MECHANISMS OF PERSISTENCE, SURVIVAL, AND TRANSMISSION OF BACTERIAL FOODBORNE PATHOGENS IN PRODUCTION ANIMALS

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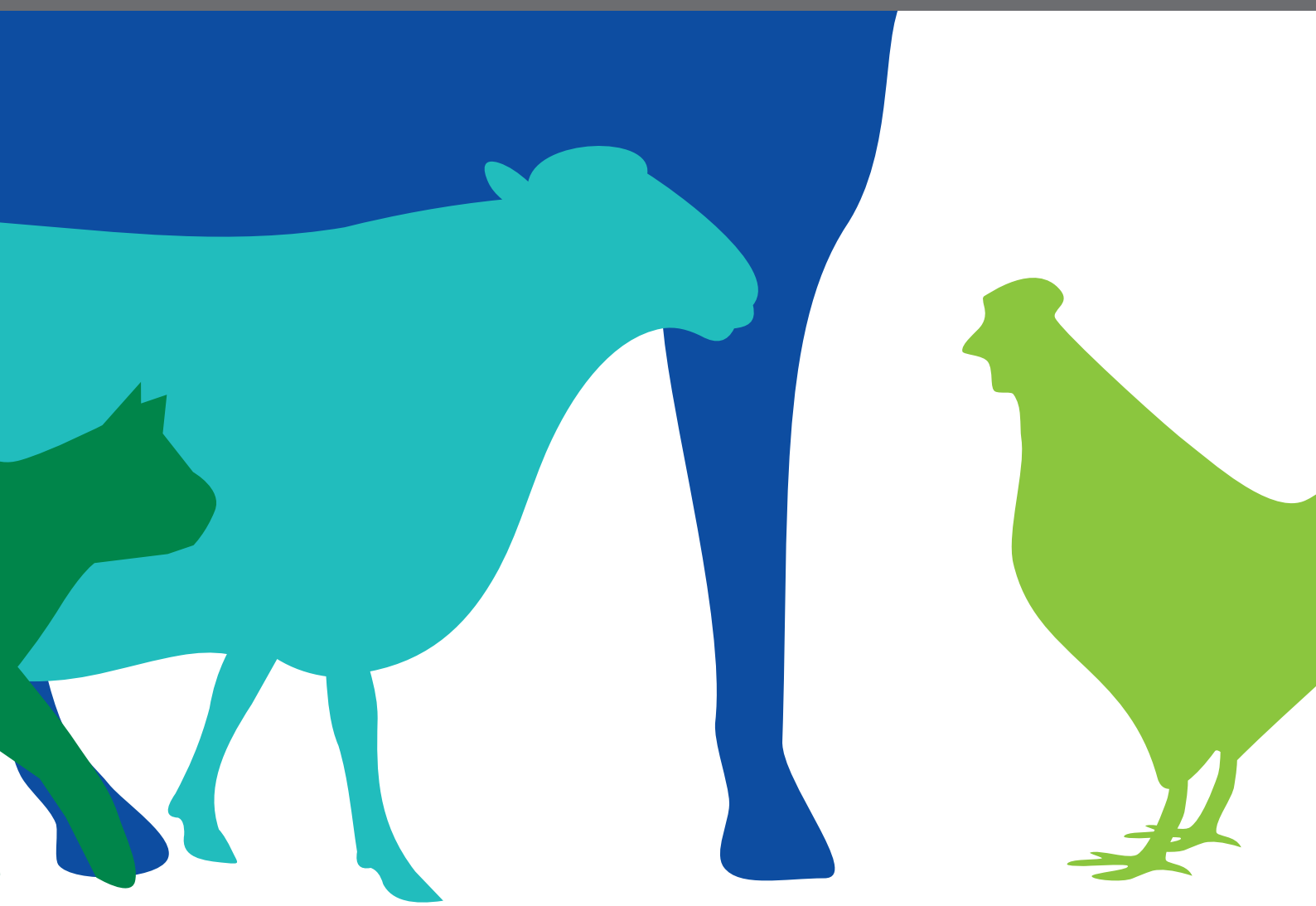

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MECHANISMS OF PERSISTENCE, SURVIVAL, AND TRANSMISSION OF BACTERIAL FOODBORNE PATHOGENS IN PRODUCTION ANIMALS

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Foodborne illness resulting from food production animals is a global health concern, and the Centers for Disease Control estimate that one in six Americans will become sick with a foodborne illness each year. Of course there are numerous causes for these outbreaks, but contamination from a food production animal is certainly one source. Understanding the host-pathogen interaction and how foodborne bacterial pathogens establish a persistent infection and evade host immune responses will be pivotal in reducing the instance of foodborne illness traced back to a food production animal source.

In this volume, we bring together original research and review articles covering some of the key issues surrounding the mechanisms of persistence, survival, and transmission of bacterial foodborne pathogens in production animals. The research focused on poultry and specifically addressed antibiotic resistance, Salmonella colonization, pathogen reduction strategies using pre- or probiotics, pathogen evasion, and post-harvest intervention and pathogen testing. The following 11 articles are fine examples of the multidisciplinary approaches that will be required to address and understand the complex interplay between food safety and animal production.

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EDITORIAL
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Editorial: Mechanisms of Persistence, Survival, and Transmission of Bacterial Foodborne Pathogens in Production Animals

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Editorial on the Research Topic

Mechanisms of Persistence, Survival, and Transmission of Bacterial Foodborne Pathogens in Production Animals

Food safety relating to animal commodities is a global matter that directly affects public health and has significant impact on international animal production industries. For years, animal food safety research focused on surveillance and prevalence of foodborne pathogens. But now, studies explore the host-pathogen interface at the molecular, biochemical, and immunological level.

Reducing antibiotic use in food producing animals has steered researchers toward novel ways to break the chain of infection, colonization, persistence, and survival and transmission of foodborne pathogens such as *Salmonella* and *Campylobacter*. Human infections associated with multidrug resistant (MDR) *Salmonella* species in poultry have become a major concern. In attempts to reduce the contamination of poultry products with MDR *Salmonella* species, researchers must characterize these infections in birds, learn how the organisms colonize, establish a "commensal" state, and are transmitted to flock members; and characterize the corresponding host immune response. The study by Liljebjelke et al. looked back at the antimicrobial resistant *Salmonella* serovars recovered from commercial broiler farms in the United States. In this retrospective study, the authors found that the reservoir for antimicrobial resistance remains in the environment; they point to the importance of additional intervention strategies to lessen the future emergence of antimicrobial resistant zoonotic bacteria from these farms. In addition to horizontal transmission of antibiotic-resistant *Salmonella*, the investigators remind us the importance of reducing bacterial loads at the breeder level to reduce future carcass contamination thereby reducing human exposure. In turkeys, researchers have found that a MDR *Salmonella* Heidelberg (*S.* Heidelberg) is easily passed among flock members during the first week-of-age, but cannot establish in the gut of 3-week-old turkeys. In addition, the host response (immune related gene expression) of turkeys to *S.* Heidelberg differs in 3-week-old turkeys vs. those within the first week post-hatch (Bearson et al.). Studies such as this in poultry and other food production species help to shed light on the dynamics of both MDR and susceptible pathogens in these animals and will lead the way to discoveries and implementation of intervention strategies that will ultimately result in the reduction of these pathogens from food production animals and subsequently reduce human infections with foodborne pathogens.

Salmonella virulence and subsequent relationships with persistence and survival in the host are firmly associated with colonization and have led researchers down various avenues of investigation including alternative housing of laying hens, the role of biofilms and *Salmonella*, and the signaling pathways associated with increased resistance against *Salmonella* colonization in broilers.

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With increasing animal welfare concerns and consumer demand, alternative farming practices including laying hens housed in enriched colony cages instead of traditional cage-based housing are being used more and more by the egg industry; however, the consequences of this transition on food safety are not fully understood. Gast et al. monitored fecal shedding of *Salmonella* Enteritidis (*S.* Enteritidis) in hens housed in traditional and enriched cages at various stocking densities and their data suggest *S.* Enteritidis colonization and fecal shedding is negatively impacted by higher stocking density regardless of the cage system. In MacKenzie et al. a review of the formation of biofilms by *Salmonella* spp. and the conditions that cause biofilm formation are compared and contrasted with *Salmonella* spp. that do not form biofilms. *Salmonella* spp. that do form biofilms appear capable of colonizing multiple host species and may "switch" to a more infectious and perhaps more virulent state once inside an acceptable host, a potential adaptation for survival and transmission. In a different approach evaluating *Salmonella* persistence, the study by Swaggerty et al. used an immune peptide array to show that the host kinome profile (protein phosphorylation patterns) in broilers with a high burden of *S.* Enteritidis is distinct from that of broilers with lower levels of colonization. As might be expected, the birds with lower loads of *S.* Enteritidis, meaning the host's immune response has restricted colonization, show increased activity in key signaling pathways associated with chemokine, Jak-Stat, MAPK, and T cell receptor signaling. These findings provide the groundwork for more in-depth studies into specific biomarkers to select individual birds that are more resistant *S.* Enteritidis colonization. Collectively, these studies have laid a solid foundation for future experiments to determine practical approaches to reduce the incidence of foodborne illnesses associated with poultry-acquired *Salmonella*.

Strategies to reduce foodborne pathogens vary widely in both their origins and their effectiveness against colonization and infection of food production species. Investigations into the administration of either pre- or probiotics to feed or water systems has become a hot topic area of research in the pursuit to find alternatives to antibiotics. In Hughes et al. researchers investigate the use of the prebiotic galacto-oligosaccharide (GOS) on host gut microbiota and gene expression and the effects of GOS on *Salmonella* gut colonization. Although GOS did not show significant reductions in *Salmonella* numbers in the gut, GOS did impact gut immune gene expression and the host microbiota compared to control birds and may offer evidence of pathways that may modulate the host response and microbiota toward reducing *Salmonella* colonization of the gut. Hayashi et al. investigated the effects of feeding *Bacillus subtilis* (*B. subtilis*) spores to broiler chickens as a probiotic aimed at *S.* Heidelberg infection and colonization. The researchers found that the *B. subtilis* spores improved performance, had immunomodulatory effects in the gut, altered the gut microflora, and reduced *S.* Heidelberg in the gut. Together, these studies show the potential impact for using pre- and probiotics as alternatives to antibiotics.

Salmonella has developed numerous mechanisms to allow it to avoid the host immune response including the ability to survive at various temperatures and develop resistance against short-chained organic acids. Dawoud et al. reviewed the link between thermal resistance and virulence in *Salmonella*. Different animal species have a wide range of body temperature, which can pose as a potential thermal stress challenge for pathogens. One of the host's primary innate immune defenses against microbial infections is merely increasing body temperature. The authors provide an extensive review of regulation of thermal stress response genes in *Salmonella* and indicate the close association between thermal resistance and virulence. One mechanism that *Salmonella* has adapted to be able to persist in a host is to readily survive over a wide range of temperatures due to the efficient expression of the heat (thermal) stress response genes. In a study highlighting another *Salmonella* defense system, Santin et al. examined a strain of *S.* Heidelberg UFPR1 found in broilers in Brazil and found susceptibility to an array of antibiotics and a *Bacillus*-based probiotic but the strain was resistant to short-chain organic acids. Further analysis showed that a comparison between the *S.* Heidelberg UFPR1 genome and the MDR SL476 strain revealed 11 missing genomic fragments and 5 insertions. The deleted genes are involved in cell cycle regulation, virulence, drug resistance, cellular adhesion, and salt efflux, which suggest that these deletions may confer *S.* Heidelberg UFPR1 strain resistance to organic acids and antibiotics. These two studies show how effective *Salmonella* is at evading the host immune response.

Salmonella and *Campylobacter* are the two leading causes of bacterial-derived foodborne illness. In the one paper looking at post-harvest intervention strategies, Li et al. looked at *Salmonella* and *Campylobacter* contamination in small-scale mobile poultry-processing units (MPPU). Carcasses treated with commercially available antimicrobials had reduced numbers of recoverable *Campylobacter jejuni* suggesting they are efficacious at reducing this key foodborne pathogen. The authors also demonstrated the importance of raising broilers on clean-shavings, instead of the common practice of using built-up-litter, as a way to also reduce *Salmonella* and *Campylobacter* contamination on MPPU-processed broiler carcasses. These two approaches provide valuable insight into ways to reduce *Salmonella* and *Campylobacter* carcass contamination.

The review by Rothrock et al. shows that in addition to *Salmonella*, *Campylobacter,* and *Escherichia coli*, *Listeria* is a major concern within the food animal industry due to their pathogenic potential to cause infection and high rates of mortality. *Listeria monocytogenes* has been isolated from all stages of poultry production/processing and outbreaks have been attributed to poultry. Since live birds are a potential vector for *Listeria* contamination, the authors suggest there is a need for genetic comparison between *Listeria* spp. and *L. monocytogenes* isolated from poultry environments and from other sources to better understand the source of listeriosis outbreaks.

Understanding the complex interplay between food safety and animal production will require a multidisciplinary approach to understand the host-pathogen interaction. This compilation of papers provides examples of the studies that will advance our knowledge and understanding of the mechanisms behind persistence, survival, and transmission of foodborne pathogens in animal agriculture.

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All authors contributed equally to the writing of the Editorial. CS was responsible for compiling each authors contributions into a single document.

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ORIGINAL RESEARCH
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Diversity of Antimicrobial Resistance Phenotypes in *Salmonella* Isolated from Commercial Poultry Farms

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Salmonella remains the leading cause of foodborne illness in the United States, and the dissemination of drug-resistant *Salmonellae* through the food chain has important implications for treatment failure of salmonellosis. We investigated the ecology of *Salmonella* in integrated broiler production in order to understand the flow of antibiotic susceptible and resistant strains within this system. Data were analyzed from a retrospective study focused on antimicrobial resistant *Salmonella* recovered from commercial broiler chicken farms conducted during the initial years of the US FDA's foray into retail meat surveillance by the National Antimicrobial Resistance Monitoring System (NARMS). Sixty-three percentage of *Salmonella* were pan-susceptible to a panel of 19 antimicrobials used by the NARMS program. Twenty-five antimicrobial resistance phenotypes were observed in *Salmonella* isolated from two broiler chicken farms. However, *Salmonella* displaying resistance to streptomycin, alone, and in combination with other antibiotics was the most prevalent (36.3%) antimicrobial resistance phenotype observed. Resistance to streptomycin and sulfadimethoxine appeared to be linked to the transposon, Tn*21*. Combinations of resistance against streptomycin, gentamicin, sulfadimethoxine, trimethoprim, and tetracycline were observed for a variety of *Salmonella enterica* serovars and genetic types as defined by pulsed-field gel electrophoresis. There were within and between farm differences in the antibiotic susceptibilities of *Salmonella* and some of these differences were linked to specific serovars. However, farm differences were not linked to antibiotic usage. Analysis of the temporal and spatial distribution of the endemic *Salmonella* serovars on these farms suggests that preventing vertical transmission of antibiotic-resistant *Salmonella* would reduce carcass contamination with antibiotic-resistant *Salmonella* and subsequently human risk exposure.

Keywords: *Salmonella,* strain type, antimicrobial resistance, poultry, vertical transmission

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INTRODUCTION

Salmonella remains the leading cause of outbreak-associated gastroenteritis in the United States, and consumption of poultry products has been implicated in several of these outbreaks (1, 2). Since implementation of the HACCP program, improvement has been made in the level of *Salmonella* contamination of processed chicken carcasses (3). However, a survey of retail meat from the Washington, DC, USA area revealed a surprising level of contamination of beef, pork, and poultry products with antibiotic-resistant *Salmonella* (4, 5). The dissemination of antibiotic-resistant *Salmonella* through the

food chain has important public health implications considering the potential for treatment failure when cases of gastroenteritis require medical intervention, especially in children, the elderly, and the immunocompromised (6). In addition, infections with antimicrobial resistant bacteria including *Salmonella* have been associated with higher rates of morbidity and mortality (7–9).

The use of antibiotics in food animal production has been implicated as a contributing factor to the emergence of drug resistance in human foodborne pathogens (6, 10). The emergence and rapid worldwide spread of the multiple drug-resistant *Salmonella enterica* Typhimurium phage-type DT104 clone and ceftriaxone-resistant *S. enterica* serovars Heidelberg, Newport, and Typhimurium have underscored the threat to both animal agriculture and human health posed by multiple drug-resistant pathogens (11–15). Antimicrobial resistance genes are widely disseminated in pathogenic, commensal, and environmental bacteria (16, 17). Furthermore, it has been shown that once antimicrobial resistance has been introduced into an ecosystem, resistance can spread and persist without continuing selection pressure from antibiotics (18, 19). In addition, the reservoir of antimicrobial resistance genes is larger than previously thought (20). It is in this environment that the potential exists for *Salmonella* to acquire antimicrobial resistance genes from resident poultry microbiota due to selection pressure from therapeutic and non-therapeutic antibiotic usage. It follows then that the longer *Salmonellae* persists in the environment of an animal production facility, the chance of acquiring resistance genes increases.

We took advantage of the integrated nature of poultry production to observe the antimicrobial resistance phenotypes acquired by salmonellae during broiler chicken production in order to identify potential critical control points for *Salmonella* contamination and antimicrobial resistance development; ultimately in order to provide information relevant to reducing the level of carcass contamination with antibiotic-resistant *Salmonella*. Data were analyzed from a retrospective study focused on antimicrobial-resistant *Salmonella* recovered from commercial broiler chicken farms conducted during the initial years of the US FDA's foray into National Antimicrobial Resistance Monitoring System (NARMS) retail meat surveillance (4). Despite the diversity of antimicrobial resistance profiles, poultry *Salmonella* recovered from these farms in 2003 were generally susceptible to the tested antimicrobials of animal and human health significance. Vertical transmission appeared to be the most important factor in chicken carcass contamination with antibiotic-resistant *Salmonella*.

MATERIALS AND METHODS

Description of Antimicrobial Usage for Two Commercial Broiler Chicken Farms in Northeast Georgia

Selection and description of study farms was as previously described (21). Approximately 17,000 chicks were placed in each house on Farm One. No litter amendments were used (22). At the hatchery, gentamicin was administered *in ovo* (0.1 mg/egg) on day 17 of development. No antibiotics were used therapeutically on this farm to treat birds during this study. Chicks were fed starter feed containing virginiamycin (10 g/ton) (25 g/ton) for the first 2 weeks. The starter feed contained coccidiostat rotated in the following order: Flock 1; diclazuril (1 g/ton), Flock 2; narasin (72 g/ton), Flock 3; monensin (100 g/ton), Flocks 4, 5; nicarbazin (82 g/ton), and Flocks 6, 7; salinomycin (60 g/ton). Flocks were fed grower feed for the next 2 weeks containing bacitracin (25 g/ton), and other coccidiostats rotated in the following order: Flock 1; salinomycin (60 g/ton), Flocks 2, 3; narasin (72 g/ton), Flocks 4, 5; lasalocid (82 g/ton), and Flocks 6, 7; diclazuril (1 g/ton). Finisher feed containing virginiamycin (15 g/ton), without coccidiostat was fed for 1–2 weeks as birds approached market weight. Withdrawal feed containing neither antibiotics nor coccidiostats was fed for the last week of grow-out. Feed was withdrawn for 16 h prior to catch.

Approximately 20,000 chicks were placed per house on Farm Two. No litter amendments were used on Farm Two (22). At the hatchery, gentamicin (0.2 mg/chick) was injected subcutaneously into day-of-hatch chicks. Chicks were reared on starter feed containing bacitracin (25 g/ton), and salinomycin (50 g/ton) for the first 2 weeks, then grower feed containing bacitracin (25 g/ton) and salinomycin (50 g/ton) for 2 weeks, then finisher feed without growth promotant or coccidiostat for 1–2 weeks. Withdrawal feed without antibiotic or coccidiostat was fed for the last week of grow-out. Feed was withdrawn for 16 h prior to shipment. *Escherichia coli* airsacculitis was diagnosed in house B during week six of Flock 3 on Farm Two, and oxytetracycline was administered in drinking water at 10.4 mg/kg weight for 1 day and at 5.1 mg/kg weight for 4 days. In this work, we sampled chick box liners, the poultry environment, and chicken carcasses. The latter was provided to us by the participating poultry companies. We did not physically interact with chickens raised on these farms and, therefore, we were exempt from university guidelines and USDA/NIH regulations regarding animal use.

Genotypic and Phenotypic Characterization of Poultry *Salmonella* Isolates

The 289 *Salmonella* strains, examined in this study, were isolated, serotyped, phage-typed, and strain-typed as previously described (21). Presence of *aadA1* and *merA* was determined as described by Bass et al. (23).

