The Contribution of Eimeria Coinfection and Intestinal Inflammation to Cecal Colonization and Systemic Spread of Salmonella Typhimurium Deficient in Tetrathionate Reductase or Type III Secretion Systems Salmonella Pathogenicity Island 1 or 2

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SUMMARY. Intestinal inflammation may provide a growth advantage for *Salmonella* and enhance its systemic spread in chickens. *Salmonella* triggers intestinal inflammation in the host by using type III secretion systems (T3SS) and produces the inflammatory end product tetrathionate. In mice, tetrathionate respiration confers a growth advantage for *Salmonella* Typhimurium over the competitive microbiome in the inflamed intestine. Coccidia also promote intestinal inflammation and enhance *Salmonella* intestinal growth and systemic spread in chickens. The objective of this study was to evaluate the contribution of inflammation, induced by *Eimeria* spp. or *Salmonella* Typhimurium, to *Salmonella* colonization and dissemination in chickens. In addition, the fitness costs associated with defects in tetrathionate reductase and T3SS associated with *Salmonella* Pathogenicity Island 1 or 2 (SPI-1 or SPI-2) were evaluated in *in vivo* competition experiments with wild-type *Salmonella* strain, with or without *Eimeria* coinfection. One-day-old specific-pathogen-free chickens were orally inoculated with a sham inoculum or with 4×10^2 *Eimeria* oocysts cocktail of *Eimeria tenella*, *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria mitis*. At 6 days of age, birds were orally administered a 1:1 ratio of *Salmonella* Typhimurium wild-type and mutant deficient in tetrathionate reductase, SPI-1, or SPI-2 (10^8 colony forming units/bird). Ceca, livers, and drumsticks were collected at 3, 7, 14, and 42 days after *Salmonella* infection, for bacteriology. Intestinal inflammation was scored by histology. Significantly higher intestinal inflammation was observed in challenge groups compared with the control. However, there were no significant differences in intestinal inflammation scores between groups coinfected with both *Eimeria* spp. and *Salmonella* Typhimurium and birds infected with *Salmonella* alone, and *Eimeria* coinfection did not increase *Salmonella* prevalence or abundance. Contrary to mouse studies, tetrathionate reductase did not enhance *Salmonella* Typhimurium cecal colonization or systemic spread in chickens. SPI-1 and SPI-2 played a significant role in *Salmonella* dissemination and cecal colonization in chickens, respectively.

RESUMEN. Contribución de la coinfección por *Eimeria* y de la inflamación intestinal a la colonización cecal y a la propagación sistémica de *Salmonella* Typhimurium deficiente en tetrationato reductasa o de sistemas de secreción de tipo III de islas de patogenicidad 1 o 2 de *Salmonella*.

La inflamación intestinal puede proporcionar una ventaja para el crecimiento de Salmonella y aumentar su propagación sistémica en pollos. *Salmonella* desencadena la inflamación intestinal en el huésped mediante el uso de sistemas de secreción tipo III (T3SS) y produce el producto final inflamatorio, tetrationato. En ratones, la respiración con tetrationato confiere una ventaja de crecimiento para *Salmonella* Typhimurium sobre el microbioma competitivo en el intestino inflamado. Coccidia también promueve la inflamación intestinal y mejora el crecimiento intestinal de *Salmonella* y la propagación sistémica en pollos. El objetivo de este estudio fue evaluar la contribución de la inflamación, inducida por *Eimeria* spp. o *Salmonella* Typhimurium, en la colonización y diseminación de *Salmonella* en pollos. Además, se evaluaron los costos de aptitud asociados con defectos en la tetrationato reductasa y T3SS asociados con las islas de patogenicidad 1 o 2 de *Salmonella* (SPI-1 o SPI-2) mediante experimentos de competencia *in vivo* con cepas de *Salmonella* de tipo silvestre, con o sin coinfección con *Eimeria*. Pollos libres de patógenos específicos de un día de edad se inocularon por vía oral con un inóculo falso o con 4×10^2 de un coctel de ooquistes de *Eimeria* que incluyó *Eimeria tenella*, *Eimeria acervulina*, *Eimeria maxima* y *Eimeria mitis*. A los seis días de edad, se les administró a las aves administró por vía oral una proporción 1: 1 de *Salmonella* Typhimurium de tipo silvestre o tipo mutante que es deficiente de tetrationato reductasa, SPI-1 o SPI-2 (10^8 unidades formadoras de colonias/ave). Se recolectaron ciegos, hígados y pernas a los tres, siete, catorce y 42 días después de la infección por *Salmonella*, para bacteriología. La inflamación intestinal se calificó por histología. Se observó inflamación intestinal significativamente mayor en los grupos de desafío en comparación con el control. Sin embargo, no hubo diferencias significativas en las puntuaciones de inflamación intestinal entre los grupos coinfectados con *Eimeria* spp. y *Salmonella* Typhimurium y las aves infectadas con *Salmonella* por si sola y la coinfección con *Eimeria* no aumentó la prevalencia o abundancia de *Salmonella*. A diferencia de los estudios en ratones, la tetrationato reductasa no mejoró la colonización cecal de *Salmonella* Typhimurium o la diseminación sistémica en pollos. Las islas de patogenicidad SPI-1 y SPI-2 jugaron un papel importante en la diseminación de *Salmonella* y en la colonización cecal en pollos, respectivamente.

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Nontyphoidal *Salmonella* cause an estimated 1.03 million illnesses annually in the United States, including 19,336 hospitalizations and 378 deaths (1). According to the Centers for Diseases Control and Prevention, consumption of chicken was the most common source of *Salmonella* outbreaks in the United States in 2013 and 2014 (2).

