a grant (2010-01346) from the USDA National Integrated Food Safety Program.

We thank Narayan Rath from the USDA-ARS, AR, for providing us with the HTC cell line for the macrophage survival assay.

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