Antibiotic susceptibility was determined for the 289 archived *Salmonella* isolates (21). The minimum inhibitory concentration (MIC) of the antimicrobial agents tested was determined with the Sensititre® automated antimicrobial susceptibility system (Trek Diagnostic Systems, Westlake, OH, USA) and interpreted according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines for microbroth dilution methods (24, 25). Sensititre® susceptibility testing was performed according to the manufacturer's instructions, and susceptibility and resistance were reported as MIC (μg/ml). Three-letter abbreviations and resistance breakpoint concentration are in parentheses. The antimicrobials assayed were as follows: amikacin (AMI > 64 μg/ml), amoxicillin/clavulanic acid (AUG > 32/16 μg/ml), ampicillin (AMP > 32 μg/ml), apramycin (APR 32 μg/ml), ceftriaxone (AXO > 64 μg/ml),

cefazolin (CEF 32 μg/ml), cefoxitin (FOX > 32 μg/ml), ceftiofur (TIO > 8 μg/ml), cephalothin (CEP > 32 μg/ml), chloramphenicol (CHL > 32 μg/ml), ciprofloxacin (CIP > 4 μg/ml), kanamycin (KAN 64 μg/ml), gentamicin (GEN > 16 μg/ml), imipenem (IMP > 4 μg/ml), nalidixic acid (NAL > 32 μg/ml), streptomycin (STR > 64 μg/ml), sulfadimethoxine (SMX > 512 μg/ml), tetracycline (TET > 16 μg/ml), and trimethoprim/sulfamethoxazole (TMS > 4/76 μg/ml). The antibiotics bacitracin and virginiamycin were not included with this panel as there is no breakpoint for *Salmonella* as their activity is specifically directed toward Gram-positive bacteria and it is used to prevent *Clostridium perfringens* infections in chickens.

This study was performed in 2003, early in the US Food and Drug Administration's survey of antimicrobial-resistant foodborne bacteria recovered from retail meats, using the same methods and antimicrobial resistance break points recommended by NCCLS (Clinical and Laboratory Standards Institute) at that time.

Statistical Analysis

The Fisher's exact test with $\alpha = 0.05$ and Mantel–Haenszel chi-squared test were used to test for non-random associations between specific data values. *Salmonella* Typhimurium PFGE types 1.1, 1.2, and 1.3 were ranked with regard to multiple drug resistance as determined by fitting linear model: $\log(\mu_i) = \beta_0 = \beta_1 * \text{PFGE type}_i$, μ_i = mean number antimicrobial resistances or resistance type, with assumption that data conformed to Poisson distribution.

RESULTS

Antibiotic Susceptibility and Diversity of Antimicrobial Resistance Phenotypes in Poultry *Salmonella*

There is ample opportunity for antibiotic-resistant *Salmonella* to emerge on poultry farms due to the combination of on farm antibiotic usage and the significant reservoir of antimicrobial resistance genes present in poultry litter. We examined the antibiotic susceptibility of *Salmonella* collected from two commercial broiler farms in northeast Georgia in relation to on-farm antibiotic usage. The majority of *Salmonella* isolates (62.6%; $n = 172$) were susceptible to all 19 antimicrobials tested, with the remainder displaying resistance to streptomycin (30.9%), gentamicin (12.6%), sulfadimethoxine (20.9%), tetracycline (13.9%), and trimethoprim/sulfamethoxazole (8.6%) (**Table 1**). *Salmonella* resistance to streptomycin alone was the most prevalent antimicrobial resistance phenotype (30.9%) (**Tables 1** and **2**).

A diversity of antimicrobial resistance phenotypes ($n = 25$) was observed among the *Salmonella* isolated from commercial broiler chicken farms (**Table 2**). Twenty percentage of our poultry *Salmonella* isolates were resistant to three or more antibiotics (**Table 1**). The most common antimicrobial resistance phenotypes identified were to streptomycin (36.28%); streptomycin and sulfadimethoxine, alone or in combination with other antibiotics (41.59%); and streptomycin, sulfadimethoxine, and gentamicin, alone or in combination with other antibiotics (28.32%) (**Table 2**). There was a statistically significant association between *Salmonella* isolates displaying resistance to streptomycin and sulfadimethoxine; and streptomycin, sulfadimethoxine, and gentamicin (chi-squared test: $p < 0.05$). While antimicrobial resistance phenotype diversity was high (Reciprocal Simpson's Index: 1.20), evenness in distribution of these phenotypes among *Salmonella* was low (0.26). The low evenness score may be a reflection of the broad distribution of certain antimicrobial resistance phenotypes compared to others [streptomycin resistance, alone (41 strain types); streptomycin, sulfadimethoxine, and gentamicin resistance (17 strain types); sulfadimethoxine, trimethoprim/sulfamethoxazole, and tetracycline resistance (11 strain types); streptomycin, sulfadimethoxine, gentamicin, and tetracycline resistance (8 strain types); streptomycin, sulfadimethoxine trimethoprim/sulfamethoxazole, and tetracycline resistance (7 strain types)].

TABLE 1 | Most prevalent antimicrobial resistance phenotypes observed in the *Salmonella* serovars isolated from production and processing of seven consecutive commercial broiler flocks.

Salmonella enterica serovar (*n*=)	% Sensitive[a]	% STR[a]	% GEN[a]	% SMX[a]	% TET[a]	% TMS[a]	% AMP[a]	% Multidrug resistant[b]
Farm One								
S. Typhimurium (153)	66.6[d]	36.6	9.8	12.4	5.9	1.9	0.6	11.1
S. Enteritidis (28)	92.8[d]	3.6	3.6	3.6	0	0	3.6	3.6
S. Montevideo (22)	40.9	18.2	0	54.5	59.1	59.1	0	53.8
S. Kentucky (13)	23.1[c]	61.5	61.5	76.9	7.7	7.7	0	53.8
S. Heidelberg (6)	33.3	50.0	33.3	33.3	16.7	16.7	16.7	66.7
All isolates (241)	60.7	35.4[c]	13.8	23.4[c]	13.3	10.4[c]	0.9	22.7
Farm Two								
S. Kentucky (13)	100[c]	0	0	0	0	0	0	0
S. Mbandaka (9)	55.6	11.1	11.1	11.1	33.3	0	0	11.1
S. Typhimurium (6)	66.6	33.3	16.7	16.7	16.7	0	0	16.7
S. Ohio (5)	80.0	20.0	0	0	0	0	0	0
S. Senftenberg (4)	75.0	0	25.0	25.0	25.0	0	25.0	25.0
All isolates (48)	72.3	8.5[c]	6.4	8.5[c]	17.0	0[c]	4.3	10.6

[a]*Resistance profiles to the following antibiotics: AMP, ampicillin; GEN, gentamicin; STR, streptomycin; TET, tetracycline; SMX, sulfadimethoxine; and TMS, trimethoprim/sulfamethoxazole. Sensitive: susceptible to the 19 antibiotics tested.*

[b]*Resistance to three or more antibiotics.*

[c]*Farm differences in isolate or serovar susceptibility to antibiotics as determined by chi-squared test ($p < 0.05$).*

[d]*Salmonella serovar differences in susceptibility to antibiotics as determined by chi-squared test ($p < 0.05$).*

TABLE 2 | Diversity of antimicrobial resistance phenotypes in *Salmonella* isolated from two commercial poultry farms.

Antimicrobial resistance phenotypes[a]	Strain type[b]	Total[c]		
STR	5	41	(36.28)	
STR SMX GEN	5	17	(15.04)	
SMX TMS TET	4	11	(9.73)	
STR SMX GEN TET	3	8	(7.08)	
STR SMX TMS TET	5	7	(6.19)	
STR SMX	2	3	(2.65)	
STR SMX GEN TMS TET	2	3	(2.65)	
CHL	2	2	(1.77)	
STR SMX GEN CEP	1	2	(1.77)	
STR TET CHL	1	2	(1.77)	
TET	2	2	(1.77)	
TET CEP CHL	2	2	(1.77)	
AMP	1	1	(0.88)	
STR SMX CEP	1	1	(0.88)	
STR SMX AXO FOX TIO AMI APR NAL	1	1	(0.88)	
STR SMX GEN AMP	1	1	(0.88)	
SMX GEN	1	1	(0.88)	
STR GEN	1	1	(0.88)	
STR SMX GEN TET AMP	1	1	(0.88)	
SMX TET TMS CEP CHL KAN	1	1	(0.88)	
STR SMX TET TMS CHL	1	1	(0.88)	
CEP AMP	1	1	(0.88)	
SMX TMS	1	1	(0.88)	
TMS TET	1	1	(0.88)	
SMX	1	1	(0.88)	
"STR SMX" alone or with another antimicrobial resistance		47	(41.59)	$p < 0.05$[d]
"STR SMX GEN" alone or with another antimicrobial resistance		32	28.32%	$p < 0.05$[e]
Diversity (Reciprocal Simpson's Index) = 1.20 Evenness = 0.26				

[a]*Resistance profiles to the following antibiotics: AMP, ampicillin; AUG, augmentin; FOX, cefoxitin; CEP, cephalothin; GEN, gentamicin; KAN, kanamycin, STR, streptomycin; AMI, amikacin; NAL, nalidixic acid; TET, tetracycline; SMX, sulfadimethoxine; TMS, trimethoprim/sulfamethoxazole; and CHL, chloramphenicol.*

[b]*Number of different S. enterica serovar or strain type with antimicrobial resistance phenotype.*

[c]*( ) Percentage of total antimicrobial resistance phenotypes identified.*

[d]*Linkage of STR with SMX as determined by the chi-squared test.*

[e]*Linkage between STR and SMX with GEN as determined by the chi-squared test.*

Two of the common antimicrobial resistances identified, streptomycin and sulfadimethoxine resistance, are commonly associated with the transposon, Tn*21*. The resistance genes *merA* and *aadA1* are resident on this mobile genetic element and the distribution of these loci was 17.86 and 10.56%, respectively, in the recovered poultry isolates. There was a significant association between these resistance genes and resistance to streptomycin or sulfadimethoxine (chi-squared test; $p < 0.05$).

Farm Variability in Antimicrobial Susceptibilities of Poultry *Salmonella*

Differences were observed within and between poultry farms in antibiotic susceptibilities of *Salmonella* isolates. Antimicrobial susceptibility patterns differed between farms as *Salmonella* isolates from Farm One were more likely to be resistant to streptomycin, sulfadimethoxine, and trimethoprim/sulfamethoxazole compared to those recovered from Farm Two (chi-squared test: $p < 0.05$) (**Table 1**). There were also differences in antibiotic susceptibilities among certain *Salmonella* serovars within farms as well as between farms. *S.* Typhimurium isolated from Farm One were less susceptible to antibiotics, tested in this study, than *S.* Enteritidis isolated from the same farm. *Salmonella* Kentucky isolated from Farm One exhibited significantly more antimicrobial resistance than other *Salmonella* isolated from the same farm as well as *S.* Kentucky isolated from Farm Two (**Table 1**). Following tetracycline treatment on Farm Two, *Salmonella* isolates were less likely to be resistant to tetracycline, as determined using one-sided, Fisher's exact test at $\alpha = 0.05$ ($p = 0.0046$), or to other antibiotics (Cochran–Mantel–Haenszel method, $p = 0.0046$). The therapeutic treatment of *E. coli* airsacculitis with tetracycline did not seem to selectively enrich for antimicrobial resistance in *Salmonella* isolated from subsequent flocks. In addition, there was no statistically significant difference in *Salmonella* isolates displaying resistance to tetracycline between the two poultry farms (chi-squared test; $p = 0.34$).

Is Horizontal or Vertical Transmission Responsible for Spread of Antibiotic-Resistant *Salmonella* to Poultry Meat?

S. Typhimurium ($n = 159$) was the most prevalent serovar isolated in this study, and this serovar was frequently isolated from Farm One. Serovar Typhimurium isolates were largely pan-susceptible (66.6%); however, the most prevalent antimicrobial resistance phenotypes were to streptomycin (6.6%), sulfadimethoxine (12.4%), gentamicin (9.4%), and tetracycline (6.4%) (**Table 3**). Resistance to the other 14 antimicrobials tested was not observed that often (≤5%). Eleven percentage of *S.* Typhimurium isolates were resistant to three or more antibiotics. The most prevalent *S.* Typhimurium resistance phenotypes observed were as follows: streptomycin alone (23.7%) and the multi-drug resistant phenotype to streptomycin, gentamicin, sulfadimethoxine, and tetracycline (5.3%). A diversity of antimicrobial resistance phenotypes ($n = 9$) was observed for the three related *S.* Typhimurium strain types identified by PFGE (**Table 3**). Combinations of resistance against streptomycin, gentamicin, sulfadimethoxine, and tetracycline, accounted for 85.3% of the resistance phenotypes (**Table 3**). There was no significant difference in resistance phenotypes between the three *S.* Typhimurium genetic types isolated from Farm One with the exception that PFGE type T1.3 was significantly more likely to be ampicillin resistant ($\alpha = 0.05$).

Of the three S. Typhimurium strain types (T1.1, T1.2, and T1.3) present on Farm One, there were three instances where two of these strain types were present with chicks on the broiler chicken farm (T1.1 and T1.2) and chicken carcasses derived from these flocks (**Table 4**). There were also three other situations where these same *S.* Typhimurium strain types were only isolated from the farm environment and then chicken carcasses at processing. The only antibiotic resistant *S.* Typhimurium strain types found on chicken carcasses matched with those isolated from chicks at farm placement indicating that resistant *S.* Typhimurium strains were likely vertically transferred from the breeder flock.

TABLE 3 | Antimicrobial resistance phenotypes of *Salmonella enterica* serovars and strain types isolated from commercial broiler chicken farms.

Salmonella serovar (phage type)[a,b]	PFGE type[b]	Antimicrobial resistance phenotype[c]	Number of isolates
S. Enteritidis (PT8)	E1.1	Sensitive	18
		AMP	1
		STR SMX GEN	1
	E1.2	Sensitive	8
S. Typhimurium (DT193)	T1.1	Sensitive	50
		STR	16
		STR SMX	1
		STR SMX GEN TET	3
		STR SMX TET TMS	1
		STR SMX GEN TET TMS	2
(DT107)	T1.2	Sensitive	47
		STR	21
		STR SMX CEP	1
		STR SMX GEN	5
		STR SMX GEN TET	3
		STR SMX AXO FOX TIO AMI APR NAL	1
(U302)	T1.3	Sensitive	5
		STR GEN SMX AMP	1
(NT)	T2	Sensitive	1
	T3	Sensitive	1
S. Montevideo	V1.1	Sensitive	6
	V1.2	SMX TET TMS	3
	V1.3	SMX TET TMS	1
	V1.5	SMX TET TMS	6
		STR SMX TET TMS	3
	NT	Sensitive	1
		STR	1
		CHL	1
S. Kentucky	NT	Sensitive	16
		STR SMX	2
		GEN SMX	1
		STR SMX GEN	6
		STR SMX GEN TET TMS	1
S. Senftenberg	S1	Sensitive	3
		STR GEN	1
		STR SMX GEN	4
		STR SMX GEN TET AMP	1
		STR SMX GEN CEP	2
S. Gaminara	G1.1	SMX TET TMS CEP CHL KAN	1
		STR TET CHL	2
		STR SMX TET TMS CHL	1
	G1.2	Sensitive	2
	G2.1	CEP AMP	1
	G3.1	Sensitive	1
S. Mbandaka	M1	Sensitive	4
		STR SMX GEN TET	2
	NT	Sensitive	1
		TET	1
S. Anatum	A1	STR SMX TET TMS	1
	A2	STR SMX TET TMS	1
		SMX TET TMS	1
	A3	Sensitive	1
		STR SMX TET TMS	1
S. Ohio	O1	STR	1
	NT	Sensitive	3
S. Tennessee	T1	SMX TMS	1
		TET TMS	1
S. California	C1	Sensitive	1
S. Heidelberg	H1	Sensitive	3
		STR	2
		STR SMX GEN	1
		AMP CEP AUG FOX	1
S. Jerusalem	J1	TET	1
S. Lille	L1	TET CEP CHL	1
	NT	TET CEP CHL	1
		CHL	1
S. Muenchen	U1	SMX	1

[a]Phage typing was done only for S. Enteritidis and S. Typhimurium isolates. ( ) = phage type.

[b]NT = not typable by phage typing (column 1) or PFGE (column 2).

[c]Resistance profiles to the following antibiotics: AMP, ampicillin; AUG, augmentin; FOX, cefoxitin; CEP, cephalothin; GEN, gentamicin; KAN, kanamycin, STR, streptomycin; AMI, amikacin; NAL, nalidixic acid; TET, tetracycline; SMX, sulfadimethoxine; TMS, trimethoprim/sulfamethoxazole; and CHL, chloramphenicol. Sensitive: susceptible to the 19 antibiotics tested.

DISCUSSION

The antibiotic susceptibility and profiles of *Salmonella* isolated from two poultry farms mirrored antimicrobial resistance data reported in other studies. The majority (51.6%) of *Salmonella* isolates, from a 2001 NARMS survey, were also pan-susceptible. The most commonly identified resistances were to the antibiotics tetracycline (26.7%), streptomycin (23.7%), sulfadimethoxine (9.1%), gentamicin (6.3%), and ampicillin (15.1%) (26). A 2002 NARMS retail survey also reported that *Salmonella* isolated from chicken meat were largely pan-susceptible (66.6%), with the most prevalent resistance observed for sulfadimethoxine (18.7%), streptomycin (32.3%), gentamicin (3.4%), ampicillin (5.1%), trimethoprim/sulfamethoxazole (1.7%), and tetracycline (34.3%) (4). The 2003 NARMS retail meats survey, contemporary with the sampling times of this study, reported 47% of *Salmonella* isolates as pan-susceptible; with resistances observed for tetracycline (27.4%), streptomycin (26.2%), sulfadimethoxine (14.3%), gentamicin (6.0%), and ampicillin (33.3%). In the most recent NARMS retail meats survey (2015), half of the poultry *Salmonella* isolates were pan-susceptible to a panel of 12 antibiotics. *Salmonella* isolated from retail meats, in this survey, were resistant to tetracycline (37.3%), streptomycin (37.3%), sulfadimethoxine (8.5%), gentamicin (5.1%), and ampicillin (8.5%) (27).

There was a diversity of antimicrobial resistance phenotypes identified among our poultry *Salmonella* isolates. Despite this diversity, the antimicrobial resistance phenotype: streptomycin and sulfadimethoxine resistance alone or with other antibiotics was commonly encountered in *Salmonella* isolated from the commercial poultry farms. The genes conferring resistance to these antimicrobials are frequently found residing on mobile genetic elements which are responsible for the wide-spread dissemination of antimicrobial resistance in nature. The transposon Tn*21*

TABLE 4 | Temporal and spatial distribution of resident antibiotic susceptible and resistant *S*. Typhimurium strain types during the production of seven consecutive commercial broiler flocks.

Flock[a]	*S.* Typhimurium PFGE type	Antimicrobial resistance phenotype[b]	Location[c]		
			Hatchery	House	Carcass
1	T1.1	Sensitive		2	
		STR		2	
		STR SMX TET TMS		1	
		STR SMX GEN TET TMS		2	
2	T1.1	Sensitive		5	
	T1.2	Sensitive	3	16	
		STR	2	1	1
		STR SMX GEN		2	
		STR SMX GEN TET		1	
	T1.3	Sensitive		4	
		STR SMX GEN AMP		1	
3	T1.1	Sensitive	1	4	1
		STR	1	3	5
		STR SMX		1	
	T1.2	Sensitive		4	
		STR		1	
	T1.3	Sensitive		1	
4	T1.1	Sensitive		3	14
		STR			1
5	T1.1	Sensitive		8	1
		STR		2	
	T1.2	Sensitive		4	1
		STR		5	
6	T1.1	Sensitive		2	
		STR		2	
	T1.2	Sensitive		2	
		STR		9	
		STR SMX CEP		1	
		MDR[d]		1	
7	T1.1	Sensitive		7	
		STR SMX GEN TET		3	
	T1.2	Sensitive		15	
		STR		2	
		STR SMX GEN		3	
		STR SMX GEN TET		2	

[a]*Poultry Farm One.*

[b]*Resistance profiles to the following antibiotics: AMP, ampicillin; AUG, augmentin; FOX, cefoxitin; CEP, cephalothin; GEN, gentamicin; KAN, kanamycin, STR, streptomycin; AMI, amikacin; NAL, nalidixic acid; TET, tetracycline; SMX, sulfadimethoxine; TMS, trimethoprim/sulfamethoxazole; and CHL, chloramphenicol. Sensitive: susceptible to the 19 antibiotics tested.*

[c]*Number of Salmonella isolates belonging to said strain type and antimicrobial resistance phenotype.*

[d]*Multidrug resistance (MDR) to antibiotics: STR, SMX, FOX, AMI, AXO, NAL, TIO, and APR.*

contains the mercury resistance gene *merA*; streptomycin resistance gene *aadA1*; and sulfadimethoxine resistance gene *sul1* (28). This transposon is often responsible for dissemination of mercury and antimicrobial resistance in nature (28) and is prevalent in poultry *Salmonella* and *E. coli* (23). While we observed linkage between the resistance genes *merA* and *aadA* and streptomycin/sulfadimethoxine resistance, only 17.72% of streptomycin-resistant *Salmonella* had *aadA1*, indicating that other antimicrobial resistance gene(s) are responsible for streptomycin resistance and further illustrates the diversity underlying antimicrobial resistance phenotypes observed in these isolates.