Poultry species are acknowledged as natural reservoirs for *Salmonella* spp. and as food vehicles for salmonellosis in humans (3). *Salmonella* present in chicken intestinal tract may invade the intestinal mucosa through uptake by M-cells and dendritic cells or by active invasion of the intestinal epithelium using a type III secretion system (T3SS-1) encoded by the *Salmonella* Pathogenicity Island 1 (SPI-1) (4,5). The *Salmonella* T3SS-1 is a multiprotein complex, referred to as the "injectisome," which literally injects effector proteins into the host cell cytoplasm. These T3SS effectors induce the rearrangement of the actin cytoskeleton, which in turn results in bacterial uptake by the epithelial cell (6,7). SPI-1 is a 40-kb region of DNA found in all *Salmonella* species, located at centisome 63 on the *Salmonella* Typhimurium chromosome (4). The T3SS-1 plays a role in the stimulation of inflammatory response and proinflammatory cytokine production when *Salmonella* enters the epithelial cells (8,9,10,11,12). After invasion, a second type III secretion system (T3SS-2), encoded by the *Salmonella* Pathogenicity Island 2 (SPI-2), contributes to intracellular survival and replication of S*almonella* within phagocytic cells (13,14). SPI-2 comprises a 40-kb region of DNA located at centisome 30 of the *Salmonella* Typhimurium chromosome (4). Expression of SPI-2 has been shown to reduce the oxidative stress encountered by bacteria within phagocytic cells, which results in a more hospitable environment for *Salmonella* survival, replication, and dissemination (15,16). Phagocytic cells containing live *Salmonella* can spread through blood vessels and lymphatics and reach systemic organs (17,18,19).

Coccidiosis is an ubiquitous protozoal intestinal disease of poultry caused by different *Eimeria* spp. Numerous studies have described how *Eimeria* coinfection enhances *Salmonella* intestinal colonization and systemic spread in chickens (20,21,22,23,24,25). *Eimeria* replication, as well as expression of the T3SS-1 by *Salmonella*, induces infiltration of inflammatory cells in the chicken intestine (26,27,28,29,30,31). Oxidative mechanisms induced by inflammatory cells promote oxidation of endogenous thiosulfate present in the intestinal lumen into tetrathionate (32), an electron acceptor that supports anaerobic respiration in *Salmonella* Typhimurium (33). In mice, intestinal inflammation induced by SPI-1 promotes tetrathionate formation and enhances the growth of *Salmonella* Typhimurium over the competitive, intestinal microbiome (32,34). Furthermore, infiltration of inflammatory cells induced by *Eimeria* spp. (35,36) could enhance *Salmonella* uptake from the intestinal mucosa and its dissemination to internal organs. In this study, cecal colonization and systemic spread were evaluated for wild-type *Salmonella* Typhimurium and mutant strains deficient in tetrathionate reductase, SPI-1, or SPI-2 in chickens with or without *Eimeria* coinfection.

MATERIALS AND METHODS

Bacterial strains. *Salmonella* Typhimurium strain TT26179 deficient for *ttrRSBCA* gene cluster was generously provided by Dr. Andreas J. Bäumler, University of California—Davis. The *ttrRSBCA* region was replaced with a chloramphenicol resistance cassette (32). This mutation was then moved into *Salmonella* Typhimurium SL1344 strain by P22 phage-mediated transduction (26) to create the *Salmonella* Typhimurium SL1344 Δ*ttrRSBCA*. *Salmonella* Typhimurium strains SL1344 deficient for SPI-1 and SPI-2 regions were generously provided by Dr. James W. Wilson, Villanova University, Pennsylvania, and contained a chloramphenicol resistance marker at the site of SPI-1 and SPI-2 deletions (37,38). Genes deleted were from *invH* through *sitDCBA* in the SPI-1 region and from *orf70-319-242* through *ssaVNOPQRSTU* in the SPI-2 region (37,38). *Salmonella* Typhimurium SL1344 with a rifampicin resistance marker was used as *Salmonella* Typhimurium SL1344 wild-type strain. Antibiotics were used at the indicated concentrations; chloramphenicol, 25 μg/ml; rifampicin, 64 μg/ml.

Preparation of *Salmonella* Typhimurium strains inocula for oral animal challenge. *Salmonella* Typhimurium SL1344 Δ*ttrRSBCA*, Δ*SPI-1*, Δ*SPI-2*, and wild-type strains were grown static, separately, in 20 ml of Luria-Bertani broth at 37 C for 16 hr. The bacterial cell density was estimated for each strain from the optical density $(OD)_{600}$ of the cell suspensions ($\sim$0.5 OD_{600} or 4.0×10^8 cells/ml). Equal volumes of *Salmonella* Typhimurium mutant and wild-type were mixed together, pelleted, and suspended in 5 ml of buffered saline gelatin (BSG) to make strain combinations for oral challenge. The final bacterial count was confirmed by plating 10-fold serial dilutions from each inoculum on XLT4 agar (Difco, Sparks, MD) with chloramphenicol (25 μg/ml) and on XLT4 agar with rifampicin (64 μg/ml) to enumerate mutant and wild-type, respectively. After 24-hr incubation at 37 C, colony forming units (CFU) were determined for each inoculum.

***Eimeria* spp. inoculum.** The *Eimeria* spp. inoculum was generously provided by Lorraine Fuller, Poultry Science Department, College of Agricultural and Environmental Sciences, the University of Georgia. The inoculum was tested by nested-PCR for the presence of all seven chicken *Eimeria* species: *Eimeria acervulina*, *Eimeria necatrix*, *Eimeria maxima*, *Eimeria mitis*, *Eimeria praecox*, *Eimeria brunetti*, and *Eimeria tenella* (39,40,41,42). Oocysts were homogenized using ceramic beads in a FastPrep-24™ 5G Instrument (Thomas Scientific; Swedesboro, NJ) at 6 m/s for 20 sec. DNA from the oocysts was extracted using DNAzol (ThermoFisher Scientific, Waltham, MA), per the DNAzol protocol. Following extraction, the DNA was purified using the PowerClean Pro DNA Clean-Up Kit (Qiagen; Germantown, MD). One microliter of DNA was used in a nested-PCR procedure designed to detect all major *Eimeria* spp. PCR products were electrophoresed on a 1% agarose submarine gel in Tris–Acetate–ethylenediaminetetraacetic acid (EDTA) buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) with ethidium bromide at $\sim$80 V for approximately 45 min. Positive bands were excised and DNA purified with the Zymoclean Gel DNA recovery kit (Zymo Research, Irvine, CA). Approximately 100 μg of DNA was used as template for each nested PCR. The PCR thermoprofile was used as follows for the internal transcribed spacer 1 primers: initial heat activation of polymerase at 95 C for 5 min; 35 cycles of denaturation at 95 C for 30 sec, annealing at 50–58 C for 30 sec, and extension at 72 C for 60 sec; and a final extension at 72 C for 3 min (39,41,42). The PCR thermoprofile was used as follows for the cytochrome C oxidase 1

Table 1. Experimental groups used in this study.