Despite the high prevalence of Tn*21* in these poultry isolates, antimicrobial resistance phenotypes were not uniformly distributed among *Salmonella* serovars within as well as between the two commercial broiler chicken farms. Certain *Salmonella* serovars differed in their antibiotic susceptibility patterns. *Salmonella* Enteritidis tended to be pan-susceptible while *S.* Typhimurium exhibited a diversity of antimicrobial resistance phenotypes. Similar trends have been observed for these *Salmonella* serovars reported in NARMS retail meats (2003, 2015) and HACCP (2003, 2014) surveys (27). Even within *S.* Typhimurium, there were differences in antibiotic susceptibilities among strain types. The *S.* Typhimurium PFGE subtype T1.1 from Farm One (21) was identified as phage type (PT) 193, a PT commonly associated with illnesses in humans (29–39). This *Salmonella* PT has also been isolated from cattle (38, 40, 41),

poultry (31, 42), pigs (31, 40), and dogs (40). Like *S.* Typhimurium DT104, PT 193 isolates generally exhibit resistance to three or more antibiotics, but resistance phenotypes reported have been variable (40, 43). The majority (68.0%) of our *S.* Typhimurium PT DT193 isolates from Farm One were pan-susceptible, with 32% possessing the following resistance phenotypes to: streptomycin alone; streptomycin, sulfadimethoxine, tetracycline, and trimethoprim/sulfamethoxazole; streptomycin, sulfadimethoxine, gentamicin, tetracycline, and trimethoprim/sulfamethoxazole. The other *S.* Typhimurium PFGE types, T1.2 and T1.3, were identified, respectively, as PTs DT107 and U302 (21). The *S.* Typhimurium PTs DT107 and DT193 from this study appear to be genetically related as determined by PFGE (44). Close genetic-relatedness as determined by PFGE among different *S.* Typhimurium and *S.* Enteritidis PTs has been reported by others (45, 46). The *S.* Typhimurium DT107 isolates were similar to the *S.* Typhimurium DT193 isolates, in that the majority were pan-susceptible (59.6%), with the most prevalent antimicrobial resistance phenotype being resistance to streptomycin only (25.4%).

Poultry litter contains a large reservoir of antimicrobial resistance genes. We had shown in a previous study that many of these antimicrobial resistance genes are shared among diverse bacterial species in poultry litter (ex. *aadA1* in *Corynebacterium* and *Salmonella*) (20). Therefore, the potential exists for environmental transfer of antimicrobial resistance to *Salmonella* and subsequent horizontal transmission of emergent resistant *Salmonella* strains to poultry in this environment. Of the eight antibiotic resistant phenotypes solely present in *S.* Typhimurium isolated from the farm environment, none were identified in *S.* Typhimurium isolated from processed chicken carcasses. This finding suggests that despite the diversity of antibiotic-resistant *S.* Typhimurium resident in the broiler house environment, none of these antibiotic resistant strains were being transmitted through the processing plant to the poultry carcass. Only those antibiotic-resistant strain types present with the chicks at placement remained on birds at processing. Therefore, our data support the importance of vertical transmission routes in the dissemination of antibiotic-resistant *Salmonella* through the food chain.

CONCLUSION

Therapeutic tetracycline antibiotic usage was not a significant predictor of emergent antimicrobial resistance in *Salmonella*. This result is not surprising, considering that the all-in, all-out production method used in the commercial poultry industry is designed to break disease cycles and should minimize antimicrobial resistance development, as long as pathogen persistence from flock-to-flock is prevented (47). However, the reservoir for antimicrobial resistance remains within the farm environment. Additional measures involving litter management and pest control may be needed to prevent future emergence of antimicrobial resistance zoonotic bacteria on treated farms. In addition, the prevalence of streptomycin resistance in poultry *Salmonella* was surprisingly high considering that streptomycin is rarely used in poultry production medicine and to our knowledge had not been used at these farms. This is most likely due to linkage of streptomycin resistance gene(s) with other resistance genes, or competitively advantageous genes (bacteriocins, siderophores, etc.); or its integration into the chromosome that has maintained streptomycin resistance in *Salmonella*, even in the absence of antibiotic selection (19). However, gentamicin is commonly used with *in ovo* poultry vaccines as a prophylaxis against peritonitis in chicks and therefore may explain, in part, the level of resistance to this antibiotic in *Salmonella*. The physical linkage of resistance genes associated with gentamicin with streptomycin resistance may also explain the persistence of streptomycin resistance in the absence of usage (19). As gentamicin was used by both poultry companies, it is uncertain whether gentamicin resistance in *Salmonella* will persist with time. The other antibiotics used by the poultry farms in this study, bacitracin and virginiamycin, are used to control *C. perfringens* infections in poultry. While these antibiotics do not affect *Salmonella* or other Gram-negative enterics, they do have an impact in the Gram positive, intestinal microbiota of chickens (48). It is currently not known how changes to the chicken intestinal microbiota, in response to bacitracin and virginiamycin, affect *Salmonella* prevalence, abundance, or antibiotic resistance patterns.

Vertical transmission from the breeder flock, rather than horizontal transmission from the environment, appears to play a significant role in carcass contamination with antibiotic-resistant *Salmonella*. If antibiotic usage is involved in the emergence and spread of antibiotic-resistant *Salmonella* to chicken meat, it may exist at the breeder, not broiler level of poultry production. One way to block transmission of antimicrobial-resistant *Salmonella* would be to apply an intervention such as competitive exclusion or vaccination at the breeder level (49, 50). The poultry integrator for Poultry Farm One has recently instituted a company-wide *Salmonella* vaccination program at the broiler-breeder level. It will be interesting to see if this mitigation strategy has significantly changed antimicrobial resistance profiles of *Salmonella* isolated from broiler chicken farms and poultry charges, especially on Poultry Farm One.

AUTHOR CONTRIBUTIONS

JM, CH, DW, and ML contributed to the conception and design of this study. KL and SA were responsible for the acquisition of data analyzed in this study. JM, KL, and DW were involved in the analysis and interpretation associated with this work. KL was responsible for writing the first draft. All the authors were involved in manuscript revisions and final approval of the version to be published.

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Characterization of a Multidrug-Resistant *Salmonella enterica* Serovar Heidelberg Outbreak Strain in Commercial Turkeys: Colonization, Transmission, and Host Transcriptional Response

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In recent years, multidrug-resistant (MDR) *Salmonella enterica* serovar Heidelberg (*S.* Heidelberg) has been associated with numerous human foodborne illness outbreaks due to consumption of poultry. For example, in 2011, an MDR *S.* Heidelberg outbreak associated with ground turkey sickened 136 individuals and resulted in 1 death. In response to this outbreak, 36 million pounds of ground turkey were recalled, one of the largest meat recalls in U.S. history. To investigate colonization of turkeys with an MDR *S.* Heidelberg strain isolated from the ground turkey outbreak, two turkey trials were performed. In experiment 1, 3-week-old turkeys were inoculated with 10^8 or 10^{10} CFU of the MDR *S.* Heidelberg isolate, and fecal shedding and tissue colonization were detected following colonization for up to 14 days. Turkey gene expression in response to *S.* Heidelberg exposure revealed 18 genes that were differentially expressed at 2 days following inoculation compared to pre-inoculation. In a second trial, 1-day-old poults were inoculated with 10^4 CFU of MDR *S.* Heidelberg to monitor transmission of *Salmonella* from inoculated poults (index group) to naive penmates (sentinel group). The transmission of MDR *S.* Heidelberg from index to sentinel poults was efficient with cecum colonization increasing 2 Log10 CFU above the inoculum dose at 9 days post-inoculation. This differed from the 3-week-old poults inoculated with 10^{10} CFU of MDR *S.* Heidelberg in experiment 1 as *Salmonella* fecal shedding and tissue colonization decreased over the 14-day period compared to the inoculum dose. These data suggest that young poults are susceptible to colonization by MDR *S.* Heidelberg, and interventions must target turkeys when they are most vulnerable to prevent *Salmonella* colonization and transmission in the flock. Together, the data support the growing body of literature indicating that *Salmonella* establishes a commensal-like condition in livestock and poultry, contributing to the asymptomatic carrier status of the human foodborne pathogen in our animal food supply.

Keywords: *Salmonella enterica* serovar Heidelberg, multidrug-resistant, foodborne outbreak, turkey, colonization, transmission, gene expression

INTRODUCTION

Food-producing animals such as swine, cattle, and poultry are a major reservoir of the human foodborne pathogen *Salmonella* (1, 2). While some *Salmonella* serovars can cause disease in food-producing animals, most serovars colonize these animals asymptomatically, resulting in the hosts becoming carriers and intermittent shedders of *Salmonella* (1). Poultry (turkey and chicken) are frequent carriers of *Salmonella*, and poultry products represent about 58% of the salmonellosis cases associated with products regulated by the Food Safety Inspection Service (3). The prevalence of foodborne disease outbreaks caused by *Salmonella enterica* serovar Heidelberg (*S.* Heidelberg) has increased over the last decade (4). One of the largest meat recalls in U.S. history resulted from a multistate outbreak of multidrug-resistant (MDR) *S.* Heidelberg in 2011 that caused 136 confirmed cases of human foodborne disease (39% hospitalization rate) and the recall of 36 million pounds of ground turkey meat (5–7). Other recent outbreaks of foodborne illness involving *S.* Heidelberg include contact with dairy bull calves (https://www.cdc.gov/salmonella/heidelberg-11-16/index.html), an international in-flight catered meal (8), and chicken that sickened 634 case patients in 29 states and Puerto Rico (38% hospitalization rate) (9). Analysis of invasive non-typhoidal *Salmonella* isolated in the U.S. from 1996 to 2007 indicated that ~14% of *S.* Heidelberg isolates were from human bloodstream infections with resistance to one or more antimicrobial agents being associated with increased risk for invasive disease (10). *S.* Heidelberg is responsible for 7% of human deaths due to non-typhoidal *Salmonella* in the U.S. (11), the second most frequent serovar causing mortality following serovar Typhimurium. The prevalence of multidrug resistance (resistance to three or more antibiotic classes) in *S.* Heidelberg has increased 2.6-fold since 2004 (12), including resistance to ampicillin, gentamicin, streptomycin, tetracycline, chloramphenicol, kanamycin, and sulfisoxazole (5, 9). Based on 2013 data from the National Antimicrobial Resistance Monitoring System, ~33% of *S.* Heidelberg isolates that cause human foodborne disease are MDR (13).

Understanding the interactions of a foodborne pathogen with its food animal host is important for managing food safety risk; therefore, we investigated the pathogenicity, colonization and transmission potential of an MDR *S.* Heidelberg strain from the 2011 ground turkey outbreak in a natural poultry host—commercial turkeys. The MDR *S.* Heidelberg strain colonized the spleen and tissues of the digestive tract of the turkey without causing noticeable clinical symptoms. Gene expression analysis of blood from 3-week-old turkeys at two days post-inoculation (dpi) suggested only a mild response to the 10^{10} CFU challenge, with 18 genes identified as differentially expressed. In young poults less than one week old, MDR *S.* Heidelberg from inoculated poults was efficiently transmitted to naive poults; these data suggest that young poults are susceptible to colonization by MDR *S.* Heidelberg which may allow for the development of an asymptomatic carrier state in turkeys, thereby confirming this vulnerability as a critical control point to reduce food safety risk in poultry. Collectively, the lack of clinical symptoms and limited gene expression in 3-week-old turkeys in response to the MDR *S.* Heidelberg outbreak strain paired with the efficient transmission, colonization, and proliferation of the strain in newly hatched poults provide insight into potential factors that contribute to the successful colonization of turkey farms with MDR *S.* Heidelberg thereby leading to the recent outbreaks with this human foodborne pathogen.

MATERIALS AND METHODS

Bacterial Strains and Selective Medium

An MDR *S.* Heidelberg strain BSX 126 (2011K-1138; CVM41579) isolated from ground turkey and associated with a 2011 ground turkey outbreak was used for this study (6). Strain BSX 126 is resistant to ampicillin, tetracycline, streptomycin, and gentamicin. In experiment 1 (described below), BSX 126 was inoculated into a turkey, isolated from the spleen at 7 days post-inoculation (dpi) and designated strain SB 395. Growth of *S.* Heidelberg on XLT-4 medium indicated that this serovar is a weak H_2S producer. Similar to our investigation of *S.* Choleraesuis (14), reducing the tergitol concentration in XLT-4 (Becton, Dickinson and Co., Sparks, MD, USA) to 25% of the normal level allowed *S.* Heidelberg to produce H_2S, resulting in the visualization of black colonies following 48 h of incubation. Therefore, the bacterial growth medium for culture of *S.* Heidelberg from turkeys was XLT-4 containing 25% tergitol (1.15 ml/l), tetracycline (15 μg/ml), streptomycin (50 μg/ml), and novobiocin (40 μg/ml).

Animal Trials and Sample Processing

Experiment 1

Sixteen 1-day-old tom (male) turkey poults were group housed for two weeks. Fecal samples from the group pen tested negative for *Salmonella* twice using qualitative bacteriology as previously described (15). Turkeys were separated in individual pens and inoculated by oral gavage with 10^8 ($n = 8$) or 10^{10} ($n = 7$) CFU of MDR *S.* Heidelberg strain BSX 126 at 3 weeks of age. Cloacal temperatures were measured using a Medline thermometer, model # MDS9850B (Mundelein, IL, USA) at 0, 1, 2, and 3 days post-inoculation (dpi). *Salmonella* levels in the feces were determined at 0, 1, 2, 3, 7, 10, and 14 dpi using quantitative and qualitative bacteriology as previously described (16). At 7 dpi, four turkeys from the 10^8 CFU inoculated group and four turkeys from the 10^{10} CFU inoculated group were euthanized, and tissues [crop, liver, spleen, small intestine (near the cecum), cecum, and cloaca] were collected for *Salmonella* enumeration as previously described (16). At 14 dpi, the remaining turkeys (3–4) were euthanized and evaluated as described earlier.

Experiment 2

Thirty-nine 1-day-old tom (male) turkey poults were group housed for the trial. Fecal samples obtained from the shipping crate tested negative for *Salmonella* using qualitative bacteriology. On the day of arrival at NADC, 20 poults (index birds) were inoculated with 2×10^4 CFU SB 395 in 0.25 ml PBS by oral gavage and 19 poults (sentinel birds) received 0.25 ml PBS. At 8 days following MDR *S.* Heidelberg inoculation into the index birds, 10 turkeys from each group (index and sentinel) were euthanized,

and the cecum and spleen were collected for *Salmonella* enumeration. The remaining turkeys in both experimental groups were euthanized at 9 days following MDR *S.* Heidelberg inoculation of index birds and the tissues harvested for *Salmonella* enumeration.

RNA Isolation and Sequencing from Turkey Blood

Using the LeukoLOCK™ Fractionation & Stabilization Kit (ThermoFisher Scientific), blood was collected and fractionated from the wing vein of 3-week-old turkeys (from experiment 1) before inoculation as well as 2 dpi with 10^{10} CFU *S.* Heidelberg following NCAH SOP-ARU-0300. RNA from the leukocyte population (white blood cells) was extracted using the LeukoLOCK™ Total RNA Isolation System (ThermoFisher Scientific). RNA quality and quantity were analyzed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). Libraries were constructed using the Illumina TruSeq RNA Sample Prep Kit v2 and were sequenced on an Illumina HiSeq 2500 in a 100-cycle paired-end sequencing run (Illumina Inc., San Diego, CA, USA) at the Iowa State University DNA core facility. Sequence data were imported, quality trimmed in CLC Genomic workbench V 9.5.2, and mapped to the *Meleagris gallopavo* reference assembly 5.0 (17). Expression values were calculated only using uniquely mapped reads. Empirical analysis of differential gene expression was performed using the *EdgeR* statistical test, implemented in CLC Genomic workbench, on the raw unique reads (18). Gene expression differences greater than 1.5-fold with false discovery rate-adjusted *P*-values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Fecal Shedding, Tissue Colonization, and Transmission of an MDR *S.* Heidelberg Outbreak Strain in Turkeys

To evaluate the pathogenicity of an MDR *S.* Heidelberg outbreak strain in turkeys, 3-week-old turkey poults were inoculated with 10^8 or 10^{10} CFU and monitored for fecal shedding of *Salmonella* and changes in body (cloacal) temperature. Using Bonferroni's multiple comparison test, no significant difference in average body temperatures at 1, 2, or 3 dpi compared to pre-inoculation was observed at either inoculation dose (data not shown); thus, no fever was induced in the turkeys following *S.* Heidelberg challenge. Fecal shedding of *Salmonella* was detected out to 10 dpi for the 10^8 CFU inoculated turkeys and to 14 dpi for the 10^{10} CFU inoculated turkeys (**Figure 1**). During the first week post-inoculation, an ~1-log difference in *Salmonella* shedding between the 10^8 and 10^{10} CFU inoculated birds was measured, and an ~1-log reduction, regardless of inoculation dose, occurred each day in the turkeys for the first 3 days.

Tissue colonization of *S.* Heidelberg was determined at 7 and 14 dpi for the crop, liver, spleen, small intestine (near the cecum), cecum, and cloaca (**Figure 2**). At 7 dpi, *S.* Heidelberg was detected in the crop (10^8 dose; 2/4 birds), spleen (10^8 and 10^{10} doses; 1/4 and 4/4 birds, respectively), small intestine (10^{10} dose; 1/4 birds), cecum (10^8 and 10^{10} doses; 2/4 and 3/4 birds, respectively), and cloaca (10^8 and 10^{10} doses; 2/4 and 3/4 birds, respectively). At 14 dpi, *Salmonella* was only detected in the cecum (2/4 birds) and cloaca (1/4 birds) samples in the turkeys inoculated with *S.* Heidelberg at 10^8 CFU. *S.* Heidelberg was not detected in the liver at 7 dpi and was therefore not evaluated at 14 dpi.

In a separate study, 1-day-old poults ($n = 20$) were inoculated with 2×10^4 CFU of the MDR *S.* Heidelberg outbreak strain and group housed with mock-inoculated poults ($n = 19$). At 8 and 9 dpi, all poults were euthanized, and spleen and cecal colonization was determined. The spleens from five index and seven sentinel poults were positive for MDR *S.* Heidelberg whereas the cecum in all poults were colonized by *Salmonella* (**Figure 3**). For the index and sentinel poults, *Salmonella* levels were 5.6 and

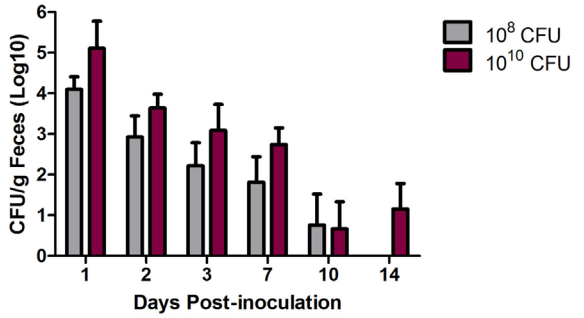

FIGURE 1 | Multidrug-resistant (MDR) *Salmonella enterica* serovar Heidelberg (*S.* Heidelberg) fecal shedding from turkeys. At 3 weeks of age, individually housed turkeys were inoculated with 10^8 ($n = 8$) or 10^{10} ($n = 7$) CFU of MDR *S.* Heidelberg. Feces was collected at the indicated time points, and quantitative and qualitative bacteriology was performed to determine fecal shedding of MDR *S.* Heidelberg. Four turkeys from each group were euthanized at day 7 dpi resulting in smaller groups for the remaining time points. Error bars indicate SEM.

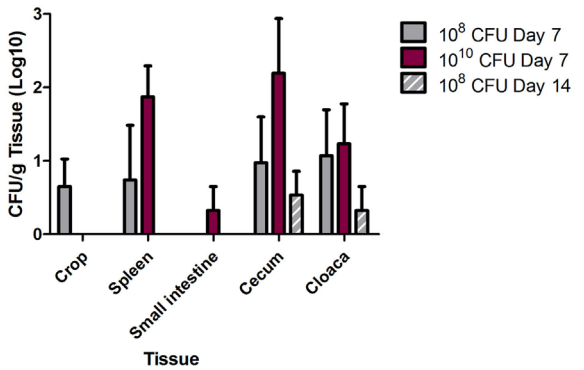

FIGURE 2 | Multidrug-resistant (MDR) *Salmonella enterica* serovar Heidelberg (*S.* Heidelberg) tissue colonization in turkeys. At 3 weeks of age, individually housed turkeys were inoculated with 10^8 ($n = 8$) or 10^{10} ($n = 7$) CFU of MDR *S.* Heidelberg. At day 7 following *Salmonella* inoculation, four turkeys from each group were euthanized, and the remaining turkeys were euthanized at 14 dpi. Tissues (crop, spleen, small intestine, cecum, and cloaca) were harvested following euthanasia for quantitative and qualitative bacteriology to determine tissue colonization by MDR *S.* Heidelberg. Error bars indicate SEM.

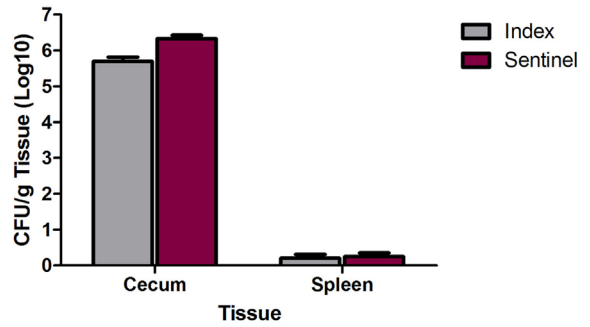

FIGURE 3 | Cecum and spleen colonization by multidrug-resistant (MDR) *Salmonella enterica* serovar Heidelberg (*S.* Heidelberg) in index and sentinel poults. At 1 day of age, 20 poults (index) were directly inoculated with 2×10^4 CFU of MDR *S.* Heidelberg and group housed with 19 poults (sentinel). At days 8 and 9 following index inoculation, 10 poults from each group were euthanized the first day, and the remaining birds were euthanized the second day to harvest tissues. Quantitative and qualitative bacteriology was performed on the cecum and spleen to determine MDR *S.* Heidelberg tissue colonization and *Salmonella* transmission from index to sentinel poults. Error bars indicate SEM.

6.3 Log10 CFU/g cecum tissue, respectively (**Figure 3**). Thus, exposure to MDR *S.* Heidelberg earlier in life (1-day-old poults) resulted in higher cecal colonization rates in poults compared to challenge at a later time in life (3 weeks, **Figure 2**), even with a lower dose of ~10^4 CFU (compared to the 10^{10} dose at 3 weeks of age). Interestingly, at 8/9 days following oral inoculation of the 1-day-old turkey poults with MDR *S.* Heidelberg, cecum colonization was increased ~2 Log10 CFUs compared to the inoculum dose regardless of whether birds were directly inoculated (index) or following transmission (sentinel) from inoculated poults. In our experience with challenging swine or turkeys with various *Salmonella* serovars, we typically observe a considerable decrease in *Salmonella* CFUs for fecal shedding and tissue colonization by 7 dpi compared to the initial inoculation dose, not an increase as measured in this study. However, our previous experiments were performed with pigs or turkeys that were 3 weeks of age or older. This suggests that the development and maturation of host factors such as immunity and/or the intestinal microbiota play an important role in limiting *Salmonella* colonization in older swine and poultry. Our results with MDR *S.* Heidelberg in turkeys are consistent with an experiment by Menconi et al. who demonstrated that day-of-hatch turkey poults inoculated with ~10^6 CFU *S.* Heidelberg were colonized with 7.04 and 6.05 Log10 CFU/g cecal contents at 24 and 72 h following inoculation, respectively, with *Salmonella* present in the cecal tonsils of all poults (20/20) at both time points (19). These results indicate that the level of *S.* Heidelberg in the cecal contents of poults at 72 h did not decrease from the inoculum level. The findings of Menconi et al. in turkeys were not replicated in two trials in which day-of-hatch broiler chicks were inoculated with either 10^5 or 10^6 CFU of serovar Heidelberg (19); at 72 h following inoculation of chicks with *Salmonella*, the cecal contents were colonized with either 1.08 or 2.96 Log10 CFU *S.* Heidelberg/g tissue. Thus, whereas in turkeys the inoculum dose and the level of cecal content colonization at 72 h were similar, in broiler chicks the colonization level decreased compared to the inoculation dose of *S.* Heidelberg. The authors specifically noted this difference indicating that turkey poults were more susceptible to serovar Heidelberg colonization compared to broiler chicks (19). *S.* Heidelberg colonization of the turkey cecal tonsils and cecal contents could be reduced by inoculating poults with a mixed culture of lactic acid bacteria (LAB) 1 h after *Salmonella* inoculation. The inoculation of poults with LAB reduced *S.* Heidelberg cecal content colonization by ~4 Log10 CFU/g content and cecal tonsil colonization by 55% at 72 h following *Salmonella* inoculation in comparison to inoculation with *S.* Heidelberg alone (19). This supports a role of the turkey intestinal microbiota in limiting colonization of *S.* Heidelberg either due to direct inoculation (e.g., probiotic administration) or maturation of the microbial community with age. The quantity of *S.* Heidelberg in the cecum or cecal tonsils in both our experiment and the trial by Menconi et al. indicates similar levels of colonization (10^6 CFU), potentially suggesting a threshold for niche colonization in the turkey cecum. Our results further extend the findings of Menconi et al. by demonstrating that in newly hatched turkey poults, *S.* Heidelberg can efficiently colonize the cecum through transmission of the pathogen within the flock. Efficient colonization of young turkey poults may contribute to lifelong colonization of turkeys with *S.* Heidelberg and a human foodborne risk for consumption of turkey meat.

Transcriptional Response of Commercial Turkeys to an MDR *S.* Heidelberg Outbreak Strain

Gene expression analysis of 3-week-old turkeys in response to an MDR *S.* Heidelberg outbreak strain was conducted by RNA-Seq using total RNA isolated from peripheral blood before and 2 days after inoculation (day 2/day 0). Eighteen genes were differentially expressed at 2 dpi compared to pre-inoculation (**Table 1**). The expression of three genes was significantly upregulated (*RUFY3*, *LOC104911311*, and *SERPINB10*). The gene ontology biological process annotation of RUFY3 suggests a role in positive regulation of cell migration; overexpression of RUFY3 caused the formation of F-actin-protrusive structures (invadopodia) and the induction of migration and invasion in human gastric cancer cell line SGC-7901 (20). The predicted gene description for LOC104911311 is "cytokine receptor common subunit beta-like." SERPINB10 (a.k.a. PI10, bomapin) is a member of the superfamily of serine proteinase inhibitors (serpins) that are key regulators in biological processes ranging from complement activation, coagulation, cellular differentiation, tumor suppression, apoptosis, and cell motility (21). A study by Schleef and Chuang described a role for PI10 in the inhibition of tumor necrosis factor alpha-induced cell death (22).

Fifteen genes were significantly downregulated in response to MDR *S.* Heidelberg challenge. A range of predicted functions for these genes includes ABC transporter (*ABCB10*), heme biosynthetic pathway (*UROD*, *UROS*), glutathione transferase (*GSTA3*), polyamine biosynthesis pathway (*ODC1*), calcium binding (*CETN3*), respiratory chain (*TTC19*), and voltage-gated potassium channel activity (*KCNAB1*). *LOC100547913* is predicted to encode aquaporin-3 (AQP3). Aquaporins are involved

TABLE 1 | Differentially expressed turkey genes in response to multidrug-resistant *Salmonella enterica* serovar Heidelberg (2 dpi/0 dpi).

Gene symbol	Gene description	Fold change	False discovery rate-adjusted *P*-value	Ensembl[a]	Source
RUFY3	RUN and FYVE domain containing 3	2.36	3.4957E−05	09878	HGNC Symbol; Acc:HGNC:30285
LOC104911311	Predicted: cytokine receptor common subunit beta-like	1.94	4.50681E−05		RefSeq: XM_019611421
SERPINB1	Serpin family B member 10	1.81	0.000464941	04373	HGNC Symbol; Acc:HGNC:8942
CETN3	Centrin 3	−1.73	−3.35822E−05		RefSeq: XM_010726066
LOC100545668	Aldose reductase-like	−1.76	−8.46444E−05	13513	RefSeq: XM_003202470
UROS	Uroporphyrinogen III synthase	−1.80	−4.95335E−05	11725	HGNC Symbol; Acc:HGNC:12592
TTC19	Tetratricopeptide repeat domain 19	−1.86	−3.24345E−05	06277	HGNC Symbol; Acc:HGNC:26006
ABCB10	ATP binding cassette subfamily B member 10	−1.88	−0.000176598		RefSeq: XM_010707019
GSTA3	Glutathione S-transferase Alpha 3	−1.94	−0.000161676	13935	UniProtKB/TrEMBL; Acc: D4N2R6
LOC100547913	Aquaporin-3	−2.04	−0.000245187	01744	RefSeq: XM_010725198
CMBL	Carboxymethylenebutenolidase homolog	−2.08	−6.24874E−05	06110	HGNC Symbol; Acc:HGNC:25090
FAM207A	Family with sequence similarity 207 member A	−2.09	−0.000101129		RefSeq: XM_010713129
UROD	Uroporphyrinogen decarboxylase	−2.25	−5.47793E−05	10326	HGNC Symbol; Acc:HGNC:12591
ODC1	Ornithine decarboxylase 1	−2.36	−0.000127647	14011	HGNC Symbol; Acc:HGNC:8109
DYX1C1	Dyslexia susceptibility 1 candidate 1	−4.39	−1.13695E−06	05949	HGNC Symbol; Acc:HGNC:21493
SCG3	Secretogranin III	−7.55	−2.57004E−05	06516	HGNC Symbol; Acc:HGNC:13707
KCNAB1	Potassium voltage-gated channel subfamily A member regulatory beta subunit 1	−9.43	−7.36884E−06	10715	HGNC Symbol; Acc:HGNC:6228
DAAM2	Disheveled associated activator of morphogenesis 2	−12.53	−6.76183E−07	10544	HGNC Symbol; Acc:HGNC:18143

[a]*The Ensembl number is proceeded by ENSSSCG000000.*

in transepithelial fluid transport and have been implicated in cell migration by a mechanism that facilitates water transport in lamellipodia of migrating cells (23). In this regard, AQP3 has been associated with macrophage immune function *via* a cellular mechanism involving water and glycerol transport that results in subsequent phagocytic and migration activity (24). AQP3$^{-/-}$ mice had an impaired mucosal innate immune response to *Citrobacter rodentium*, as demonstrated by reduced crypt hyperplasia, decreased epithelial expression of IL-6 and TNF-α, and diminished bacterial clearance (25). If LOC100547913 is AQP3, downregulation of the gene may contribute to the limited immune response observed in the turkeys. The gene with the greatest reduction in expression was *DAAM2*, predicted to encode a key effector in the canonical Wnt signal transduction pathway involved in gene expression regulation during embryonic development and regenerative myelination (26).

Similar to our results, a study of the chicken response to *S. enterica* serovar Enteritidis identified *SERPIN B* as upregulated and *AQP8* (an aquaporin) as downregulated (27). However, the number of differentially expressed genes in the MDR *S.* Heidelberg-challenged turkeys seemed minimal compared to other gene expression studies of poultry in response to *Salmonella* (28). Moreover, a recent study by our group profiling the transcriptome of 3-week-old commercial turkeys in response to *S.* Typhimurium challenge identified over 1,000 differentially regulated genes (manuscript in preparation). In a comparison of the gene expression changes in response to the two *Salmonella* serovars, 17 of the 18 genes differentially expressed in turkeys following *S.* Heidelberg challenge were similarly differentially expressed in response to *S.* Typhimurium challenge (data not shown). Taken together, this MDR *S.* Heidelberg outbreak strain appears capable of colonizing turkeys without inducing a strong host response (transcriptionally or clinically), conceivably due to the commensal-like state of this human foodborne pathogen in turkeys.

In summary, *S.* Heidelberg has been isolated from most food-producing animals, shown increased resistance to antimicrobial agents, and is among the top 5 serovars associated with human foodborne illness (29). Understanding the commensal state established by *S.* Heidelberg (and other *Salmonella* serovars) in livestock and poultry requires investigating the complex interactions of the virulence mechanisms of the particular *Salmonella* serovar and the host's response to not only initial colonization but also the subsequent establishment of a persistently colonized condition. Numerous host factors play a role in this response including animal genetics, age of exposure, health and immune status, farm husbandry practices, and the microbial composition of the gastrointestinal tract. Our colonization data provide insight into the ability of this serovar to effectively evade host response systems, because the 3-week-old turkeys appeared unaffected by the inoculation with 10 billion *Salmonella* Heidelberg, both clinically and transcriptionally. Furthermore, the efficient transmission, colonization, and proliferation of MDR *S.* Heidelberg from index to sentinel poults during the first week of life suggests that limiting the introduction of *Salmonella* into turkey flocks during the establishment of the intestinal microbiota is critical for control of this human foodborne pathogen. Efficient colonization of turkeys at a young age by serovar Heidelberg may help explain the prevalence of this serovar in human foodborne disease including outbreaks.

ETHICS STATEMENT

Procedures involving animals followed humane protocols as approved by the USDA, ARS, National Animal Disease Center Animal Care and Use Committee in strict accordance with the recommendations in the Guide for the Care, and Use of Laboratory

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AUTHOR CONTRIBUTIONS

BB and SB designed the experiments; BB, SB, and DS were involved in acquisition of the experimental data. BB, SB, TL, GC, and DS performed data analysis and interpretation. The manuscript was drafted and revised for important intellectual content by BB, SB, TL, GC, and DS, as well as, final approval of the version to be published with agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Frequency and Duration of Fecal Shedding of *Salmonella* Enteritidis by Experimentally Infected Laying Hens Housed in Enriched Colony Cages at Different Stocking Densities

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Human infections with *Salmonella* Enteritidis are often attributed to the consumption of contaminated eggs, so the prevalence of this pathogen in egg-laying poultry is an important public health risk factor. Numerous and complex environmental influences on *Salmonella* persistence and transmission are exerted by management practices and housing facilities used in commercial egg production. In recent years, the animal welfare implications of poultry housing systems have guided the development of alternatives to traditional cage-based housing, but their food safety consequences are not yet fully understood. The present study assessed the effects of different bird stocking densities on the frequency and duration of fecal shedding of *S*. Enteritidis in groups of experimentally infected laying hens housed in colony cages enriched with perching and nesting areas. In two trials, groups of laying hens were distributed at two stocking densities (648 and 973 cm^2/bird) into enriched colony cages and (along with a group housed in conventional cages at 648 cm^2/bird) orally inoculated with doses of 1.0×10^8 cfu of *S*. Enteritidis. At 10 weekly postinoculation intervals, samples of voided feces were collected from beneath each cage and cultured to detect *S*. Enteritidis. Fecal shedding of *S*. Enteritidis was detected for up to 10 weeks postinoculation by hens in all three housing treatment groups. The overall frequency of positive fecal cultures was significantly ($P < 0.05$) greater from conventional cages than from enriched colony cages (at the lower stocking density) for the total of all sampling dates (45.0 vs. 33.3%) and also for samples collected at 4–9 weeks postinfection. Likewise, the frequency of *S*. Enteritidis isolation from feces from conventional cages was significantly greater than from enriched colony cages (at the higher hen stocking density) for the sum of all samples (45.0 vs. 36.7%) and at 6 weeks postinoculation. Moreover, the frequency of *S*. Enteritidis fecal recovery from enriched colony cages at the higher hen stocking was significantly greater than from similar cages at the lower stocking density for all 10 sampling dates combined (39.4 vs. 33.3%). These results suggest that stocking density can affect *S*. Enteritidis intestinal colonization and fecal shedding in laying hens, but some other difference between conventional and enriched colony cage systems appears to exert an additional influence.

Keywords: *Salmonella* Enteritidis, laying hens, conventional cages, enriched colony cages, stocking density, fecal shedding

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INTRODUCTION

Although substantial government and agricultural industry resources have been invested in controlling food-borne diseases, the incidence of human *Salmonella* infections in the United States has not declined significantly over time (1, 2). Eggs contaminated by *Salmonella enterica* subspecies *enterica* serovar Enteritidis (*S*. Enteritidis) are internationally prominent sources of human illness (3, 4). Both active disease surveillance and retrospective epidemiologic analysis show an association between the frequency of human infections with this pathogen and its prevalence in commercial egg-laying poultry (5, 6). A survey of 24 European countries identified laying hens as the leading reservoir for human salmonellosis (especially due to *S*. Enteritidis), accounting for 42% of all cases (7). However, the sustained participation of egg producers in comprehensive *S*. Enteritidis flock testing and risk reduction programs (8) has recently been linked to decreased incidences of both egg contamination and human illness in several nations (9–11).

The edible interior contents of eggs (yolk or albumen) become contaminated with *S*. Enteritidis because this pathogen is able to colonize reproductive tissues (ovaries and oviducts) in infected hens (12, 13). Because salmonellae can be highly persistent in the environment of poultry houses, the opportunities for hens to be exposed and infected (and thus to lay contaminated eggs) can extend over a prolonged period of time (14, 15). Testing to detect *S*. Enteritidis in environmental samples from laying houses is often utilized as the initial screening step for identifying infected flocks (16, 17). Fecal shedding of S. Enteritidis by infected hens is a principal source of environmental contamination, often reaching peak levels just before egg production begins in commercial flocks and then declining steadily thereafter (18, 19). Experimental oral infection of chicks or hens with *S*. Enteritidis can cause intestinal colonization and associated bacterial shedding in feces for several months (20, 21).

The diverse available housing systems for commercial egg-laying hens have been extensively examined and evaluated in recent years in the contexts of their animal welfare and economic implications, but their public health consequences remain unresolved (22). Each of these housing options incorporates unique and complex facility characteristics and management practices, which might influence the persistence and transmission of *S*. Enteritidis infections in laying flocks. However, the published scientific literature does not provide any singular or definitive perspective about the food safety effects of poultry housing (23). Comparisons of conventional cage-based (battery) systems, cage-free systems, and intermediate alternatives such as enriched (furnished) colony cages or aviaries have yielded variable results, which do not document any consistent superiority of particular housing systems in the persistence of salmonellae in infected chickens or their housing environment (22). In a recent multi-institutional field study, the *Salmonella* prevalence in both environmental and eggshell samples was similar among several different hen housing systems, although unique inherent management challenges for sanitation and pathogen control were identified within each system (24). One characteristic parameter of poultry housing systems that might influence the introduction and perpetuation of *Salmonella* infections is the stocking density of hens (the amount of floor space available per bird). The objective of the present study was to determine the effects of two different bird stocking densities on the frequency and duration of fecal shedding of *S*. Enteritidis in groups of experimentally infected laying hens housed in colony cages enriched with perching and nesting areas.

MATERIALS AND METHODS

Experimental Housing of Laying Hens

In each of 2 similar trials, 142 laying hens were obtained from the specific pathogen-free flock of Single Comb White Leghorn chickens maintained at the U. S. National Poultry Research Center in Athens, GA, USA. These hens (31- and 41-week-old at the beginning of the first and second trials, respectively) were distributed into three separately housed groups in different rooms of a disease-containment facility (biosafety level 2) containing cage systems designed to simulate commercial conditions. In one room, 42 hens were housed in conventional laying cages (6 hens per cage), which provided 648 cm^2 of floor space per bird. Hens in the other two rooms were housed in enriched colony laying cages, each of which included access to two perches and a single enclosed nesting area. In one enriched colony room, 40 hens were housed (20 per cage) at a stocking density of 973 cm^2 of floor space per bird. In the other enriched colony room, 60 hens were housed (30 per cage) at a stocking density of 648 cm^2 of floor space per bird. All hens were provided with water (*via* two automatic nipple-type drinkers in each conventional cage and six in each enriched colony cage) and feed (a pelleted, antibiotic-free layer–breeder ration) *ad libitum*.

Experimental Infection of Laying Hens with *S*. Enteritidis

In each trial, all hens were orally inoculated with a measured dose of *S*. Enteritidis, consisting of a mixture of strains of phage types 8 and 13a. Each *S*. Enteritidis strain was resuscitated by transfer into tryptic soy broth (Acumedia, Neogen Corp., Lansing, MI, USA) for two successive cycles of 24-h incubation at 37°C. After cell numbers in each incubated culture were estimated by determining their optical density at 600 nm, equal numbers of the two inoculum component strains were combined, and further serial 10-fold dilutions in 0.85% saline produced a final cell concentration in each oral dose of approximately 1.4×10^8 cfu (confirmed by subsequent plate counts).

Fecal Samples

Immediately before inoculation and at 10 weekly postinoculation intervals, sterile cotton swabs were used to collect samples of voided feces from polystyrene trays (food-grade but not sterile), which had been placed under each cage 1 day earlier. A total of 36 samples per room were collected on each sampling date, evenly distributed among all occupied cages (6 samples per conventional cage and 18 samples per colony cage). Feces selected for sampling were visibly moist (recently voided) and dark in color (characteristic of cecal discharge). Each sample was collected into 10 ml

of buffered peptone water (Acumedia) and incubated for 24 h at 37°C. A 0.1-ml portion of each culture was then transferred into 10 ml of Rappaport-Vassiliadis broth (Acumedia) and incubated for 24 h at 41.5°C. A 10-µl portion from each of these broth cultures was then streaked onto brilliant green agar (Acumedia) supplemented with 0.02 mg/ml of novobiocin (Sigma Chemical Co., St. Louis, MO, USA) and incubated for 24 h at 37°C. The identity of presumptive colonies of *Salmonella* was confirmed biochemically and serologically (25).

Statistical Analysis

Within each trial, between the two trials, and for both trials combined, significant differences ($P < 0.05$) between housing systems, hen stocking densities, or sampling dates in the mean frequencies of *S.* Enteritidis isolation from voided fecal samples were determined by Fisher's exact test. Because the two replicate trials did not differ significantly in the frequency of *S.* Enteritidis recovery from fecal samples, their results were combined for analysis and presentation. Data were analyzed with InStat biostatistics software (GraphPad Software, San Diego, CA, USA).