| Groups[A] | Inocula combination 0.1 ml per inoculum: oral route | |
	Day 1	Day 5
G1 (Negative control)	BSG[B]	BSG
G2	*Eimeria* spp.	BSG
G3	BSG	*Salmonella* Typhimurium SL1344 Δ*ttrRSBCA*/WT[C]
G4	*Eimeria* spp.	*Salmonella* Typhimurium SL1344 Δ*ttrRSBCA*/WT
G5	BSG	*Salmonella* Typhimurium SL1344 Δ*SPI-1*/WT
G6	*Eimeria* spp.	*Salmonella* Typhimurium SL1344 Δ*SPI-1*/WT
G7	BSG	*Salmonella* Typhimurium SL1344 Δ*SPI-2*/WT
G8	*Eimeria* spp.	*Salmonella* Typhimurium SL1344 Δ*SPI-2*/WT

[A]Sample size per group = 18 birds.
[B]Buffered saline gelatin.
[C]Wild-type.

primers: initial heat activation of polymerase at 96 C for 10 min; 35 cycles of denaturation at 94 C for 30 sec, annealing at 48–64 C for 30 sec, and extension at 72 C for 60 sec; and a final extension at 72 C for 10 min (40). The present inoculum was PCR-positive for *E. tenella*, *E. mitis*, *E. acervulina*, and *E. maxima* species.

The inoculum was diluted in a saturated sodium chloride solution (366 g of NaCl per liter of deionized water) in a 1 to 10 ratio and transferred to a McMaster counting chamber (Chalex Corp., Portland, OR) for enumeration. *Eimeria* spp. oocysts were differentiated by morphology and size using a bright field microscope (43) and counted using the formula (number of oocysts) × (10) × (6.67) (44). The inoculum contained a total of 400 oocysts with approximately 300 oocysts of *E. tenella*, 40 oocysts of *E. acervulina*, 40 oocysts of *E. mitis*, and 20 oocysts of *E. maxima* per 0.1-ml inoculum dose.

Oral challenges of specific-pathogen-free, white leghorn chickens. Specific-pathogen-free (SPF) eggs were purchased from an external supplier (Charles River Laboratories, Wilmington, MA), and incubated and hatched at the Poultry Diagnostic and Research Center, College of Veterinary Medicine, the University of Georgia. On the day of hatch, 1-day-old specific-pathogen-free white leghorn chickens were housed in biosafety level 2, HEPA-filtered, Horsfall isolator units at the Poultry Diagnostic and Research Center, College of Veterinary Medicine, the University of Georgia. Paper was placed on wire-bottom isolators to facilitate fecal–oral transmission of *Salmonella* Typhimurium strains and *Eimeria* spp. Two hundred and forty, 1-day-old SPF chickens were randomly divided into seven treatment groups and one control group. Bird density was 12 to 14 birds per isolator unit for the first week of age, 9 to 13 birds per isolator during the second week, six to eight birds per isolator from weeks 3 to 5, and finally four to five birds per isolator from week 6 until the end of the study.

Control and treatment groups consisted of 30 chickens each. Chicks were orally inoculated by crop gavage at 1 day of age either with 0.1 ml of sterile buffered saline gelatin (groups 1, 3, 5, 7) or with 4×10^2 oocysts/0.1 ml of *Eimeria* spp. (*E. tenella*, *E. acervulina*, *E. maxima*, and *E. mitis*; groups 2, 4, 6, 8). Chicks received the inoculum before their placement into the isolator units. Five days later, challenge groups (groups 3 to 8) were orally administered with $(3.5–4) \times 10^8$ CFU/0.1 ml of a 1:1 mix of *Salmonella* Typhimurium mutant and wild-type strains (Table 1). Three inocula were made from an equal amount of *Salmonella* Typhimurium SL1344 mutant strain (Δ*ttrRSBA*, Δ*SPI-1*, or Δ*SPI-2*) and wild-type strain. Treatment groups consisted of birds infected either with *Salmonella* Typhimurium strains (groups 3, 5, 7) or coinfected with *Eimeria* spp. and *Salmonella* Typhimurium strains (groups 4, 6, 8; Table 1). Oral challenges of *Salmonella* Typhimurium strains were performed using a pipette tip carefully placed in the oral cavity of the birds. All birds were given *ad libitum* access to water and to an unmedicated feed, 18.00% crude protein content (All Grain Start-N-Grow, Crumble; Southern States Cooperative Inc., Richmond, VA) starting after the oral inoculation of *Eimeria* spp. Chickens were brooded on a 12-hr lighting program, following standard temperature and humidity regimens according to the age of the birds. Chickens were observed for clinical signs twice a day. Experiments were conducted under strict adherence to Institutional Animal Care and Use Committee guidelines at the University of Georgia.

Prior to *Eimeria* inoculation on day 1 and prior to *Salmonella* inoculation on day 5, three birds per group were euthanatized by carbon dioxide followed by cervical dislocation. Ileum and ceca were aseptically collected and divided into two equal parts. One part was placed into 10% buffered formalin for intestinal inflammation scoring by histology. The other part was tested by culture for *Salmonella*. Three birds per group were euthanatized by carbon dioxide followed by cervical dislocation on days 3, 7, 14, and 42 after *Salmonella* inoculation. The body of the chickens was sprayed with 70% ethanol prior to necropsy. Ceca with terminal ileum, drumstick (muscle with tibiotarsus bone), and liver samples were aseptically collected and placed individually into 532-ml Nasco Whirl-Pak bags for *Salmonella* culture. Samples of ileum and ceca were also placed into 10% buffered formalin for intestinal inflammation scoring by histology.

Enumeration of *Eimeria* spp. oocysts. Within each group, four to five fecal droppings were collected daily from day 4 to day 21, and on days 32 and 38 after *Eimeria* spp. inoculation. Fecal droppings were placed into 532-ml Nasco Whirl-Pak bags for *Eimeria* spp. oocyst counts. Oocysts present in feces were enumerated using the McMaster chamber counting method as previously described (44). Fecal droppings were weighed and diluted 10-fold in deionized water. After 24 to 48 hr at 4 C, fecal solutions were homogenized and then filtered through a double layer of grade-40 cheesecloth. Fifteen milliliter conical centrifuge tubes (Thermo Scientific, Rochester, NY) were filled with filtered fecal homogenate and centrifuged for 5 min at $302 \times g$ at room temperature to pellet *Eimeria* spp. oocysts. After centrifugation, the supernatant was discarded and pelleted oocysts were suspended into a 15-ml saturated sodium chloride solution (366 g of NaCl per liter of deionized water), homogenized, and transferred to a McMaster counting chamber (Chalex Corp.). Oocysts were counted using a bright field microscope, and numbers of oocysts per gram of fecal material were calculated for each group as (number of oocysts) × (10) × (6.67) (44).