RESULTS

None of the fecal samples collected before inoculation in either trial were positive for *Salmonella*. For both replicate trials combined, *S.* Enteritidis was recovered from 86.1% of fecal samples from hens in conventional cages, 95.8% of samples from hens in enriched colony cages at the higher stocking density, and 97.2% from hens in enriched colony cages at the lower stocking density at 1-week postinoculation (**Table 1**). The frequency of *S.* Enteritidis isolation from fecal samples collected in conventional cages declined significantly ($P = 0.0014$) to 59.7% at 3 weeks postinoculation, further to 40.3% at 5 weeks ($P = 0.0344$), and again to 19.4% by 9 weeks ($P = 0.0112$). In samples from enriched colony cages at the higher hen stocking density, *S.* Enteritidis recovery decreased significantly ($P < 0.0001$) to 62.5% at 3 weeks postinoculation, again to 27.8% at 5 weeks ($P < 0.0001$), and then to 12.5% by 8 weeks ($P = 0.0378$). For feces from enriched colony cages at the lower hen stocking density, the frequency of *S.* Enteritidis contamination dropped significantly ($P = 0.0014$) to 75.0% at 2 weeks postinoculation, again to 56.9% at 3 weeks ($P = 0.0439$), then to 36.1% at 4 weeks ($P = 0.0219$), to 18.1% at 5 weeks ($P = 0.0251$), and finally to 5.6% at 9 weeks ($P = 0.0370$). On the last sample collection date at 10 weeks postinoculation, *S.* Enteritidis was still found in 16.7% of fecal samples from hens in conventional cages, 8.3% of samples from hens in enriched colony cages at the higher stocking density, and 5.6% from hens in enriched colony cages at the lower stocking density.

For both trials combined, the frequency of positive results for *S.* Enteritidis recovery from fecal samples was significantly greater for conventional cages than for enriched colony cages at the lower stocking density at 4 weeks (59.7 vs. 36.1%; $P = 0.0089$), 5 weeks (40.3 vs. 18.1%; $P = 0.0061$), 6 weeks (43.1 vs. 16.7%; $P = 0.0011$), 7 weeks (31.9 vs. 15.3%; $P = 0.0313$), 8 weeks (23.6 vs. 6.9%; $P = 0.0099$), and 9 weeks postinoculation (19.4 vs. 5.6%; $P = 0.0217$), as well as for the overall total of all 10 sampling dates (45.0 vs. 33.3%, $P < 0.0001$). The frequency of *S.* Enteritidis isolation from feces collected in conventional cages was significantly greater than from enriched colony cages at the higher hen stocking density at 6 weeks postinoculation (43.1 vs. 23.6%; $P = 0.0228$) and for the sum of all sampling dates (45.0 vs. 39.4%, $P = 0.0219$). The frequency of *S.* Enteritidis fecal recovery from enriched colony cages at the higher hen stocking was significantly greater than from enriched cages at the lower stocking density for the total of all 10 sampling dates (39.4 vs. 33.3%, $P = 0.0185$).

DISCUSSION

Fecal shedding is a consequence of *Salmonella* adherence to avian intestinal cells (26). Intestinal colonization by salmonellae typically declines steadily during the initial weeks after experimental infection of mature hens (21, 27), although highly persistent

TABLE 1 | Recovery of *Salmonella* Enteritidis[1] from voided fecal samples of experimentally infected laying hens in different housing systems and stocking densities.[2]

Weeks postinoculation	Conventional cages (high stocking density)	Enriched colony cages (high stocking density)	Enriched colony cages (low stocking density)
	S. Enteritidis-positive/total (%)		
1	62/72 (86.1)^{a,A}	69/72 (95.8)^{a,A}	70/72 (97.2)^{a,A}
2	54/72 (75.0)^{a,A,B}	62/72 (86.1)^{a,A}	54/72 (75.0)^{a,B}
3	43/72 (59.7)^{a,B}	45/72 (62.5)^{a,B}	41/72 (56.9)^{a,C}
4	43/72 (59.7)^{a,B}	34/72 (47.2)^{a,b,B}	26/72 (36.1)^{b,D}
5	29/72 (40.3)^{a,C}	20/72 (27.8)^{a,b,C}	13/72 (18.1)^{b,E}
6	31/72 (43.1)^{a,C,D}	17/72 (23.6)^{b,C,D}	12/72 (16.7)^{b,E,F}
7	23/72 (31.9)^{a,C,D}	16/72 (22.2)^{a,b,C,D,E}	11/72 (15.3)^{b,E,F}
8	17/72 (23.6)^{a,C,D}	9/72 (12.5)^{a,b,D,E}	5/72 (6.9)^{b,E,F}
9	14/72 (19.4)^{a,D}	6/72 (8.3)^{a,b,E}	4/72 (5.6)^{b,F}
10	12/72 (16.7)^{a,D}	6/72 (8.3)^{a,E}	4/72 (5.6)^{a,F}
All	328/720 (45.0)^{a}	284/720 (39.4)^{b}	240/720 (33.3)^{c}

[1]After oral inoculation of all hens with approximately 10^8 cfu of an equal mixture of phage type 8 and 13a strains of S. Enteritidis.

[2]High stocking density = 648 cm^2 of floor space per hen; low stocking density = 973 cm^2 of floor space per hen.

[a,b]Values in rows that share no common lower-case superscripts are significantly ($P < 0.05$) different.

[A,B,C,D,E,F]Values in columns that share no common upper-case superscripts are significantly ($P < 0.05$) different.

colonization has also been observed (18, 21). Following experimental oral infection with large doses of *S*. Enteritidis, a small percentage of hens housed in either conventional or enriched colony cages in the present study continued shedding the pathogen in their feces for at least 10 weeks. These results correspond with those of a prior study, which reported that inoculation with $\geq 10^6$ cfu of *Salmonella* led to fecal shedding for at least 8 weeks (21). Such prolonged shedding could extensively contaminate the housing environment and perpetuate opportunities for infection to spread. In some egg collection systems, contaminated feces can also introduce salmonellae onto egg shells. Nevertheless, interpolation from experimental infection data to predict housing and management influences on *Salmonella* shedding in commercial poultry must also account for some distinguishing characteristics of naturally occurring infections. The observed prevalence of *Salmonella* fecal shedding in commercial laying flocks sometimes fluctuates over time (28, 29). Both the frequency and duration of fecal shedding by orally infected hens are directly related to the *S*. Enteritidis exposure dose (21, 30, 31). Commercial laying hens are likely exposed to relatively low doses of salmonellae from environmental sources or *via* horizontal contact transmission, generally resulting in infrequent infection and egg contamination (9, 32).

Persistent environmental contamination in commercial poultry facilities serves as a potential reservoir for the infection of successive laying flocks with *S*. Enteritidis (8, 33, 34). Feces and dust, which are widely distributed throughout laying houses, sometimes remain contaminated with *S*. Enteritidis for many months (35). The ability of *S*. Enteritidis isolates to survive adverse environmental conditions may also correlate with their pathogenicity for chickens (36). High populations of rodent or insect vectors can sustain or amplify *Salmonella* levels in poultry flocks (37). The prevalence of *Salmonella* in laying house environments has been linked to several management-associated risk factors, including larger flock size, greater flock age, housing in older facilities, access to outdoor areas, and multiple-age stocking (38–41). Once introduced from environmental sources, *Salmonella* infection can rapidly and extensively spread within flocks (42). The susceptibility of chickens to horizontal transmission of *S*. Enteritidis can be increased by stressors such as feed deprivation, water deprivation, or exposure to extreme environmental temperatures (43–45).

Numerous and complex environmental influences on *Salmonella* persistence and transmission are exerted by management practices and housing facilities used in commercial egg production (46). However, prior investigations of the food safety consequences of poultry housing systems have yielded diverse and sometimes contradictory results (22, 23). For example, conventional cage-based housing systems for egg-laying flocks have sometimes been associated with higher frequencies of *Salmonella* infection or environmental contamination, especially when rodent population levels are elevated (41, 47, 48). Alternatively, other researchers have linked cage-free housing systems to higher *Salmonella* prevalence in egg shell and environmental samples and with greater horizontal dissemination of infection within laying flocks (49–51). Additionally, some studies have found no significant differences in the frequencies of either *Salmonella* infection or environmental contamination between cage and cage-free systems (52, 53) or between conventional and enriched colony cage systems (54, 55). Recently, a large field survey conducted under commercial egg production conditions found similar overall *Salmonella* prevalence in both egg shell and environmental samples from hens in conventional cage, enriched colony cage, and aviary housing systems, although salmonellae were isolated significantly more often from hens in conventional cages than from the other systems when internal organs were sampled after flock depopulation (24, 48). Unique *Salmonella* reservoirs and risk factors, attributable to the distinctive facility design features and management practices that are characteristic of individual poultry housing systems, may require correspondingly specific *Salmonella* risk reduction strategies for each system (24, 56).

In a series of previous experimental infection studies, *S*. Enteritidis was isolated significantly more often from internal organs and voided feces from hens in conventional cages than from hens in enriched colony cages, although no corresponding differences were reported for either horizontal transmission of infection or egg contamination (42, 57–59). Because the two housing systems in these trials differed in the amount of floor space provided per hen, a subsequent experiment (60) compared the effects of two stocking densities on the consequences of *S*. Enteritidis infection of hens in enriched colony cages. In this latter study, *S*. Enteritidis was found at higher frequencies in livers and ovaries of hens housed in enriched colony cages at a higher stocking density than at a lower one, but *S*. Enteritidis was also recovered at a higher frequency from spleens of hens in conventional cages than from enriched colony cages when both groups were housed at the higher density. In the present investigation, housing infected hens in enriched colony cages at a higher stocking density was associated with more frequent fecal shedding of *S*. Enteritidis than was detected at a lower stocking density, but hens in conventional cages (at the higher stocking density) shed the pathogen in their feces at a significantly higher overall frequency than either enriched colony cage group. These results suggest that the susceptibility of hens to intestinal colonization by *S*. Enteritidis can be influenced by stocking density, although some other characteristic of conventional cage housing appears to exert an additional effect.

Possible explanations for stocking density effects on the susceptibility of laying hens to *S*. Enteritidis infection include diminished immune responses or increased opportunities for horizontal contact exposure to the pathogen. Housing chickens in crowded and unsanitary conditions was previously reported to decrease their resistance to *S*. Enteritidis infection (61). Stress caused by high stocking densities has been found to suppress both humoral and cellular immunity, thereby facilitating increased invasion of internal organs by *S*. Enteritidis (62). Any disruptions of the complex regulatory circuitry, which coordinates immune responses, could reduce the effectiveness of host defenses against infection (63, 64). Stronger antibody and cellular immune responses were mounted by laying hens housed in enriched colony cages than by hens in conventional cages when subjected to social stress (65). Differences between housing systems and stocking densities in the frequency of colonization of the spleen (an important secondary

lymphatic organ) in experimentally infected hens are consistent with a stress-mediated explanation (57, 60). Stress-related impairment of mucosal IgA secretion or other lymphocyte functions in intestinal lymphoid tissues could compromise effective clearance of *Salmonella* colonization (66, 67).

The carefully controlled conditions under which experimental infection studies are conducted are useful for evaluating the effects of narrowly defined treatments, but they cannot account for all of the complex management and environmental influences, which affect commercial egg production flocks and facilities. Accordingly, a comprehensive understanding of the public health impacts of different laying hen housing options can only be assembled by integrating experimentally derived data about housing system effects on hens' susceptibility to *Salmonella* infection with applicable field data regarding the introduction, transmission, and persistence of this pathogen in commercial hens and their environment. Currently available information, encompassing both these research approaches, does not document any consistent overall pattern of significant differences between housing systems in their food safety consequences but instead suggests that effective control of egg-transmitted salmonellae may best be attained by addressing the specific risk factors inherent to each system.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Institutional Animal Care and Use Committee and Institutional Biosafety Committee of the U. S. National Poultry Research Center and U.S. Department of Agriculture, Agricultural Research Service.

AUTHOR CONTRIBUTIONS

RKG was lead investigator and principal author; RG, DJ, KA, and DK were collaborators and coauthors.

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frontiers in Veterinary Science

REVIEW
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Examining the Link between Biofilm Formation and the Ability of Pathogenic *Salmonella* Strains to Colonize Multiple Host Species

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Salmonella are important pathogens worldwide and a predominant number of human infections are zoonotic in nature. The ability of strains to form biofilms, which is a multicellular behavior characterized by the aggregation of cells, is predicted to be a conserved strategy for increased persistence and survival. It may also contribute to the increasing number of infections caused by ingestion of contaminated fruits and vegetables. There is a correlation between biofilm formation and the ability of strains to colonize and replicate within the intestines of multiple host species. These strains predominantly cause localized gastroenteritis infections in humans. In contrast, there are salmonellae that cause systemic, disseminated infections in a select few host species; these "invasive" strains have a narrowed host range, and most are unable to form biofilms. This includes host-restricted *Salmonella* serovar Typhi, which are only able to infect humans, and atypical gastroenteritis strains associated with the opportunistic infection of immunocompromised patients. From the perspective of transmission, biofilm formation is advantageous for ensuring pathogen survival in the environment. However, from an infection point of view, biofilm formation may be an anti-virulence trait. We do not know if the capacity to form biofilms prevents a strain from accessing the systemic compartments within the host or if loss of the biofilm phenotype reflects a change in a strain's interaction with the host. In this review, we examine the connections between biofilm formation, *Salmonella* disease states, degrees of host adaptation, and how this might relate to different transmission patterns. A better understanding of the dynamic lifecycle of *Salmonella* will allow us to reduce the burden of livestock and human infections caused by these important pathogens.

Keywords: *Salmonella*, biofilms, curli, cellulose, gastroenteritis, host adaptation

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INTRODUCTION

Salmonella Nomenclature, Disease States, and Worldwide Impact of Infections

The current system of *Salmonella* nomenclature is based on layers of genetic, biochemical, and serological classification. New *Salmonella* strains can be categorized into species and subspecies according to DNA relatedness at the genomic level, originally shown through DNA–DNA hybridization, and the presence or absence of 11 biochemical traits (1, 2). Two species have been

defined, *Salmonella bongori* and *Salmonella enterica*, of which *S. enterica* is further split into six subspecies that are designated by a Roman numeral and name (I, *enterica*; II, *salamae*; IIIa, *arizonae*; IIIb, *diarizonae*; IV, *houtenae*; and VI, *indica*) (**Figure 1**) (3). *Salmonella* are further subdivided into serovars using the classical Kauffman and White classification system (4). Serovars are representative of a unique combination of flagellar antigens (H1 and H2) and (lipopolysaccharide) oligosaccharide (O) or capsular polysaccharide (K) antigens (5). Using this classical system, more than 2,600 serovars have been identified, with their given name reflecting their combination of antigens, or in the case of serovars in subspecies *enterica*, a name representing their associated disease, host specificity, geographic origin, or relationship to other identified serovars (5, 6). Whole genome sequencing (WGS) is increasingly being used for classification, such as the sequencing of every *Salmonella* isolate by Public Health England (7) or the new typing scheme developed for *S. enterica* serovar Typhi (8). Strain typing *via* WGS has so far demonstrated promising results, both by supporting the current structure of *Salmonella* serovar nomenclature and by providing improved resolution of the phylogenetic relationship between *Salmonella* isolates (7). In regards to infection, subspecies I, or *S. enterica* subspecies *enterica* is the most well-represented among serovars and disease, accounting for approximately 60% of all serovars identified and greater than 95% of *Salmonella* isolates obtained from humans and domestic mammals (9, 10). In contrast, *Salmonella* isolates belonging to the remaining species and subspecies are normally obtained from cold-blooded hosts, and are only occasionally able to cause infections in humans (11).

Pathogenic *Salmonella* strains cause three main types of infections in humans. Gastroenteritis (150 million annual cases) is caused by many of the serovars in subspecies *enterica*, with serovars Typhimurium and Enteritidis being the most common (12, 13). In immunocompetent individuals, gastroenteritis infections involve the short-term colonization of the pathogen within the gastrointestinal (GI) tract, resulting in a localized inflammatory immune response accompanied by profuse diarrhea (14). The *Salmonella* serovars causing gastroenteritis are collectively referred to as non-typhoidal *Salmonella* (NTS). Enteric or typhoid fever (26.9 million annual cases) is caused by *S. enterica* serovars Typhi and Paratyphi, which are known as typhoidal *Salmonella* (15). This distinct disease involves invasion of the extra-intestinal compartment, often without the induction of inflammation or diarrhea (16). While such infections can occur asymptomatically, clinical manifestations of typhoidal *Salmonella* infections may include a persistent and gradual fever that elevates in a stepwise manner, as well as other symptoms, such as headache, chills, nausea, coughing, malaise, or a rapid pulse (16). The yearly mortality attributed to typhoidal *Salmonella* is estimated at ~145,000 deaths, which is more than double the number of deaths associated with gastroenteritis (15). After spreading systemically in their host, typhoidal *Salmonella* have the potential to persist for several weeks to years as a result of the pathogen's intracellular association with monocytes and macrophages and potential long-term colonization of the gall bladder (17). The third human disease is caused by a group of NTS strains that cause systemic infections and have an increased association with bloodstream infections in sub-Saharan Africa (18). Like typhoidal *Salmonella* infections, invasive non-typhoidal *Salmonella* (iNTS) disease frequently lacks diarrheal symptoms, with febrile illness being the dominant clinical presentation in 95% of cases (19). This disease has a huge burden of mortality in the hardest hit areas,

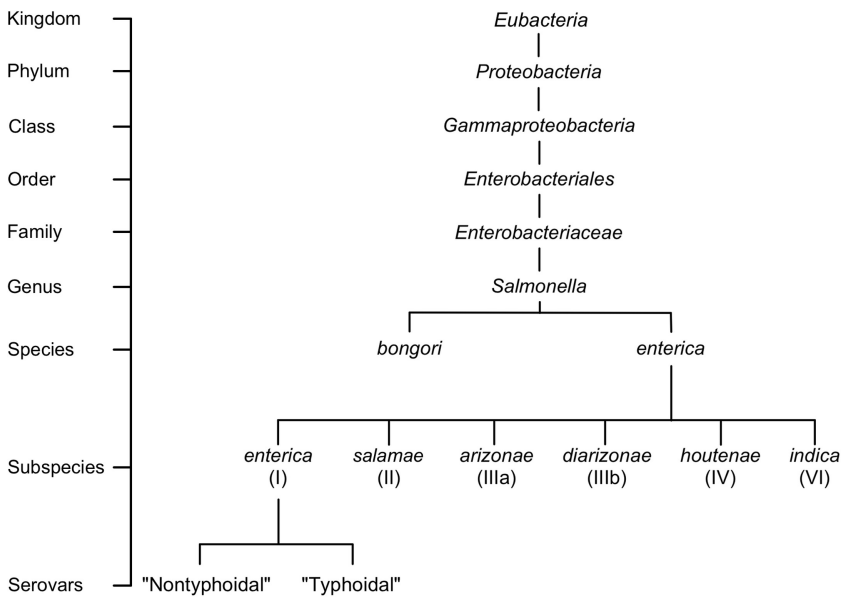

FIGURE 1 | *Salmonella* taxonomy and general classifications. The genus *Salmonella* is classified into species, subspecies, and serovars based on the White–Kauffman–Le Minor scheme. Serovars are often grouped into non-typhoidal or typhoidal categories; however, this referencing approach is not a part of the official *Salmonella* classification scheme. For a expanded version of the taxonomical distribution of *Salmonella*, readers are referred to Ref. (9).

with an estimated 681,000 deaths per year. Due to non-specific symptomology and multidrug resistance of iNTS strains, there are often poor clinical outcomes despite correct diagnosis (18). A review of several clinical studies has revealed a significant association between invasive infections with NTS and immunocompromised populations, particularly children with malnutrition or severe malaria and adults with advanced infections of human immunodeficiency virus (HIV) (18–20). Failure of the immune system to maintain the intestinal epithelial barrier or to control intracellular *Salmonella* infections in these individuals provides a unique opportunity for NTS serovars to persist in the host (19).

Salmonella species continue to have a significant impact on global health. In a recent study analyzing the impact of 22 of the world's most important foodborne pathogens, non-typhoidal, and typhoidal *Salmonella* were listed #1 and #2 in terms of disability adjusted life years (DALY) (15). The DALY metric is a measure of the overall disease burden, or the number of years lost due to ill-health, disability or early death. However, it does not take into account the economic impact of disease. In the United States of America alone, the impact of *Salmonella* infections related to hospital time, treatment costs, and lost work productivity have been estimated in the billions of dollars each year (21, 22). In addition to these estimates, *Salmonella* also presents as a significant economic challenge for producer groups and governments that are tasked with screening for and eliminating these pathogens within livestock species. Taken together, the different measures of impact demonstrate why it is so important to understand the complete *Salmonella* life-cycle, including how cells survive, persist and are transmitted between hosts.

BIOFILM FORMATION IN *Salmonella*

The majority of bacterial life in nature is thought to exist in biofilms, a mode of growth where cells aggregate and become embedded in a self-produced extracellular matrix, usually in contact with a physical surface. Up to 40% of human and livestock diseases are thought to be biofilm-related and have enormous medical and economic impacts (23, 24). In addition, most of these biofilms are polymicrobial in nature (25). The exact reasons why bacteria aggregate together are not fully understood. There are examples of emergent behaviors associated with aggregation of larger numbers of cells, such as the enhanced breakdown of chitin by *Vibrio cholerae* (26). There is also the possibility that polymer production within the biofilm is the result of cells competing with each other for access to oxygen or nutrients (27). The presence of extracellular polymers themselves can create a unique microenvironment for cells within a biofilm, by inducing potential oxygen gradients (28, 29), or signaling nutrient limitation (30). An example of this was demonstrated for *Pseudomonas aeruginosa*, where the presence of DNA in the extracellular matrix imposed a cation restriction on the cells inside the biofilm, which in turn led to increased antibiotic resistance in the biofilm cells (31). Thus, the characteristic properties of a biofilm may be due in part to the physical barriers provided by the matrix polymers and also to the microenvironments induced by growth at high cell densities within the matrix.