Enumeration of *Salmonella* Typhimurium strains in ceca. Ceca with terminal ileum samples were placed into sterile 532-ml Nasco Whirl-Pak bags, weighed, and an equal volume of BSG, by weight (1 ml per gram of sample), was added into each bag. Samples were homogenized by pressing the bags between a porcelain mortar and a pestle to obtain a fluid mixture. Samples were then serially diluted 10-fold in BSG (final dilution 10^{-9}) for enumeration on XLT4 agar with chloramphenicol (25 μg/ml) and on XLT4 agar with rifampicin (64 μg/ml). Plates were incubated at 37 C for 24 hr, and *Salmonella* Typhimurium mutant and wild-type strains were detected and enumerated (CFU/g cecal content).

Detection of *Salmonella* Typhimurium strains in tissues with enrichment. Ceca with terminal ileum, liver, and drumstick samples placed in sterile 532-ml Nasco Whirl-Pak bags were weighed, and buffered peptone water (BPW; Difco) was added into each sample in a 1 to 10 ratio. Ceca and liver samples were homogenized with a stomacher (Stomacher80 Seward; Worthing, U.K.) for 1 min. Drumstick samples were manually macerated through the bag. Tissue homogenates were incubated at 37 C for 24 hr. After incubation, 0.1 ml of BPW cultures was transferred into a 10-ml glass tube of
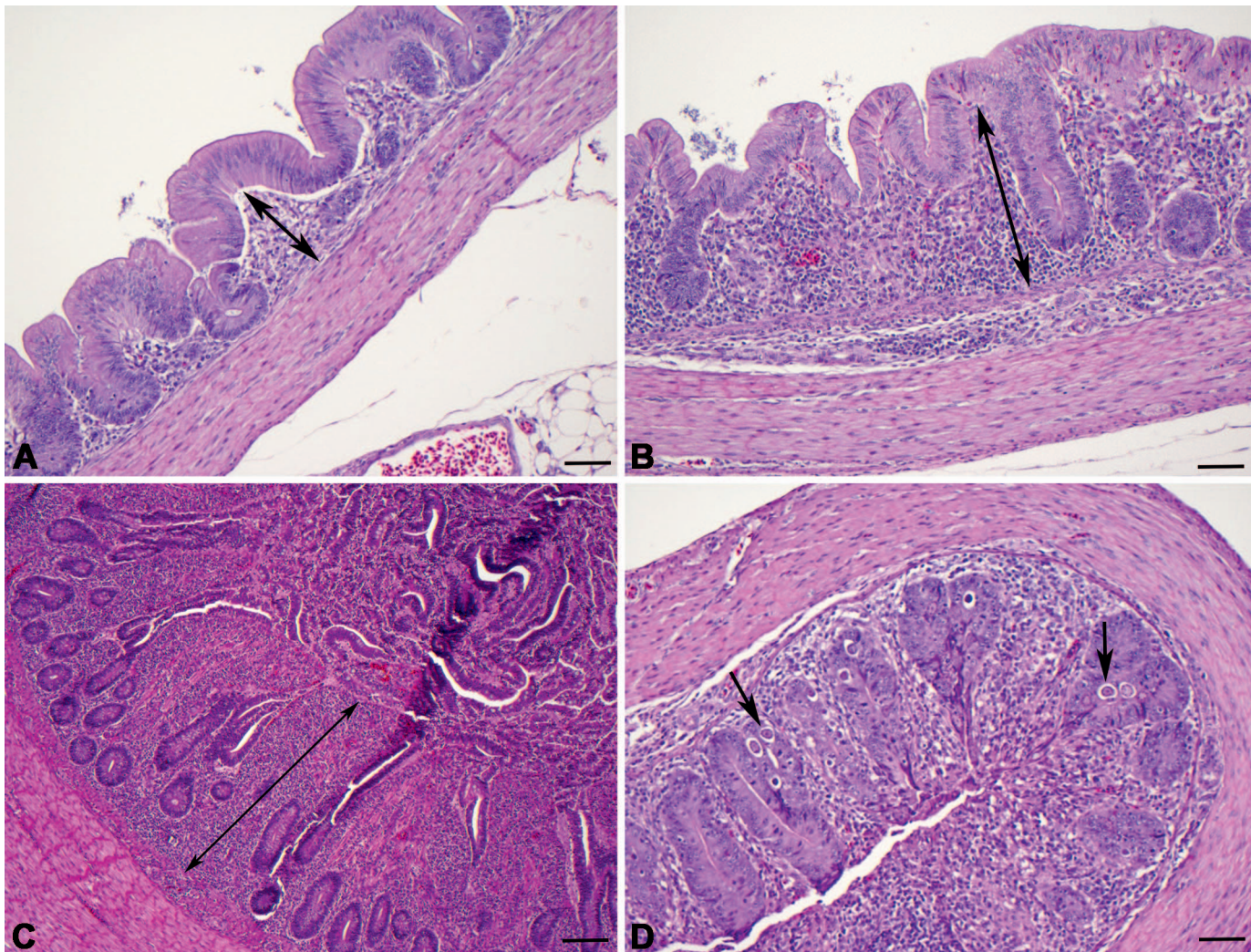
Fig. 1. Histological scoring system for intestinal inflammation. **(A)** Score 0: no inflammation or intestinal inflammation within the normal limits, bar = 50 μm. No or very few isolated inflammatory cells are present within the lamina propria (double-headed arrow). **(B)** Score 1: mild intestinal inflammation, bar = 50 μm. Mild infiltration of isolated or clustered inflammatory cells. The lamina propria is less than twice expanded (double-headed arrow). **(C)** Score 2: moderate intestinal inflammation, bar = 50 μm. Moderate infiltration of inflammatory cells within the intestinal mucosa, usually organized in clusters. The lamina propria is two to three times expanded (double-headed arrow). **(D)** Score 2 with intracellular coccidian protozoan in various developmental stages (arrows) associated with lymphocytic infiltration within the lamina propria, bar = 50 μm.