General Description of *Salmonella* Biofilm Types

For *Salmonella*, the best studied biofilm phenotype has been termed the rdar morphotype, named for the *r*ed, *d*ry, *a*nd rough appearance of colonies grown on agar plates containing Congo red dye (32, 33). Congo red accumulates within the rdar colony due to the presence of the proteinaceous curli fimbriae, which are functional amyloid structures that are resistant to detergents, pH, and proteases (32, 34), and cellulose, the β1-4-linked glucose polymer, which is another resistant polymer (35). These two components function as the extracellular matrix scaffold, with curli providing short-range interactions between cells and cellulose providing long-range interactions over the distance of the entire colony (36). Together their production leads to a rough and dry colony appearance (37) (**Figure 2A**). There are other polymers known to be present within the rdar extracellular matrix, such as the O-antigen capsule (38) and polysaccharides yet to be fully characterized (39), as well as proteins, such as flagella, that contribute to the architecture of the resulting colony (40). In standing liquid cultures, biofilms with a matrix comprised of curli and cellulose have been described as pellicles, which refer to the film of cell growth that appears at the air–liquid interface (33, 41, 42) (**Figure 2B**). BapA, a large *Salmonella* protein containing numerous repeated sequences, has been shown to contribute to the strength and integrity of these pellicles (43). There are also biofilms formed at the air–liquid interface in severely nutrient-limited liquid media that are composed of cellulose, but not curli (42, 44). We recently developed an *in vitro* flask model for studying *Salmonella* biofilm development, where the cells in the culture differentiate into two distinct populations: multicellular aggregates and planktonic cells. The multicellular aggregates produce the same polymers as standard biofilms (45) and accumulate in the bottom of the flask, whereas the planktonic cells remain suspended in the growth media (**Figure 2C**). The proportion of each cell type within replicate flask populations is relatively stable (**Figure 2D**).

Each of the biofilms described above, except for cellulose-dominated biofilms formed on glass, are related in regulatory mechanisms and are activated in a similar way (**Figure 3A**). Regulation feeds through CsgD, a transcriptional regulatory protein that activates the biosynthesis of the majority of biofilm polymers described above (46, 47). The favored growth conditions for biofilm formation are media of low osmolarity, at temperatures below 30°C, and in the presence of gluconeogenic substrates, such as amino acids (33). Nutrient limitation is known to activate polymer production, but there are many inputs into the *csgD* promoter, which is part of one of the most complex regulatory networks in *Salmonella* [see Ref. (47) or (48) for a comprehensive review]. Important biofilm-activating factors include microaerophilic oxygen levels (49), iron limitation (33) and the presence of bis-(3′–5′)-cyclic dimeric guanosine monophosphate, or cyclic-di-GMP (50). Cyclic-di-GMP is a bacteria-specific secondary messenger molecule that is known

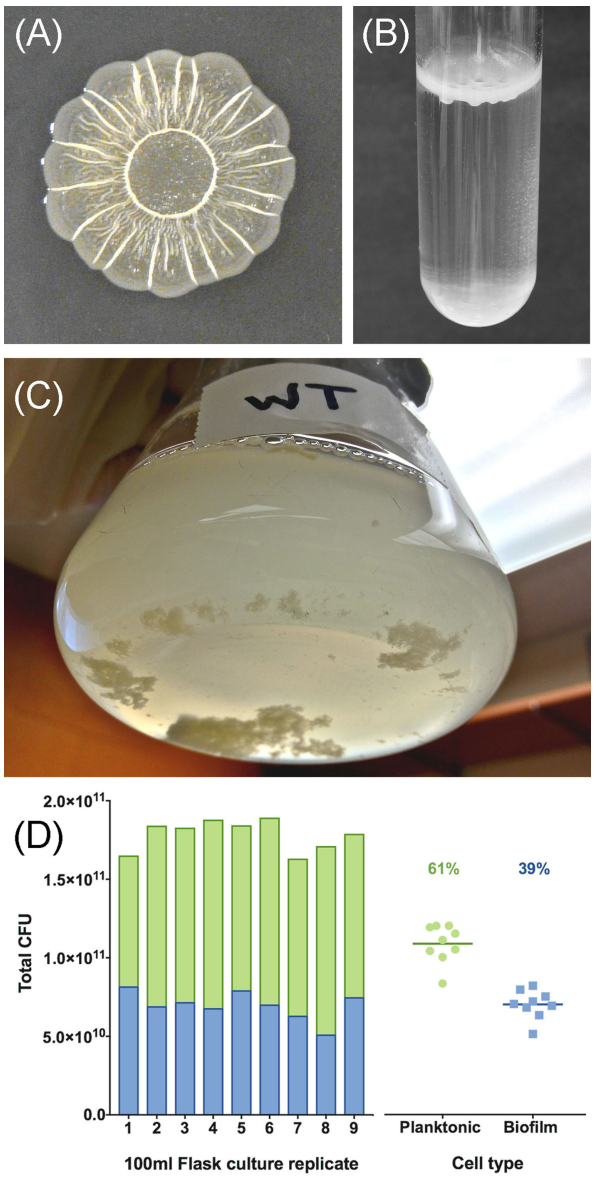

FIGURE 2 | Examples of *Salmonella* biofilm formation. **(A)** Colonies grown for 48 h at 28°C on solid 1% tryptone media form the characteristic surface patterns of the *r*ed, *d*ry, *a*nd *r*ough (rdar) morphotype. The colony appears red when the media is supplemented with the dye Congo red. **(B)** Pellicle formation at the air–liquid interface of a 1% tryptone liquid culture [adapted from Ref. (46)]. **(C)** *Salmonella* form multicellular aggregates and planktonic cells within the bulk liquid phase of a flask culture. **(D)** The number of colony forming units (CFU) present in aggregate or planktonic cell subpopulations from **(C)** was calculated using conversion factors determined from serial dilution plating after homogenization (1.92×10^9 CFU per 1.0 OD_{600} for planktonic cells; 1.73×10^8 CFU/mg for aggregates). The green bars and blue bars represent the proportion of planktonic cells and aggregates comprising the total number of cells in the population; points on the right side of the graph represent total CFU values for each cell type from nine replicate flask cultures. The percentage values represent the average proportion of each cell type.

to activate biofilm formation when produced at high levels, almost universally in all bacterial species where it has been examined [see Ref. (51) for a review]. In *Salmonella*, there are 22 enzymes that can potentially regulate c-di-GMP levels, but only a subset affect *csgD* expression directly (44, 52). Finally, the presence of glucose has been shown to repress biofilm formation and *csgD* transcription (53, 54). This indicates that biofilm formation is a choice heavily influenced by the growth potential in the environment surrounding the cells.

S. enterica serovar Typhi has been shown to form unique biofilms on the surface of gallstones (55, 56). These biofilm-coated gallstones have been observed in association with chronic human Typhi carriers (57) and the presence of bile is a key inducing factor (56). Curli fimbriae are not involved in the formation of these biofilms, although the O-antigen capsule and flagella have been implicated (58, 59). These biofilms are distinct from the rdar morphotype and are specific to serovar Typhi; they are likely to have a unique function in the *Salmonella* lifecycle and as such, are not discussed extensively in this review.

Biofilm as a Survival Advantage

In the early days of genome-wide comparisons in *Salmonella*, researchers had identified multiple different fimbrial types, each with scattered distribution between the different *S. enterica* serovars (60). For the current picture of fimbrial distribution, see Ref. (61, 62). Due to pioneering work with Type I and P fimbriae in *E. coli* (63, 64), it was predicted that the presence of different fimbrial types would allow *Salmonella* cells to attach to the intestinal epithelium of specific host species (65). One example was the association of SEF14 fimbriae with poultry-associated *S. enterica* serovars (60, 66). Curli fimbriae were unique in that they were conserved in the *Salmonella* genus (60); the *csgA* gene coding for the major curli subunit (formerly *agfA*) was developed as an early *Salmonella* diagnostic, detectable in 603 of 604 tested strains (67). Curli fimbriae and the corresponding biosynthetic operons were also detected in *E. coli* (60, 68, 69), which had a last common ancestor with *Salmonella* approximately 100 million years ago (70). Hammar et al. identified the presence of two polycistronic *csg* operons responsible for curli biosynthesis in *E. coli* (71), which were later identified in *Salmonella* (72) and subsequently shown to be interchangeable cross-species (69). These findings brought up interesting questions about curli fimbriae and why they would be conserved in *Salmonella* strains that were capable of colonizing so many different host species. It indicated that curli fimbriae were involved in a common aspect of the *Salmonella* lifecycle that was shared by many diverse strains.

One clue about curli function came as a result of the unusual protocol for curli purification. After the majority of *S. enterica* serovar Enteritidis cellular material was solubilized or had been removed by enzymatic digestion, purified curli were isolated from the top of a preparative SDS-PAGE, as the material that did not enter the gel (32). Purified curli fibers remained intact after boiling in the presence of SDS, exposure to sodium hydroxide, or digestion with proteinase K, treatments that would depolymerize or degrade most if not all other fimbrial types (32). Resuspension in >70% formic acid was the only treatment found to depolymerize the curli fibers into their structural subunits (32, 73); it should be noted that no improved alternative to this procedure has been published in the last 25 years. We know now that curli fimbriae are a functional amyloid with extensive cross-β structure (74, 75),

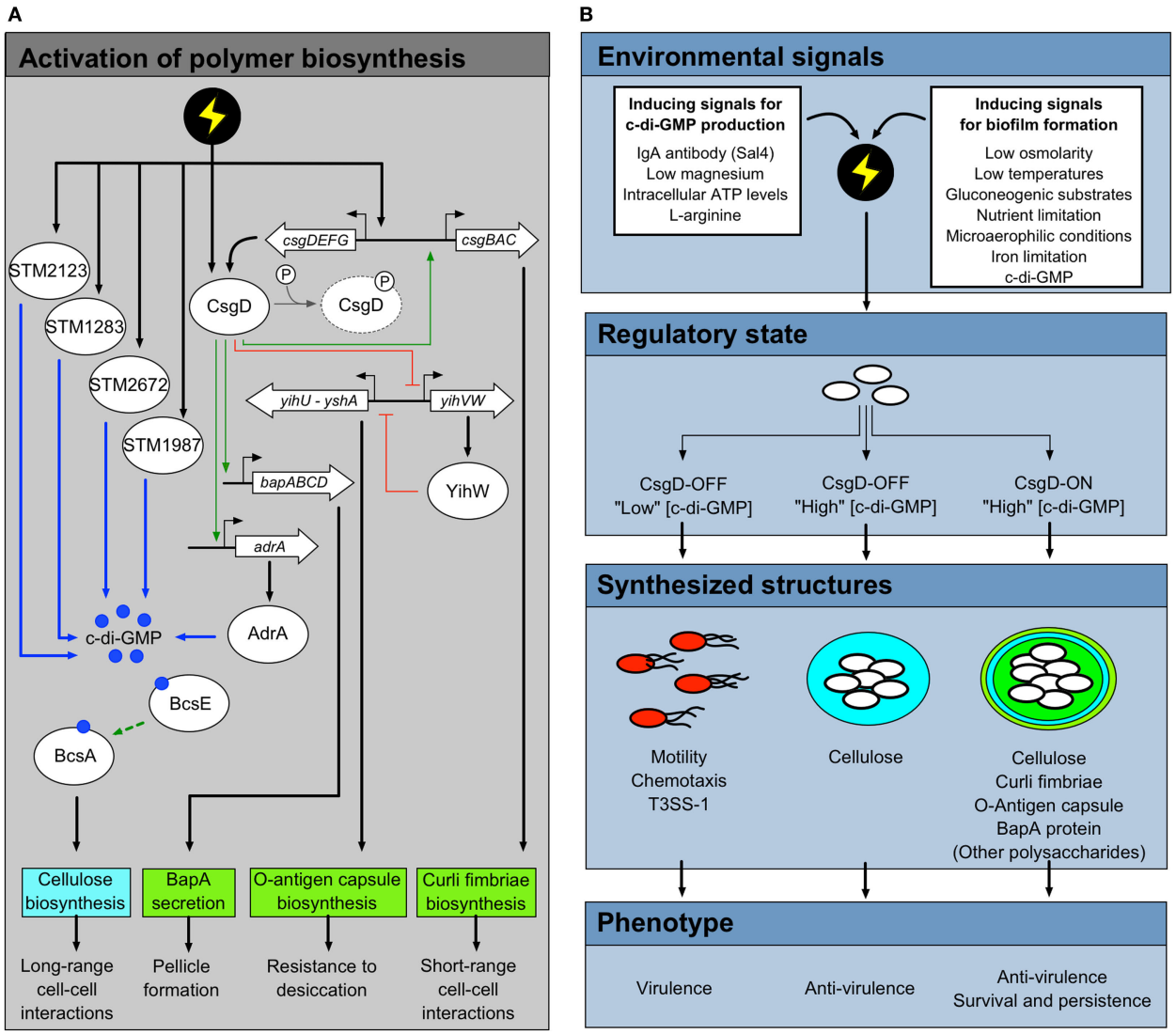

FIGURE 3 | *Salmonella* phenotypes that result from the activity of CsgD and c-di-GMP. **(A)** Synthesis of biofilm-associated polymers is regulated at the genetic level by CsgD and the secondary messenger molecule c-di-GMP. Multiple environmental conditions act as inducing signals for *csgD* expression, CsgD synthesis, and c-di-GMP production. These environmental conditions (represented here as a lightning bolt) are transduced into intracellular signals *via* outer membrane proteins, two-component signal transduction systems, regulatory proteins, and enzymes associated with c-di-GMP production. A select set of diguanylate cyclases (STM1283, STM2123, STM2672, and STM1987) can contribute to the c-di-GMP pool that induces BcsE and BcsA activity, resulting in cellulose biosynthesis. CsgD is the master transcriptional regulator associated with *Salmonella* biofilm formation. In its unphosphorylated active state, CsgD promotes the expression of *adrA*, a potent diguanylate cyclase associated with promoting cellulose biosynthesis. CsgD is additionally responsible for activating the transcription of genes and operons associated with the biosynthesis of curli fimbriae, O-antigen capsule, and BapA protein. Genes and operons are shown as open arrows, proteins as ovals, and c-di-GMP molecules as dark blue circles. Positive regulation is denoted as green arrows, while regulatory inhibition is shown as flat-headed red arrows. Activation *via* c-di-GMP molecules is shown as blue arrows. **(B)** Multiple environmental signals can induce the biosynthesis of biofilm polymers. However, some conditions can activate c-di-GMP production and cellulose biosynthesis independently from other biofilm polymers. Under biofilm-inducing conditions, only a subset of *Salmonella* cells in the total population will synthesize high levels of CsgD. This subpopulation enters a CsgD-ON state, which results in significant c-di-GMP production and biosynthesis of biofilm matrix polymers. Cells within the biofilm are able to survive and persist in harsh environmental conditions. In contrast, some *Salmonella* cells in the population do not have sufficient synthesis of CsgD, resulting in a CsgD-OFF state and subsequently low intracellular concentrations of c-di-GMP. These cells remain in a planktonic state, are highly motile, and synthesize the type-three secretion system (T3SS)-1, resulting in a virulent cell subpopulation. Due to the CsgD-independent activity of some diguanylate cyclases, *Salmonella* cells can have high intracellular levels of c-di-GMP while in a CsgD-OFF state. As such, these cells may synthesize cellulose in the absence of other major biofilm matrix polymers. The relatively low expression/activity of virulence-associated factors in cells aggregated together within cellulose or other biofilm polymers is due at least in part to the state of c-di-GMP pools within the cells.

hence their extreme stability. It was apparent early on that curli fimbriae could provide physical stability and possible resistance for aggregated *Salmonella* cells.

Another indication of the potential function of curli fibers came from the optimal growth conditions for their production *in vitro*. Curli fimbriae were originally discovered within a

strain of *E. coli* isolated from cattle manure (76). The fimbriae originally discovered in *S. enterica* serovar Enteritidis by Collinson et al. were thought to be distinct from curli fimbriae in *E. coli* due to differences in amino acid residues and due to constitutive expression in serovar Enteritidis at 37°C and 28°C (32). However, the amino acid differences were based on the false identification of the curli subunit by Olsén et al. (76), who had in fact identified Crl, a biofilm transcriptional regulatory protein (named Crl for curli). The constitutive production of curli in serovar Enteritidis was found to be due to a single nucleotide polymorphism (SNP) in the promoter region that changed the transcriptional regulation (33). Most strains of *Salmonella*, it has since been found, only produce curli fimbriae at temperatures below 32°C (76). In addition, curli production requires low osmolarity and appears to be triggered by nutrient limitation (32, 33, 77). One could envision that similar conditions might naturally exist in non-host environments. Hence, researchers started to think more about curli production and *Salmonella* aggregation in the context of the environment.

Anriany et al. (78) first analyzed the survival properties of rdar colonies formed by *Salmonella* serovar Typhimurium (78), due to the apparent similarities to the "rugose" colony morphology in *V. cholerae*, which had previously been shown to enhance *Vibrio* survival (79). Cells in rdar colonies had enhanced survival upon exposure to hydrogen peroxide and to acidic pH, as compared to non-rdar colonies. Cellulose was discovered as the second major component of the *Salmonella* biofilm extracellular matrix (42, 80); it is also extremely resistant and forms extensively hydrogen-bonded sheets (35). Solano et al. (42) treated rdar colonies with sodium hypochlorite, which is used as a common waterline disinfectant, and showed that the presence of cellulose provided protection to the cells. Other researchers extended these results to include pellicle biofilms formed at the air–liquid interface in liquid cultures (81). Scher et al. also tested heat and acidity but the pellicle cells were not significantly more resistant than stationary-phase cells. We performed a series of survival experiments, taking advantage of the unique properties of rdar colonies, specifically that they can be lifted off the agar surface in one piece (33). We started placing intact colonies on plastic surfaces and allowing them to dry out before periodically inoculating pieces of these colonies into fresh liquid media. Within 1–2 months, we realized that not only were cells staying viable in this dried out state, but also there was no apparent lag-time when placed into fresh media. Therefore, we performed an experiment comparing survival of rdar colonies to colonies formed by *Salmonella* mutant strains without curli, without cellulose, or without the entire extracellular matrix (Δ*csgD*) (37). After 3 months, the rdar colonies displayed 3–10 times enhanced survival compared to the biofilm mutants. After 9 months, the difference was as high as 30 times increased survival and exposure of the dried colonies to sodium hypochlorite yielded an even bigger survival difference. At the time that these desiccation experiments were performed, collaborators had discovered a new polysaccharide capsule in *Salmonella* that was part of the biofilm extracellular matrix (38). Gibson et al. performed desiccation experiments using a lyophilizer, and proved that the O-antigen capsule was the major factor providing desiccation resistance to cells. This reinforced the role of polysaccharides in the biofilm matrix to maximize water retention, nutrient trapping, and provide buffering (82).

As a final test of the potential importance of the biofilm extracellular matrix in the survival of *Salmonella* cells, we examined the viability of dried out rdar morphotype colonies after 2.5 years (83). The recovery of these cells was problematic in that they did not grow well on a variety of selective media commonly used for *Salmonella* isolation, such as SS or XLD agar. On non-selective media, the recovery rate after 30 months was ~5% of the starting number of cells; however, when evaluating viability using a live-dead cell stain, over 50% of cells appeared to be alive (84). The discrepancy in measured cell number between plating and live-dead staining suggested that cells might be in a type of viable, non-culturable (VBNC) state (85, 86). The existence of a VBNC state would add to the difficulty in eradicating *Salmonella* in agricultural or food-processing settings. Perhaps most importantly, cells in 2.5-year old rdar colonies retained an ability to cause infections in the mouse model of infection (83). There are many differing theories about how *Salmonella* can persist in industrial and/or agricultural settings, including survival in the local rodent or insect populations (87–89). The survival results for rdar colonies indicate that *S. enterica* strains could survive on their own in dryness or without exogenous nutrients for a long period of time.

In agricultural and industrial settings, biofilm formation has long been considered a factor to explain the extreme persistence of *Salmonella*. Outbreaks of human gastroenteritis have been linked to the consumption of a wide variety of foods or food products, not just fresh foods that may come into contact with contaminated water sources but also processed foods that go through extremes of dryness (84). Often researchers have analyzed the isolates/strains that are associated with outbreaks or with agricultural/industrial persistence to check their biofilm-forming ability, which has tended to be overwhelmingly positive (90–92). However, as yet, there has been no positive confirmation that the rdar morphotype is "the" critical factor. We performed a project examining *Salmonella* colonization of egg-conveyer belts used in modern poultry barns, because one farm had their flock re-infected by the same strain of *S. enterica* serovar Enteritidis over a 3-year period and the conveyor belt was identified as the source of contamination. Isolates taken from each year all formed rdar colonies, the rdar colonies were resistant to treatment with common disinfectants used in the industry, and all isolates formed robust biofilms on pieces of egg belt (93). However, the presence of rdar biofilm had no measurable effect on survival when contaminated pieces of egg belt were treated with disinfectant. We concluded that it is difficult to recreate real-world situations in an *in vitro* setting.