Rappaport-Vassiliadis R10 broth (Difco). Rappaport-Vassiliadis R10 was chosen as an enrichment broth to allow growth of all *Salmonella* Typhimurium strains used in the present study, including the *Salmonella* Typhimurium tetrathionate reductase mutant. Tubes were incubated at 42 C for 24 hr. A loopful of the Rappaport-Vassiliadis R10 enrichment (10 μl) was streaked on XLT4 agar with chloramphenicol (25 μg/ml) and on XLT4 agar with rifampicin (64 μg/ml). Plates were incubated at 37 C for 24 hr, and *Salmonella* Typhimurium mutants and wild-type strains were detected.

Histology and intestinal inflammation scoring. Formalin-fixed samples of ileum and ceca were embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin. Three sections of intestine were blindly examined from each bird using a bright field microscope. Intestinal inflammation scores were attributed as follows: 0, no inflammation or inflammation within normal limits; 1, mild inflammation; 2, moderate inflammation; 3, severe inflammation (Fig. 1). Inflammation scoring was based on the severity of the expansion of the lamina propria and mucosa by infiltration of inflammatory cells such as heterophils, lymphocytes, and macrophages. Mucosal and submucosal changes such as ulceration, necrosis, and presence of *Eimeria* spp. organisms were also evaluated.

Statistical analysis. Bacterial numbers were logarithmically transformed before statistical analysis. The constant 1 was attributed to culture-negative samples before log-transformation. The Mann-Whitney test was used to determine whether intestinal inflammation scores differed significantly between groups. *Salmonella* Typhimurium abundance statistical comparisons between mutant and wild-type strains were analyzed using a multilevel mixed-effects model with *Salmonella* type and day as fixed effects and bird as a random effect. Pairwise comparisons were made using the Bonferroni procedure. McNemar's test with all time points combined was used to compare *Salmonella* Typhimurium mutant and wild-type strains prevalence in organs. Overall *Salmonella* Typhimurium prevalence statistical comparisons between groups were determined using Fisher's exact test. Statistical differences at probability values below 0.05 were considered significant. All tests were performed using Stata/SE 14.2 software (45).

Table 2. Prevalence of *Salmonella* Typhimurium Δ*ttrRSBCA*, Δ*SPI-1*, and Δ*SPI-2* mutants and wild-type strains in liver and drumstick of chickens, in the presence or absence of *Eimeria* coinfection. Values are presented as percentage (no. infected/total).

	3 days PSI[A]		7 days PSI		14 days PSI		42 days PSI		Overall	
Group	Liver	Drumstick	Liver	Drumstick	Liver	Drumstick	Liver	Drumstick	Liver	Drumstick
G3:ST[B]										
ttrRSBCA mutant	100 (3/3)	33.3 (1/3)	100 (3/3)	33.3 (1/3)	66.7 (2/3)	0 (0/3)	0 (0/3)	0 (0/3)	66.7 (8/12)	16.7 (2/12)
WT	100 (3/3)	33.3 (1/3)	100 (3/3)	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)	50 (6/12)	8.3 (1/12)
G4:ST + *Eimeria*[C]										
ttrRSBCA mutant	100 (3/3)	33.3 (1/3)	100 (3/3)	66.7 (2/3)	100 (3/3)	0 (0/3)	0 (0/3)	0 (0/3)	75 (9/12)	25 (3/12)
WT	100 (3/3)	33.3 (1/3)	100 (3/3)	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)	50 (6/12)	8.3 (1/12)
G5:ST										
SPI-1 mutant	33.3 (1/3)	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)	8.3 (1/12)[D]	0 (0/12)
WT	100 (3/3)	0 (0/3)	100 (3/3)	0 (0/3)	100 (3/3)	0 (0/3)	0 (0/3)	0 (0/3)	75 (9/12)[D]	0 (0/12)
G6:ST + *Eimeria*										
SPI-1 mutant	0 (0/3)	0 (0/3)	33.3 (1/3)	0 (0/3)	33.3 (1/3)	0 (0/3)	0 (0/3)	0 (0/3)	16.7 (2/12)[D]	0 (0/12)
WT	100 (3/3)	0 (0/3)	100 (3/3)	0 (0/3)	66.7 (2/3)	0 (0/3)	0 (0/3)	0 (0/3)	66.7 (8/12)[D]	0 (0/12)
G7:ST										
SPI-2 mutant	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/12)[D]	0 (0/12)
WT	66.7 (2/3)	0 (0/3)	100 (3/3)	0 (0/3)	33.3 (1/3)	0 (0/3)	0 (0/3)	0 (0/3)	50 (6/12)[D]	0 (0/12)
G8:ST + *Eimeria*										
SPI-2 mutant	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)	50 (1/2)	0 (0/3)	0 (0/3)	0 (0/3)	9.1 (1/11)	0 (0/12)
WT	66.7 (2/3)	0 (0/3)	100 (3/3	0 (0/3)	50 (1/2)	0 (0/3)	0 (0/3)	0 (0/3)	54.5 (6/11)	0 (0/12)

[A]Days after *Salmonella* infection.
[B]*Salmonella* Typhimurium mutant/wild-type inoculation dose: $(3.5–4) \times 10^8$ CFU/bird, inoculation at 5 days of age.
[C]*Eimeria* spp. (*E. tenella*, *E. acervulina*, *E. maxima*, and *E. mitis*) inoculation dose: 4×10^2 oocyst/bird, inoculation at 1 day of age.
[D]McNemar's test, $P < 0.05$.

RESULTS

***Eimeria* spp. oocyst shedding.** In all groups infected with *Eimeria* spp., the first cycle of oocyst shedding was observed between days 7 and 10 after coccidia challenge. During the first cycle, birds shed 10,000 to 44,000 oocysts per gram of feces. The second cycle occurred on days 16 and 17 with birds shedding between 13,000 and 59,000 oocysts per gram of feces. *Salmonella* culture and intestinal inflammation scoring were performed during the first peak of *Eimeria* oocyst shedding, on day 3 after *Salmonella* infection. Next, samples were collected between the first and the second cycle of oocyst shedding, on day 7 after *Salmonella* infection. Finally, samples were collected after the second replication cycle, on days 14 and 42 after *Salmonella* infection (Supplemental Fig. S1).