Several studies have provided convincing evidence to implicate biofilm formation as an important factor in the interaction between *Salmonella* and plants [reviewed in Ref. (94)]. Curli fimbriae, cellulose, and O-antigen capsule are involved in different stages of plant colonization and *Salmonella* persistence within or on plant tissue (95–97). In their laboratory study, Lapidot and Yaron reported the observation of multicellular aggregates beneath the surface of parsley leaves grown in soil irrigated with

contaminated water (98). Perhaps, the closest real-world evidence of enhanced biofilm formation having a biological impact was with the recent *E. coli* outbreak in Germany in 2011. The O104:H4 strain that was associated with contaminated fenugreek seeds caused the highest rates of hemolytic–uremic syndrome ever recorded and displayed clinical evidence of increased biofilm formation (99). Analysis of this strain showed that it had acquired a unique gene that led to enhanced biofilm formation (100). It is possible that biofilms were involved in the initial attachment to the fenugreek seeds, as previously demonstrated for alfalfa seeds (101), which would have facilitated both the dissemination and persistence of the outbreak strain.

Biofilm as an Anti-Virulence Trait

Collinson et al. first proposed that aggregation could provide *Salmonella* with a mechanism for surviving the harsh conditions of the host intestinal tract to ensure that a "viable and sufficient" inoculum could reach the epithelial layer (32). This seemed a valid hypothesis because although the dogma was that a high infectious dose was required for *Salmonella* infections, there were multiple epidemiological trace-backs to outbreaks that seemingly had a low inoculum (102). One could envision small aggregates being taken up by a host and the presence of resistant extracellular matrix polymers shielding cells during passage through the stomach. This would lower the infectious dose required to cause infections. The biofilm flask cultures described (**Figure 1**), provided an opportunity to test this hypothesis. Cultures of wild-type *S. enterica* serovar Typhimurium were competed in mouse infections with an isogenic, curli-negative mutant that was unable to aggregate. To our surprise, the non-aggregated mutant strain consistently outcompeted the wild-type strain (45). In our more recent studies, planktonic and aggregate cell types were isolated from the biofilm flask cultures, and competed during co-infections of mice. Again, the non-aggregated cells consistently outcompeted the aggregates (103). There is other recently published evidence that the presence of extracellular matrix factors reduce *Salmonella* virulence in the mouse model of infection (104). The results from these studies have established that the formation of rdar biofilms is not a virulence adaptation.

The results above created a conundrum because curli fimbriae have been well established as potent inducers of the innate immune system. Curli represent a pathogen-associated molecular pattern (PAMP) that causes activation of toll-like receptors 1 and 2 (105, 106), as well as intracellular NOD-like receptors (107). There is also evidence that curli can bind to multiple host proteins, such as contact-phase proteins or extracellular matrix proteins fibronectin and laminin [reviewed in Ref. (34)]. Although this research would seem to indicate that curli are produced *in vivo*, studies have yet to show that CsgD and curli are produced during infection. Deletion of *csgBA* (formerly *agfBA*), encoding the main curli subunit proteins, caused no noticeable impairment of *Salmonella* virulence (108). We monitored the expression of a curli reporter fusion *in vivo* using a whole animal imager, but we did not detect expression in any of the 13 mice that were screened (45). We hypothesized that the host immune system does interact with *Salmonella* rdar biofilms during an infection, perhaps immediately after ingestion of *Salmonella* biofilm cells.

Cellulose, the other main structural component of rdar biofilms, may have an important role in host–pathogen interactions during *Salmonella* infection. In contrast to curli biosynthesis, which is regulated at the transcriptional level by CsgD (69, 72), the *bcs* operons encoding the cellulose biosynthesis enzymes are transcriptionally independent of CsgD and the rdar morphotype (80). Activation of cellulose production is largely dependent on allosteric activation of the BcsA subunit by c-di-GMP (51). CsgD is responsible for inducing the expression of *adrA*, the first diguanylate cyclase enzyme to be associated with cellulose biosynthesis in *Salmonella* (36, 50). The transcriptional uncoupling of cellulose biosynthesis from CsgD regulation thus provides an opportunity for cellulose production to occur independently of the biofilm phenotype. As stated above, the *Salmonella* genome contains multiple diguanylate cyclase genes. The evidence for a CsgD-independent pathway for cellulose production was first provided by Ref. (42) and has since been demonstrated in *E. coli* (109). In a pair of subsequent studies, Lasa and colleagues performed systematic deletion and re-integration of the genes for each c-di-GMP synthase enzyme, resulting in the identification of four c-di-GMP synthesizing enzymes that can each independently induce *Salmonella* biofilm formation at 37°C (44, 110) (**Figure 3A**).

Within the host, there are potentially multiple cues that can induce c-di-GMP-based activation of cellulose biosynthesis. Co-incubation of *Salmonella* with Sal4, a protective IgA antibody secreted into the intestinal lumen, has been shown to increase c-di-GMP levels and cellulose production through activation of YeaJ (STM1283) (111), one of the enzymes identified by Lasa and colleagues. Cellulose has also proven important for attachment of *E. coli* and *Salmonella* to the surface of intestinal cells, although the exact c-di-GMP synthase enzymes responsible for this process are still unknown (112, 113). Further, *Salmonella* have been shown to produce measurable amounts of cyclic-di-GMP and cellulose while inside macrophages in response to low intracellular concentrations of magnesium and to changes in intracellular ATP levels (114). These authors discovered the production of cellulose *in vivo* by studying a *mgtC* mutant strain. *mgtC* is a key virulence gene that is expressed inside macrophages; deletion of *mgtC* caused attenuation of virulence and an increase in cellulose production. However, the *mgtC* mutant strain was no longer attenuated if the accumulation of cellulose was prevented, suggesting that cellulose was impeding virulence. Most surprisingly, these authors found that a *Salmonella* cellulose synthase mutant was hypervirulent, and killed mice faster than the wild-type strain (114). Ahmad et al. also recently published evidence that cellulose impedes *Salmonella* virulence; synthesis of BcsZ was necessary to reduce cellulose production *in vivo* and to maintain virulence (115). Mills et al. (116) identified L-arginine as another potential signal that is present inside the macrophage and is able to induce c-di-GMP biosynthesis and cellulose production. Finally, previous work out of the Romling lab demonstrated that cellulose production inhibits host cell invasion (117, 118). Altogether, the evidence indicates that *Salmonella* cellulose production plays a key role in host–pathogen interactions and that the interactions may be much more complex than initial experiments demonstrating

that cellulose production was not essential for *Salmonella* virulence (42).

CORRELATION BETWEEN BIOFILM FORMATION AND HOST SPECIFICITY

Since the discovery of curli fimbriae and description of the rdar morphotype, researchers have examined the conservation of this phenotype within the *Salmonella* genus. These data are summarized in **Table 1**. In general, the rdar morphotype is conserved in a majority of non-typhoidal *S. enterica* serovars, such as Enteritidis and Typhimurium, in strains that normally would cause gastroenteritis. We proved that *csgD* (curli) promoter function was conserved in a diverse collection of strains, representing *S. bongori* and all six *S. enterica* subspecies (119). However, many of the strains had lost biofilm formation due to *trans* changes in the rdar regulatory network likely caused by domestication of the strains (46, 120). This provided a cautionary note that these kinds of extracellular phenotypes can be easily lost during laboratory passage (120).

Römling and colleagues first identified a connection between increased host adaptation in *Salmonella* strains and an inability to form biofilms (121). One particular variant of serovar Typhimurium (i.e., var. Copenhagen) that causes a systemic disease in pigeons (129) had lost the ability to form the rdar morphotype. Their analysis was expanded to include other host-adapted serovars, such as Cholerasuis (pigs), Gallinarum (chickens), and host-restricted serovar Typhi (humans), which were almost entirely rdar negative (**Table 1**). We reported that two Typhi strains were rdar positive (37); however, this was a mistake caused by the presence of a contaminating rdar-positive isolate within the stock cultures. We subsequently tested >200 Typhi isolates and all were rdar negative (45). We also identified *S. enterica* subspecies *arizonae* (IIIa) strains as being rdar negative due to inactivating SNPs in the *csgD* (curli) promoters (46). Subspecies *arizonae* isolates rarely cause human infections, but are frequently isolated from the gut of reptiles and snakes, and therefore, may be part of the commensal microflora (130). In *S.* Typhimurium var. Copenhagen isolates, the prevalent mutation was a G to T transversion in the −35 region in the *csgD* promoter, which may partially explain the loss of the biofilm phenotype (121). For *S.* Typhi, the intergenic region between the divergent curli operons has conserved sequence, but multiple mutations exist within the curli (*csg*) and cellulose (*bcs*) biosynthesis operons, where preliminary stop codons within *csgD* and *bcsC* genes may eliminate the possibility for synthesis of curli fimbriae and cellulose altogether (121, 131). We speculated that sequence mutations or *cis* changes in the *csg* genes/promoters are indicative of a change in the lifestyle of *Salmonella* isolates (46). Each of the serovars above that are lacking rdar biofilm formation have evidence of a restricted host range.

The most recent screening efforts have focused on iNTS strains from sub-Saharan Africa. With a few exceptions, it appears that biofilm formation is impaired in these strains (**Table 1**). Ramachandran et al. (125) performed colony desiccation and sodium hypochlorite experiments with serovar Typhimurium ST313 isolates and demonstrated that they were also impaired for survival. This preliminary evidence suggests that iNTS isolates may have undergone an evolutionary change in lifestyle when compared to their gastroenteritis-causing NTS counterparts.

STRATEGIES OF *IN VIVO* INFECTION

Central to *Salmonella* pathogenesis is its ability to modify host cell biology *via* two type-three secretion systems (T3SS), T3SS-1 and T3SS-2 (132). These specialized organelles span the bacterial inner and outer membranes and allow for the delivery of effector proteins into the cytoplasm of eukaryotic cells (133, 134). Type-three secretion systems are found exclusively in Gram-negative bacteria (135, 136). For *Salmonella*, genes for the T3SS-1 or T3SS-2 apparatus, regulatory components, and nearly all associated effector proteins are found within horizontally acquired DNA regions known as *Salmonella* Pathogenicity Islands (SPIs) (134). The SPI-1 region contains genes associated with T3SS-1, is found in all serovars of *S. enterica* and *S. bongori*, and is important for the invasion of intestinal epithelial cells (137, 138). In contrast, the full-length SPI-2 region harboring genes for T3SS-2 is present exclusively in *S. enterica*, and is associated with the intracellular survival of *Salmonella* within eukaryotic cells (137).

Non-typhoidal versus Typhoidal *Salmonella*

Non-typhoidal *Salmonella* infections in immunocompetent individuals can be generalized into three important steps: invasion, inflammation, and intestinal replication. While decades of literature have been dedicated to understanding invasion and inflammation, recent research has uncovered important aspects of *Salmonella* replication and transmission in the inflamed intestine. In this section, we evaluate what is known about these important steps in gastroenteritis associated with NTS infections and consider how biofilm biology and *Salmonella* transmission may relate to our current understanding of *Salmonella* pathogenesis.

Following *Salmonella* entry into the host *via* contaminated food or water, cells travel through the digestive system and localize to the distal ileum and colon of the GI tract (139). *Salmonella* cells rely on flagellar motility and chemotaxis systems to traverse the intestinal mucus layer and identify sites on the host cell that are permissive for invasion (140). Fimbriae and other protein adhesins on the bacterial cell surface initiate association of the pathogen to the targeted epithelial cell; the needle complex of the T3SS-1 is critical for stabilizing this interaction. It is currently hypothesized that expression and synthesis of the T3SS-1 is induced by several important cues provided by the local host environment, including low-oxygen tension, high osmolarity, near-neutral pH, and acetate production levels from the resident microflora (141). The secretion apparatus is established in a step-wise manner, requiring formation of the basal body within the bacterial cell membranes to facilitate secretion of the needle complex (142). Proteins attached to the end of the T3SS-1 needle, collectively referred to as the translocon, are then inserted into the host cell membrane, creating a pore that allows for the injection of *Salmonella* effector proteins into the host cell (143). Establishment of a secretion-competent T3SS-1 is a mandatory prerequisite for the process of host cell invasion [reviewed in

TABLE 1 | Biofilm formation among host-generalist and host-adapted *Salmonella* strains.

Study	*Salmonella enterica* serovars (# strains)	Biofilm formation[a]	Strain origins	Special notes[b]
Solano et al. (42)	Enteritidis (204 strains)	40 of 56 (71%) 48 of 63 (76%) 27 of 54 (50%) 20 of 31 (65%)	Animal Clinical Environmental Food	Overall biofilm formation 198 of 204 (97%)
Römling et al. (121)	Typhimurium, Enteritidis (>800)	720 of ~800 (90%)	Animal, human	Collected at National Reference Center; Germany
Solomon et al. (122)	28 serovars (71 strains)[c]	11 of 15 (73%) 26 of 31 (84%) 14 of 25 (56%)	Clinical Meat Produce	Overall curli production was 93%
White et al. (37)	37 serovars (72 strains)	58 of 72 (80.5%)	*Salmonella* reference collection B (SARB);	Boyd et al. (123)
Malcova et al. (124)	Typhimurium (84)	76 of 84 (90%)	Animal species	Collected 2004–2007 in Czech Republic
Vestby et al. (92)	Agona (47), Montevideo (38), Senftenberg (42), Typhimurium (21)	110 of 148 (74%)	Clinical and feed and fish meal factories in Norway	Overall curli production 100%; 55% Agona and Seftenberg isolates were cellulose-negative
De Oliveira et al. (90)	Serovars undetermined (174)[d]	96 of 174 (55%)	Raw poultry isolates from Brazil	Overall biofilm formation 171 of 174 (98%)
Laviniki et al. (91)	16 serovars (54 strains)[e]	54 of 54 (100%)	Ingredients, equipment—4 feed mills in Brazil	
Ramachandran et al. (125)	Typhimurium ST19	5 of 5 (100%)	Human blood isolates in Mali + one reference	Gastroenteritis-causing isolates
White and Surette (46)	Subspecies I, II, IIIa, IIIb, IV, VI, and *S. bongori* (group V) Lab collection Remaining strains	 5 of 16 (31%) 72 of 80 (90%)	*Salmonella* reference collection C	Boyd et al. (119); IIIa strains had inactivating single nucleotide polymorphisms (SNPs) in *csgD* promoter (curli); signs of domestication in lab collection
Römling et al. (121)	Typhi Choleraesuis Typhimurium v. Copenhagen Gallinarum	0 of 19 (0%) 0 of 17 (0%) 0 of ~80 (0%) 1 of 23 (4%)	Human Animal, human Pigeon Animal, human	Several Gallinarum strains were cellulose positive at 37°C
Malcova et al. (124)	Typhimurium v. Copenhagen	0 of 10 (0%)	Pigeon, duck	These are phage type DT2 isolates
White et al. (45)	Typhi	0% of >200 isolates	Human	*Salmonella* genetic stock center
Singletary et al. (126)	Typhimurium ST313 African Lineage I African Lineage II	 3 of 3 (100%) 0 of 6 (0%)	Lineages referred to in Okoro et al. (127)	Common SNP in *bcsG* (cellulose) identified in lineage II isolates, including the type strain, D23580
Ramachandran et al. (125)	Typhimurium ST313 Typhi Paratyphi A	0 of 11 (0%) 0 of 6 (0%) 0 of 3 (0%)	Human blood isolates from Mali + reference strains	Authors suggested that ST313 isolates may not be able to persist in the environment
Ashton et al. (128)	Typhimurium ST313 African Lineage II	 0 of 16 (0%)	Human clinical isolates from UK	Genome degradation in African lineage II strain D23580 conserved in UK isolates

[a]*Most studies have tested strains for ability to form red, dry and rough (rdar) colonies on media containing Congo red; this indicates curli and cellulose production. Biofilm-negative strains were smooth and white (saw) on this media. It is typical for isolates to be rdar at 28°C and saw at 37°C. In some cases, researchers grew strains on media containing calcofluor and tested for fluorescence as confirmation of cellulose production.*

[b]*Overall biofilm results are reported in cases where researchers have tested strains in a variety of growth conditions.*

[c]*Serovars Anatum (2), Baildon (1), Branderup (1), Bredeney (1), Derby (1), Enteritidis (5), Hadar (3), Gaminara (2), Heidelberg (3), Hidalgo (1), Infantis (1), Kentucky (2), Mbandaka (2), Michigan (1), Montevideo (2), Muenchen (1), Muenster (2), Newington (1), Newport (3), Oranienburg (1), Poona (6), Reading (1), Saint Paul (3), Saphra (1), Schwarzengrund (2), Stanley (1), Thompson (3), Typhimurium (13), Worthington (1).*

[d]*Most commonly isolated Salmonella strains from poultry in Brazil are from serovar Enteritidis.*

[e]*Serovars Agona (5), Anatum (4), Cerro (1), Infantis (2), Mbandaka (1), Montevideo (18), Morehead (1), Newport (2), Orion (3), O:3,10 (2), O:16:c:- (1), Schwarzengrud (1), Senftenberg (6), Tennessee (4), Typhimurium (1), Worthington (2).*

Ref. (144)]. Pathogen cells that traverse the epithelial cell layer encounter tissue mononuclear cells (i.e., macrophages and dendritic cells) within the lamina propria, resulting in the uptake of the pathogen into a phagosome (139). *Salmonella* depend on the T3SS-2 and associated effectors to manipulate the phagosome environment and promote pathogen survival and replication within the *Salmonella*-containing vacuole (SCV). The effectors are responsible for re-modeling the SCV environment and are hypothesized to provide a potential source of nutrients during pathogen replication (144).

Detection of PAMPs and components injected by the pathogen during its uptake into host cells elicits the production

of a proinflammatory immune response (139). This response inhibits the spread of NTS past the lamina propria in three ways: (1) by activating infected macrophages and inducing killing of intracellular *Salmonella*, (2) through recruitment of neutrophils to the infection site for extracellular killing, and (3) by stimulating epithelial cells to release antimicrobial peptides into the intestinal lumen to control the replication of NTS cells (139). While host inflammation effectively controls the NTS cells that have invaded the epithelial cell layer, it acts as a potent stimulator of growth of the NTS population in the intestinal lumen (139). Recent studies have shown two mechanisms by which NTS are able to exploit the host inflammatory response. Epithelial cells release the antimicrobial agent lipocalin-2, a molecule that binds to enterochelin, an iron chelation molecule used by Gram-negative bacteria in the gut to acquire iron from the host (145). In addition to enterochelin, NTS are able to produce a second iron chelation molecule, salmochelin, which cannot be bound by lipocalin-2 (145). As a result, NTS cells in the intestinal lumen continue to replicate while the local microbiota are starved for iron. The low-oxygen conditions of the intestinal lumen promote the establishment of an anaerobic microbiota that use fermentation to derive energy from available amino acids and complex polysaccharide (139). Hydrogen sulfide is produced as a byproduct of this fermentation, which is immediately converted to thiosulfate by the epithelial cell layer of the colon (146). During inflammation, neutrophils infiltrate the intestinal lumen and release reactive oxygen species molecules as part of the mechanism for the extracellular killing of bacterial pathogens (146). The association of reactive oxygen species with thiosulfate molecules results in tetrathionate, which can be used by NTS as an electron acceptor during anaerobic respiration. In addition to activating a metabolic response that promotes growth of the pathogen, anaerobic respiration further allows NTS to utilize carbon sources that would otherwise metabolize poorly during aerobic fermentation (147). Altogether, NTS can use these mechanisms to promote their own growth at the expense of the existing host microbiota.

Two features that distinguish enteric or typhoid fever from gastroenteritis are the relative absence of inflammation and an innate immune response, and the replication of typhoidal *Salmonella* in the systemic compartment of the host. These changes in pathogenesis are linked to genomic differences between typhoidal and NTS. Approximately 200 functional genes in NTS have been inactivated or functionally disrupted in *S.* Typhi and *S.* Paratyphi A (148). Many of the mutations in *S.* Typhi affect processes used by NTS to induce intestinal inflammation, including motility and chemotaxis, adherence to and invasion of host cells, and loss of virulence factors associated with intracellular replication (148). The loss of these functions suggest that *S.* Typhi may gain access to the systemic compartment through a mechanism distinct from active invasion of intestinal epithelial cells. Although this mechanism remains elusive, it is hypothesized that microfold (M) cells that sample the intestinal lumen actively take in *S.* Typhi cells and transfer them to macrophages and dendritic cells within the gut-associated lymphoid tissue of the Peyer's patches, located in the small intestine (16). Typhoidal *Salmonella* are unique/distinct from non-typhoidal serovars in that they are able to persist in this intracellular niche without activating the immune response and infiltration of neutrophils that would otherwise restrict typhoidal infections (149). Within these immune cells, typhoidal *Salmonella* are shuttled to other sites in the body associated with the mononuclear phagocyte system (previously known as the reticuloendothelial system), taking residence in such places as the liver, spleen, mesenteric lymph nodes, bone marrow, as well as the gall bladder (17, 20). Typhoidal *Salmonella* are thought to transfer back into the duodenum *via* the biliary tract, resulting in intermittent shedding of typhoidal cells in the feces (17).