Intestinal inflammation associated with *Eimeria* or *Salmonella* infection in white leghorn chickens. Over the length of the study, intestinal inflammation scores were significantly higher in challenged groups (groups 2 to 8) compared with the control group (group 1; Supplemental Fig. S2). However, coinfection with *Eimeria* spp. (groups 4, 6, 8) did not significantly increase intestinal inflammation scores compared with infection with *Salmonella* Typhimurium strains alone (groups 3, 5, 7; Supplemental Fig. S3). In all treatment groups, intestinal inflammation was mild to moderate. Lymphocytes and macrophages were the predominant inflammatory cells observed within the lamina propria of infected birds in all groups (Fig. 1). Heterophils were also frequently observed but in fewer numbers.

Contribution of tetrathionate reductase, SPI-1, and SPI-2 to *Salmonella* Typhimurium cecal colonization with and without *Eimeria* coinfection. Cecal abundances of *Salmonella* Typhimurium wild-type and *Salmonella* Typhimurium Δ*ttrRSBCA* mutant strain were not statistically different on days 3 and 7 after *Salmonella* infection, regardless of *Eimeria* coinfection (Fig. 2A, 2B). Interestingly, 14 days after *Salmonella* challenge, *Salmonella* Typhimurium counts in ceca were significantly higher for tetrathionate mutant than for the wild-type strain (Fig. 2A, 2B). On day 42 after *Salmonella* infection, all ceca samples from all groups were culture negative for the *Salmonella* Typhimurium wild-type strain. Only the tetrathionate reductase mutant strain was recovered from ceca on day 42, from chickens coinfected with *Eimeria*.

Averaged over the length of the study, *Salmonella* numbers in ceca were lower for the SPI-1 mutant strain compared with the wild-type strain, in chickens coinfected with *Eimeria* (Fig. 2D). In the absence of *Eimeria* coinfection, cecal abundances of *Salmonella* Typhimurium SPI-1 mutant and wild-type strains were not statistically different (Fig. 2C).

Over the length of the study, *Salmonella* Typhimurium SPI-2 mutant strain was approximately 2 logs less abundant in ceca than the wild-type strain, regardless of *Eimeria* coinfection (Fig. 2E, 2F).

The role of tetrathionate reductase, SPI-1, and SPI-2 in *Salmonella* Typhimurium dissemination in chickens and the influence of *Eimeria* coinfection. Prevalence of *Salmonella* Typhimurium in liver and drumstick was determined for each strain within groups by calculating percentage of birds with culture-positive samples for *Salmonella* Typhimurium mutants and wild-type strains. Over the length of the study, prevalences of *Salmonella* Typhimurium Δ*ttrRSBCA* mutant and wild-type strains were not statistically different in liver and drumstick samples ($P > 0.05$; Table 2). Interestingly, the tetrathionate reductase mutant was recovered for a longer period from these samples as compared with the wild-type strain (Table 2). These results were independent of *Eimeria* coinfection (Table 2).

Salmonella Typhimurium Δ*SPI-1* and Δ*SPI-2* mutants were recovered at a lower rate from liver samples in comparison with the wild-type (WT) strain in groups inoculated with Δ*SPI-1*/WT, *Eimeria* + Δ*SPI-1*/WT, and Δ*SPI-2*/WT (Table 2). However, overall differences in *Salmonella* prevalence between SPI-2 mutant and wild-type strain were not statistically different for the liver of birds coinfected with *Eimeria* spp. ($P > 0.05$). In groups infected with Δ*SPI-1*/WT or Δ*SPI-2*/WT (Δ*SPI-1*/WT, *Eimeria* + Δ*SPI-1*/
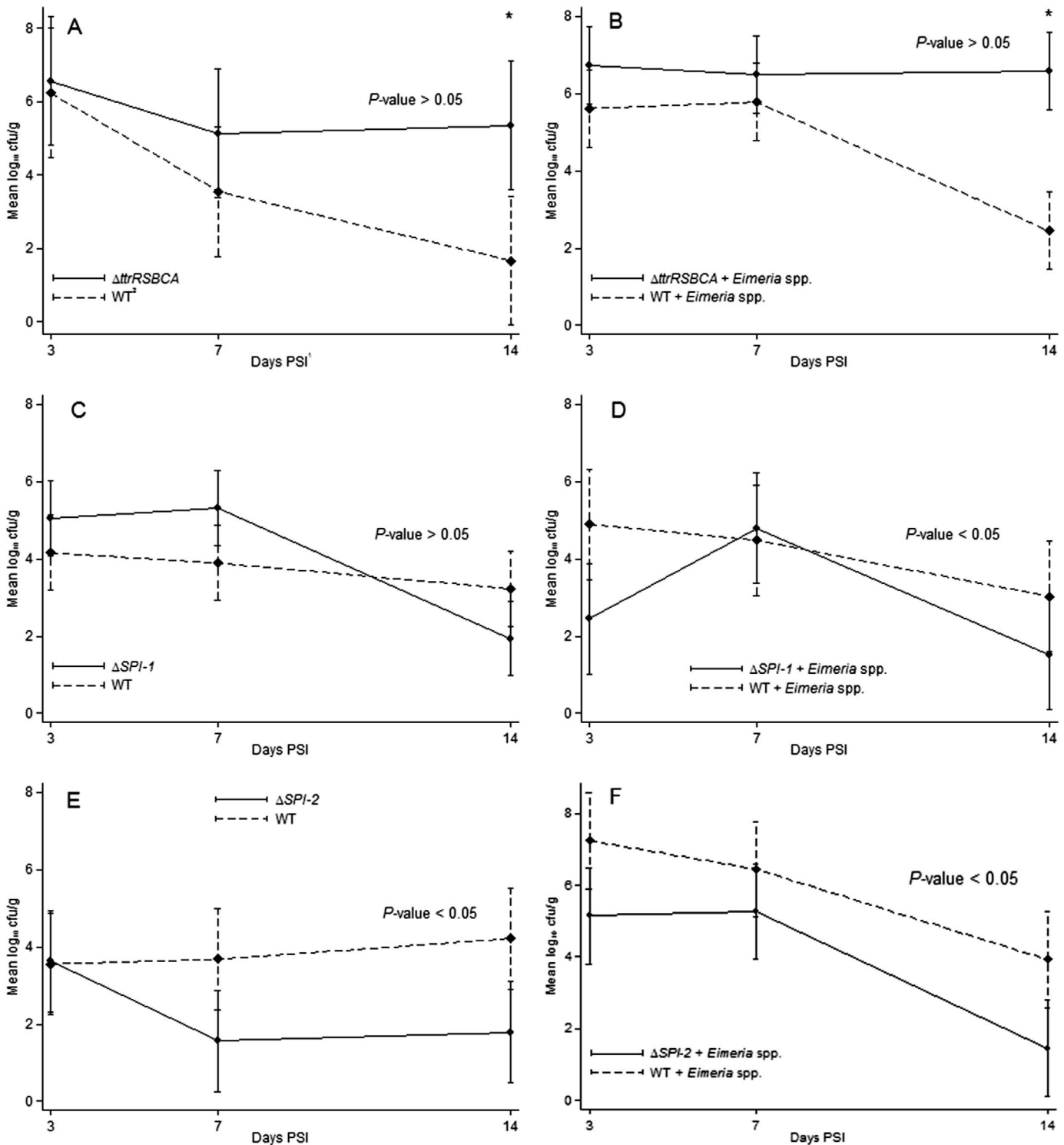
Fig. 2.   Cecal colonization of *Salmonella* Typhimurium mutants and wild-type strains in chickens in the presence or absence of *Eimeria* coinfection. Each point represents mean *Salmonella* $\log_{10}$ CFU/g of cecal content, bars represent 95% confidence interval. (**A**), (**C**), (**E**) Birds were inoculated with *Salmonella* Typhimurium mutant and wild-type strains at 5 days of age. (**B**), (**D**), (**F**) Birds were inoculated with *E. tenella*, *E. maxima*, *E. acervulina*, and *E. mitis* at 1 day of age and with *Salmonella* Typhimurium mutants and wild-type strains at 5 days of age. Over the length of the study, *P* values <0.05 show significant differences in *Salmonella* counts between mutant and wild-type strains. *P* values >0.05 mean that no significant differences in *Salmonella* counts between mutant and wild-type strains were observed, with all time points combined. At specific time points, asterisks show significant differences in *Salmonella* counts between mutant and wild-type strains. [1]Days post *Salmonella* inoculation. [2]Wild-type.

WT, Δ*SPI-2*/WT, *Eimeria* + Δ*SPI-2*/WT), *Salmonella* Typhimurium wild-type strain as well as strains deficient in SPI-1 and SPI-2 were not recovered from drumstick samples, at any time point during the experiment, regardless of *Eimeria* coinfection (Table 2).

Effect of *Eimeria* spp. coinfection on total *Salmonella* Typhimurium prevalence in ceca, liver, and drumstick. Total *Salmonella* prevalence in tissues was determined within groups by calculating the percentage of birds with culture-positive samples, regardless of *Salmonella* Typhimurium strain. Over the length of the study, total *Salmonella* prevalence in ceca, liver, and drumstick was not statistically different between groups only infected with *Salmonella* Typhimurium and groups coinfected with *Eimeria* spp. ($P > 0.05$, data not shown).

DISCUSSION

Coccidiosis is a global challenge with regard to maintaining healthy poultry flocks. Especially problematic is that total elimination of oocysts from the poultry house is never complete between flocks (45). In addition, coccidia infection makes poultry more susceptible to infections with bacterial pathogens, including *Salmonella*. In the present study, chickens were inoculated with a low dose of *Eimeria* spp. at 1 day of age in order to mimic field challenges that commercial birds face when placed on farms (46). Because prepatent periods of *Eimeria* spp. generally range from 4 to 5 days (47), birds were infected with *Salmonella* at 5 days after *Eimeria* spp. challenge, when intestinal epithelial cells are likely damaged by protozoan replication (47,48).

Contribution of tetrathionate reductase, SPI-1, and SPI-2 to *Salmonella* cecal colonization and dissemination in tissues was evaluated in the presence or absence of *Eimeria* coinfection. The tetrathionate reductase did not significantly contribute to *Salmonella* Typhimurium cecal colonization and systemic spread in chickens, regardless of *Eimeria* coinfection. Oral administration of streptomycin before a *Salmonella* Typhimurium challenge in mice disrupts the microbiome, which enables *Salmonella* to colonize and persist within the gastrointestinal tract and reproduces a characterized model of intestinal inflammation; in a mouse colitis model, numbers of *Salmonella* Typhimurium *ttrA* mutant in the colon were significantly lower than the wild-type strain (32). In that study, 4 days after oral infection with *Salmonella* Typhimurium, mice had a severe intestinal inflammation with infiltration of polymorphonuclear (PMN) leukocytes and a significant increase in the levels of neutrophil chemoattractant messenger RNA (32). In the present study, infection with *Salmonella* Typhimurium strains alone or combined with *Eimeria* spp. induced mild to moderate intestinal inflammation in chickens, and the predominant inflammatory cells observed within the intestinal mucosa were mononuclear leukocytes. Chickens lack neutrophils, and heterophil is the PMN leukocyte encountered in avian species. The inflammatory response in avian species is rapid, and heterophils can be detected in tissues within 1 to 3 hr during acute inflammation. Because samples from chickens challenged with *Salmonella* were collected at and after the third day post *Salmonella* inoculation (PSI), it is possible that sample collection timing did not reveal a more robust PMN inflammatory response in chickens.

Contrary to mouse studies, tetrathionate reductase did not enhance wild-type *Salmonella* Typhimurium cecal colonization in chickens, regardless of *Eimeria* coinfection. Others have also reported exclusion of wild-type *Salmonella* Typhimurium by tetrathionate reductase mutants (*ttrB* and *ttrS*) in the ceca of newly hatched chicks (49), and these same mutations in tetrathionate reductase did not adversely affect fecal excretion of *Salmonella* Typhimurium in chickens over a 4-wk period, after challenge (50). The SPI-2 significantly contributed to *Salmonella* Typhimurium cecal colonization in chickens, in the presence or absence of *Eimeria* coinfection. The role of SPI-1 in colonization of chicken ceca was, however, questionable. *Salmonella* Typhimurium deficient in SPI-1 was significantly less abundant than the wild-type strain in birds coinfected with *Eimeria* spp. but not in birds infected with *Salmonella* alone. Contribution of SPI-1 and SPI-2 in systemic spread and intracellular survival is well documented (5,7,13,14); whereas the role of these genes in the colonization of the chicken gastrointestinal tract is unclear (51,52). Others have evaluated the contribution of SPI-1 and SPI-2 on *Salmonella* cecal colonization in birds challenged at 1 wk of age (51,53). Dieye *et al.* demonstrated that SPI-1 but not SPI-2 contributed to *Salmonella* cecal colonization (53); whereas Rychlik *et al.* showed that SPI-1 did not significantly affect *Salmonella* colonization in chickens (54). Other studies have determined the contribution of SPI-1 and SPI-2 T3SS injectisome and effectors on *Salmonella* colonization of the chicken ceca (52,55,56,57). Morgan *et al.* showed that a single mutation in either SPI-1 *prgK* (injectisome) or *sicA* (effector), or SPI-2 *ssaQ* (injectisome) reduced *Salmonella* Typhimurium cecal colonization in chickens (56). Turner *et al.* reported SPI-1 *sipC* (effector) was necessary for *Salmonella* colonization in chickens (57); whereas Morgan *et al.* found that this same mutation did not reduce *Salmonella* Typhimurium cecal colonization in chickens (56). *HilA*, a transcriptional activator that regulates the expression of SPI-1, was required for long-term cecal shedding of *Salmonella* Enteritidis in birds challenged at 1 day of age (55). In a recent study, SPI-2 *ssaU* (injectisome) was necessary for *Salmonella* colonization in 1-wk-old chickens, but not in chickens inoculated at 1 day of age (52). *Salmonella* Typhimurium SPI-1 and SPI-2 mutant strains used in the present study encompassed the deletion for SPI-1 *prgK*, *sicA*, *sipC*, and *hilA*, as well as the deletion of SPI-2 *ssaO* and *ssaU* (37,38). The present results are consistent with the studies of Morgan *et al.* and Jones *et al.* that demonstrated that SPI-2 plays a role in *Salmonella* cecal colonization in chickens (52,56). However, contrary to previous work (55,56,57), deletion of SPI-1 locus, including *prgK*, *sicA*, *sipC*, and *hilA* genes, did not affect *Salmonella* Typhimurium cecal colonization in the present study. The discrepancies observed in the contribution of SPI-1 and SPI-2 to colonization reflects *Salmonella* serovar and strain differences.

The role of SPI-1 in *Salmonella* Typhimurium systemic dissemination is clearly demonstrated by differences in *Salmonella* prevalence in liver between SPI-1 mutant and the wild-type strains. These results were independent of *Eimeria* coinfection. In the present study, the role of SPI-2 in systemic dissemination was less obvious. *Salmonella* Typhimurium deficient in SPI-2 was recovered at a lower rate than the wild-type strain, but the difference observed was not statistically significant in birds coinfected with *Eimeria* spp. This result was likely due to a small sample size (three birds per group at each time point). Dieye *et al.* (53) previously showed that SPI-1 and SPI-2 deletion both impaired *Salmonella* Typhimurium splenic colonization in chickens; with SPI-1 contributing more than SPI-2 in systemic colonization. Mutation of *invA*, *invB*, *invC* genes of the SPI-1 region can reduce the colonization of ileal wall and spleen by *Salmonella* Typhimurium after oral inoculation of 1-day-old chickens (58). Moreover, Jones *et al.* (52) demonstrated that

spaS gene of SPI-1 and *ssaU* genes of SPI-2 played a major role in *Salmonella* Typhimurium systemic infection in 1-wk-old chickens. In birds inoculated with ΔSPI-1/WT and ΔSPI-2/WT, all drumstick samples were culture negative for *Salmonella*. These results may be due to a small sample size (3 birds per group at each time point); in the literature, *Salmonella* prevalence was 0.8% in drumstick bones of chickens collected at the processing plant (59,60). In experimental challenges, 20% of drumstick bones and 20% of drumstick muscles were detected positive with *Salmonella* in 35-day-old SPF chickens inoculated at 1 day of age (61).

The role of *Eimeria* coinfection in overall *Salmonella* Typhimurium cecal colonization and systemic spread was also evaluated. Previous works described that an oral challenge with 1×10^6 sporulated oocysts of *E. acervulina* (25) or 5×10^4 sporulated oocysts of *E. tenella* (20) increased *Salmonella* Typhimurium prevalence in ceca of coinfected birds compared with birds inoculated with *Salmonella* alone. The present results showed that a low *Eimeria* challenge dose (4×10^2 oocysts per bird) did not increase overall *Salmonella* Typhimurium prevalence in ceca, liver, and drumstick of infected birds. Other studies described an increase in *Salmonella* Typhimurium prevalence in liver of chickens coinfected with *E. tenella* (20,25), *E. necatrix* (23,24), or *E. maxima* (25) compared with birds only infected with *Salmonella* Typhimurium alone. However, these studies were conducted using higher challenge doses of *Eimeria* spp. (2×10^4 to 1×10^6 sporulated oocysts per bird) compared with the inoculum dose used in the present study (4×10^2 oocysts per bird). The effect of low dose of *E. tenella* infection on *Salmonella* invasion in systemic organs has been studied by Tellez *et al.* (62). The authors reported that, in a dose-related manner, an increase in the inoculum dose from 10 to 1×10^3 *E. tenella* sporulated oocysts resulted in a significant decrease in *Salmonella* Enteritidis prevalence in liver and spleen of infected chickens (62). The decrease in organ invasion was significantly correlated with an increase of the cecal lamina propria thickness induced by infiltration of inflammatory cells (62). A higher challenge dose of *Eimeria tenella*, which causes cecal coccidiosis, could have increased the severity of the inflammatory response and *Salmonella* Typhimurium colonization pattern in the ceca and ileocecal junction, the main colonization sites for *Salmonella* in poultry.

In summary, *Salmonella* Typhimurium tetrathionate reductase does not enhance *Salmonella* cecal colonization and systemic spread in SPF white leghorn chickens. Instead, the present results show that deletion of tetrathionate reductase operon *ttrRSBCA* may prolong cecal colonization of *Salmonella* Typhimurium. Deficiency in SPI-2 had a detrimental effect on *Salmonella* Typhimurium cecal colonization, whereas deficiency in SPI-1 impaired *Salmonella* Typhimurium dissemination to liver, and these effects were independent of *Eimeria* coinfection. A low dose of *Eimeria* spp. did not increase S. Typhimurium prevalence in ceca and did not enhance *Salmonella* dissemination in liver and drumstick of infected chickens.

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