The lack of inflammation associated with typhoidal *Salmonella* infections suggests important differences in the pathogen's surface antigens or its interactions with host cells. For example, the potential downregulation in serovar Typhi flagellar expression results in decreased inflammation (150). Other gene mutations identified in *S.* Typhi include regulatory elements affecting the O-antigen structure, which may limit exposure of this important PAMP to immune cells (151). Further, the typhoidal *Salmonella* genome also possesses 300 to 400 unique genes that are absent in NTS. Of these additional accessory genes, the Vi capsule plays an important role in reducing the host inflammatory response to the presence of *S.* Typhi cells. Production of the Vi capsule limits complement deposition on the surface of *S.* Typhi cells, masks surface antigens that would normally activate the host immune response, and provides resistance to phagocytic killing (16, 17, 152). Further, the Vi capsule has also been demonstrated to induce production of the cytokine interleukin 10, an important anti-inflammatory molecule (153). Vi-negative mutants of *S.* Typhi were unable to cause enteric fever in human infection trials (154). However, the Vi capsule cannot solely account for differences between typhoidal and NTS infections, as this capsule is not expressed by other typhoidal serovars (i.e., *S.* Paratyphi A) (149). It is likely that serovar-specific combinations of gene acquisition and gene loss are responsible for the ability of typhoidal *Salmonella* strains to evade the host immune response. There are several other reasons for the host-restriction of serovar Typhi strains, as recently discovered by Spanò et al. (155). Further study of these factors will provide insight for understanding how *Salmonella* serovars and strains progress from host-generalists to host-adapted and finally to becoming host restricted.

OUR CURRENT UNDERSTANDING ABOUT *Salmonella* TRANSMISSION

Despite their differences in host range, non-typhoidal and typhoidal *Salmonella* serovars maintain a genetic relatedness at the species level (149). Therefore, *Salmonella* pathogens present an opportunity to study the biological factors that are important for transmission (156).

Nearly all NTS serovars associated with human disease demonstrate the ability to colonize multiple host species and induce gastroenteritis. In North America, NTS infections are often associated with the ingestion of contaminated food or water, but can also be transmitted directly from zoonotic

sources, such as domestic or food animals, through the fecal–oral route (84). Outbreaks of NTS have been associated with a wide range of food products, including animal-based (meat, poultry, eggs), plant-based (tomatoes, sprouts, melons, lettuce, mangoes, raw almonds) and processed foods (powdered infant formula, dry seasonings, cereals, peanut butter) (84). However, a number of NTS outbreaks in developed countries emphasize the importance of environmental reservoirs as an intermediary step for transmission of this pathogen. Several reported cases exemplify the ability of NTS to persist in the non-host environment. Surface runoff contaminated with animal feces was suspected as the source of two separate cases in 2008 of drinking water contamination affecting communities in the United States (157, 158). Similarly, a study analyzing 288 cases of drinking water-related outbreaks in Canada between the years 1971 and 2001 noted water treatment practices and nearby wildlife as the most frequently reported sources of contamination (159). Several reports of NTS outbreaks associated with fresh produce have traced contamination to irrigation water or animal manure used to fertilize fields (160–162). Of particular interest are two separate outbreaks of NTS infections in the United States in 2002 and 2005, both of which were linked to a rare strain of *S.* Newport in tomatoes (162). In both outbreaks, investigators were able to trace back the unique strain to a Virginian farm, where the strain had been isolated from a contaminated pond used to irrigate the fields (162). The identification of this same rare strain in pond water samples taken years apart indicates the added importance of *Salmonella* persistence in environmental reservoirs. A similar case of non-typhoidal *S.* Typhimurium persistence was observed for a Danish pig farm associated with recurring infections in its herd (163). Samples collected by Baloda and colleagues over a 2-year period revealed the presence of the same *Salmonella* clone in the piggery, the feed provided to the animals, and in the pig manure used to fertilize agricultural soil (163). Farmland soil treated with manure yielded viable *S.* Typhimurium cells for 14 days following spread, providing further evidence for the survival of NTS within the non-host environment. The authors hypothesized that the persistence of *Salmonella* in this setting could result in a cycle of re-infection of the pig herd from environmental reservoirs, potentially explaining the long-term presence of NTS at the farm. While it may be logical to infer a role for biofilm formation in such cases of *Salmonella* persistence, it will be important for future research endeavors to include efforts to characterize the physiological state of *Salmonella* cells *in situ*.

For typhoidal *Salmonella,* chronic persistence within their current host increases the opportunities for subsequent transmission events. Between 5 and 10% of patients recovering from enteric fever experience a relapse in infection with the same typhoidal *Salmonella* strain, resulting in milder symptoms than before and fecal shedding of the pathogen for 3 weeks to 3 months following the initial infection (17). While the mechanism behind this short-term persistence in the host is unclear, it is hypothesized that typhoidal *Salmonella* can remain dormant within immature immune cells in the bone marrow (16). Between 2 and 4% of people living in areas endemic for enteric fever are associated with a chronic carrier state that involves asymptomatic carriage of typhoidal *Salmonella* for more than a year (16). Epidemiological studies hoping to identify factors associated with the chronic carrier state are difficult due to the asymptomatic nature of infections in these hosts (17). However, recent evidence points to persistence of typhoidal *Salmonella* within the gall bladder. It is currently hypothesized that typhoidal *Salmonella* cells first localize to the liver and replicate in the resident macrophage (Kupffer) cells before traveling to the gall bladder *via* the biliary tract (17). Bile, a digestive secretion with detergent and antimicrobial properties, contributes to the sterility of the gall bladder. In a landmark study assessing the incidence of gall bladder disease in patients associated with acute or chronic infections with typhoidal *Salmonella*, the authors detected gallstones in nearly 90% of chronically infected patients (164). Microscopic analysis of this interaction suggested that typhoidal *Salmonella* cells use fimbrial protein structures on their surface to attach to gallstones, while growth in the presence of bile stimulates the production of protective extracellular polysaccharides (59). It is currently hypothesized that short-term human carriers are mainly responsible for the transmission of enteric fever in endemic areas, while long-term chronic carriers are responsible for resurgence of infections in endemic regions despite attempts to control such infections (17). Blaser and Kischner proposed that Typhi carriers would have been necessary in hunter gatherer societies to allow the disease to spread to others even after everyone in the local family group had been infected and had acquired immunity (165). Typhoidal *Salmonella* serovars are transmitted primarily from person to person through food and water contaminated with human feces; as such, infections are more frequent in low-income countries that lack available safe water resources and have poor sanitation standards (166). Cases of typhoidal infections in high-income countries are usually the result of patients traveling to endemic areas, but can also be spread by individuals that are chronically infected with *S.* Typhi (166).

Like typhoidal serovars, strains of iNTS induce a fever-like illness and persist within the systemic compartment of infected individuals (19). As of today, humans are the only identified reservoir for invasive strains of NTS (167, 168). Thus, the speculation is that these strains are transmitted similarly to Typhi, with the human carrier being the main source of new infections. iNTS infections occur predominately in children between 6 and 18 months of age and in adults between 25 and 40 years old (166). For children, the predominant host risk factors are HIV infection, malnutrition, and malaria, while advanced HIV infection is the main risk factor in adults (19). Further, cases of iNTS disease in children and adults are strongly correlated with the rainy season in sub-Saharan Africa, which could be the result of waterborne transmission, malaria, or malnutrition during this season (20, 166).

Bistable Gene Expression and a New Perspective on Biofilm Formation

Our current understanding of *Salmonella* biofilm formation has been primarily shaped by its characterization in the laboratory. Most early comparisons between biofilm-positive

and biofilm-negative isolates were done at a population level. While this yielded valuable information, it was hard to envision how these biofilm phenotypes would manifest in nature. For example, Serra et al. showed that there was massive cell heterogeneity within rdar colonies (40), therefore, ascribing functions to the rdar colony as a whole may not be biologically accurate. Furthermore, this density of cells (i.e., 10^{10}–10^{11} cells per colony) would not be sticking together in the environment in one piece, except under very special circumstances, such as a wastewater treatment plant (169), but even in those conditions there are many different species involved so the dynamics would be different. This led us (and others) to the question: "How does the rdar morphotype appear in nature?" To simulate the environment, we attempted to induce *S. enterica* serovar Typhimurium aggregation at low cell densities in liquid cultures (45). Under these conditions, the population of cells in liquid culture differentiated into two forms: multicellular aggregates and planktonic cells. We showed that the aggregates produced curli and cellulose polymers and had many of the properties of a rdar biofilm (45). We know now that the biofilm cells and single cells arise due to bistable production of CsgD (i.e., biofilm cells are CsgD-ON; single cells are CsgD-OFF) (170) (**Figure 3B**).

In a large-scale RNA-seq experiment, we compared the gene expression profile of *Salmonella* multicellular aggregates and planktonic cells (103). Although they are formed under the same growth conditions, these clonal cell types had differential expression of over 1,856 genes, which represents approximately 35% of all genes in the serovar Typhimurium genome. Previous work had identified genes corresponding to carbon central metabolism and the general stress response being expressed during biofilm formation (54). Transcriptome analysis expanded this by identifying increased expression of genes important for the metabolism of amino acids, lipids, and nucleotides. We demonstrated that biofilm cells were more resistant to desiccation and antibiotics than the planktonic cells. The transcriptome of planktonic cells was vastly different from multicellular aggregates, with significant expression of multiple virulence traits, including the T3SS-1. In the literature, it was thought that expression of the T3SS-1 was exclusively induced by *in vivo* conditions inside the host, which is an important first step in *Salmonella* pathogenesis (133). As such, expression of T3SS-1 in biofilm-inducing conditions was highly unexpected and required rigorous functional validation. We confirmed that the proteins for the secretion apparatus and its effectors were synthesized under these atypical environmental conditions. The increased abundance of T3SS-1 provided a virulence advantage for planktonic cells compared to multicellular aggregates both for invasion of a human intestinal cell line *in vitro* and during competitive infections in mice. Determining how the T3SS-1 is induced in the planktonic cell subpopulation remains an important question for us. The relative absence of SPI-1 expression in multicellular aggregates may also provide an important clue for the molecular link between the persistence and virulence phenotypes. While it is tempting to speculate that there is a direct link between CsgD and SPI-1 expression, as implied by other studies (171), such a relationship has not yet been established. It is plausible that the presence of the extracellular matrix may provide an important feedback signal that ultimately inhibits the expression of virulence factors such as the T3SS-1. Regulation between SPI-1 and biofilm formation may also be indirect in nature. Desai and colleagues recently demonstrated that SsrB, a transcriptional regulator encoded within SPI-2, can switch between promoting expression of the T3SS-2 within the acidic macrophage vacuole and relieving H-NS silencing of *csgD* expression (172). Establishing the genetic link between persistence and virulence is an important direction for the future of *Salmonella* biofilm research.

SELECTION PRESSURES ACTING ON BIOFILM FORMATION

Immune Avoidance or Continued Transmission Success?

As described in Section "Correlation between Biofilm Formation and Host Specificity," biofilm formation is highly conserved in *Salmonella* strains associated with gastroenteritis, but is lost in *Salmonella* strains that are responsible for invasive disease or are adapted to life in a particular host. Romling and colleagues were the first to discuss this correlation in the context of *Salmonella* biology (121). They suggested that loss of biofilm formation in invasive strains/serovars/species was a pathoadaptive trait, presumably to improve the fitness of the pathogen so that it is able to survive better in host tissues (173). Part of the reasoning behind this was the knowledge that *Shigella* and enteroinvasive *E. coli*, two pathogens which breach the intestinal epithelium (174), have lost the rdar morphotype due to multiple insertions and deletions in the curli biosynthesis operons, indicating strong selection pressure against this phenotype (175). In contrast, *E. coli* strains that were commensal inhabitants of the GI tract appeared to retain an ability to express the rdar morphotype (112). We reported the same trend in *E. coli* as in *Salmonella*; among 284 *E. coli* isolates from diverse host species, we observed that host-generalist isolates were 84% rdar-positive, whereas isolates that had the largest genetic differences and were the most likely to be host-adapted were less than 50% rdar-positive (176). The 115 human isolates that we screened were only 36% rdar-positive versus 169 isolates from different animal hosts that ranged from 59 to 93% rdar-positive. We reasoned that human commensal *E. coli* were more host-adapted and would have less reliance on transmission *via* the environment as compared to transient strains that cause extra-intestinal infections and are only in the host for a short time. However, reduced rdar prevalence in our study was also correlated with an increase in the presence of virulence genes, meaning that the dynamics of *E. coli* colonization are complex (176, 177). The possibility of a pathoadaptive trait conserved between species indicates that similar selection pressures are acting on *Salmonella* and *E. coli*. Römling et al. focused on the interactions with the intestinal epithelium, arguing that when a pathogen crosses this barrier the dominant selection pressure comes from the host immune system (121). While the

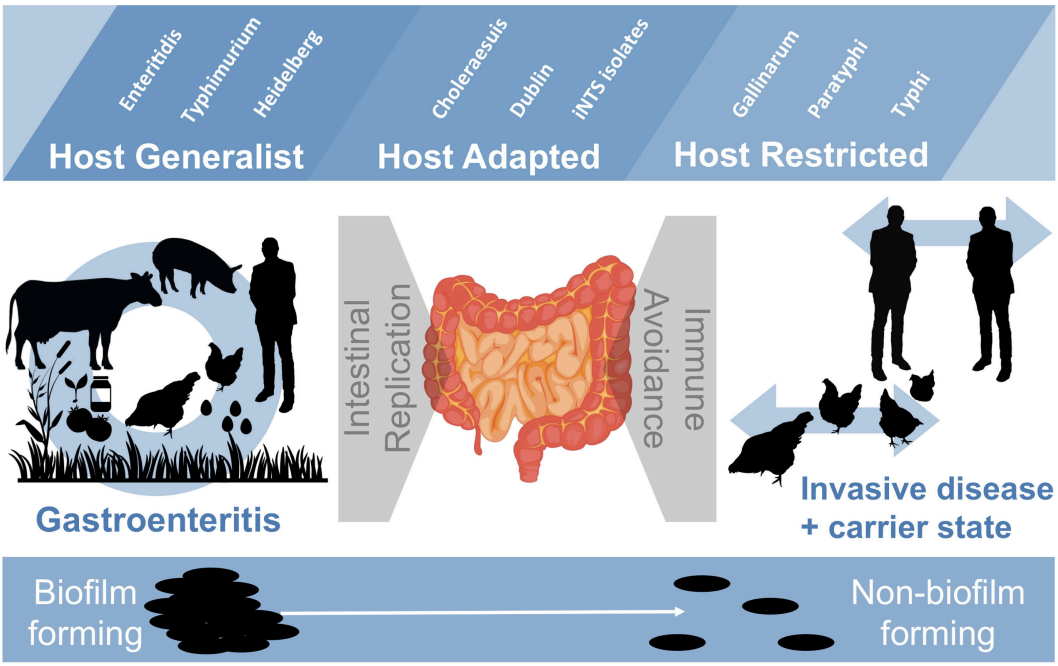

FIGURE 4 | Host interactions, lifecycles, and biofilm-forming ability of *Salmonella* strains. Host-generalist *Salmonella* strains have a varied lifecycle, in which several host species and environments are encountered, and zoonotic transfer to humans may occur. Transfer may also occur through ingestion of contaminated vegetables (i.e., tomatoes, sprouts) or processed foods. Infections are localized to the intestine and the selection pressures are on intestinal replication and transmission. In contrast, host-adapted and host-restricted *Salmonella* strains have an evolutionary narrowed lifecycle, in which transmission is primarily between individual hosts. Selection pressures are on immune avoidance with the objective of long-term persistence within the host. The loss of biofilm formation in host-adapted and host-restricted strains is thought to reflect a shift in selection pressures caused by a change in lifecycle.

immune system undoubtedly plays an important role, there are also strong pressures on pathogens to maintain their transmission cycles (156). Therefore, loss or impairment of the rdar morphotype could also reflect a change in strain transmission patterns (125). At present, we do not know which aspect of the *Salmonella* lifestyle has the greatest evolutionary influence on the relationship between host adaptation and ability to produce rdar biofilms.

CONCLUSION AND PREDICTIONS

Bistable CsgD expression and analysis of the aggregate and planktonic cell subpopulations has shifted our perception of *Salmonella* biofilm formation and the process of pathogen transmission. What once was considered a population-level phenotype is now understood as a regulatory phenomenon mediated at the single cell level (170). What is the purpose of this phenotype switching in the life cycle of *Salmonella*? Is there an advantage for *Salmonella* to express virulence factors in a non-host setting? Compared to the host niche, where the conditions of host–pathogen interactions are relatively defined, life in non-host environments and the process of transmission are unpredictable. In bacteria, bistable genetic networks are often associated with the formation of two distinguishable phenotypes within a clonal population (178), which is thought to allow genotypes to persist in fluctuating environments (179). Based on this theory and our characterization of the planktonic and aggregated cell subpopulations, we hypothesize that "phenotype switching" improves the overall chances for *Salmonella* transmission. In a scenario where *Salmonella* immediately encounters its next host, planktonic cells would be able to instigate a new infection. In contrast, if a host were not encountered, *Salmonella* biofilm cells would be prepared to survive in non-host environments for a long time until an opportunity for infection arises. This unpredictable step in the *Salmonella* life cycle places equal selection pressure on virulence and persistence phenotypes.

Since non-host environments have unpredictable conditions, it would be a poor evolutionary choice for *Salmonella* cells to adapt once they arrive there. Rather, *Salmonella* likely requires anticipatory genetic regulation where cells pre-emptively express the phenotype that is necessary for the next step in their life cycle (180). If bistable CsgD expression is necessary for cells to prepare for transmission, conditions within the GI tract may provide important cues to induce this differentiation. In the GI niche, *Salmonella* cells are exposed to host temperatures, low pH, high osmolarity, bile acids, antimicrobial peptides, iron limitation, and in some cases, nutrient limitation (141, 181). Some of these conditions (i.e., temperature and osmolarity) may favor the expression of virulence factors such as the T3SS-1 (141), while repressing *Salmonella* biofilm formation (33, 49). Conversely, exposure to stresses such as bile, antimicrobial peptides, iron limitation, or poor nutrient availability

would promote *csgD* expression and the biofilm phenotype. Regulation of *csgD* expression and synthesis is incredibly complex, and few studies have attempted to understand the hierarchy of this regulation (33, 49, 77, 182). For example, iron limitation has been shown to over-ride the normal temperature shut-off of biofilm formation at 37°C (33). It is possible that microenvironments within the intestinal niche provide strong activating signals required to generate the CsgD-ON state. In the lumen of the small intestine, high bile concentrations have been shown to increase the intracellular concentration of cyclic-di-GMP in the enteropathogen *V. cholerae* [reviewed in Ref. (181)] and to influence biofilm formation (183). For *Salmonella*, high concentrations of c-di-GMP activate *csgD* expression and promote the biofilm phenotype (51). Expression of the T3SS-1 is known to be location-dependent; *Salmonella* cells positioned at the surface of the intestinal epithelial layer were 100% positive for T3SS-1 expression, while the majority of cells in the lumen were negative for this virulence trait (184). We predict that biofilm expression may also be induced by signals produced toward the end of host–pathogen interactions. These signals may include intestinal inflammation and the associated rapid replication of *Salmonella* within the lumen (139, 146, 147, 185). The reactive oxygen species produced by neutrophils undoubtedly provides an important cue for activation of the bacterial cell stress response. Metabolic cues, such as the pathways involved with anaerobic respiration during this stage [i.e., ethanolamine and 1,2-propanediol (186)] or the nutrient limitation caused by rapid *Salmonella* replication following inflammation, may also add a temporal element to the anticipatory regulation of *Salmonella* biofilm formation. Consistent with this, our transcriptomic analysis revealed that genes for the ethanolamine and 1,2-propanediol metabolic pathways have increased expression within the CsgD-ON multicellular aggregates (103).

Our ability to elucidate the *in situ* regulation of *Salmonella* biofilm formation is limited by the reality that *Salmonella* biofilms have yet to be observed in nature. However, the enteric bacteria *V. cholerae* has been shown to exist both as planktonic cells and in multicellular aggregates in human stool samples (187, 188). We previously investigated *csgD* expression and the biofilm phenotype in *Salmonella* during murine infection by using luciferase reporters fused to biofilm-related gene promoters. *csgD* expression was activated within the mouse intestine during the course of infection, but the expression of curli biosynthesis genes (*csgBAC*) was only observed within fecal pellets that had passed out of the infected mice (45). From this experiment, we concluded that synthesis of the extracellular matrix only occurs after passage from the host. However, we have since learned that there is a limitation in the ability to detect bacterial luciferase during the course of infection (189). Heavy bacterial loads were required for consistent luciferase detection, despite expression from a strong constitutive promoter. This means that if activation of extracellular matrix production was limited to only a small subpopulation of cells as part of CsgD bistability, signaling from the *csgBAC* promoter would not be detectable using bioluminescence as a marker. Work in our laboratory is currently underway to determine if curli fimbriae and multicellular aggregates can be observed during murine infections by using single cell detection techniques (i.e., tissue sectioning and confocal microscopy).

The lifecycle of *Salmonella* involves exposure to both the host and natural (extracorporeal) environments (190). Evidence from our own and other research groups suggests that the rdar biofilm morphotype controlled by CsgD is crucial to the transmission success of *Salmonella*, allowing cells to survive the natural environment that is encountered between host infections (45, 77, 83, 103). Similarly, it may be possible that *Salmonella* produces a cellulose-based biofilm during host infection to mitigate stresses that arise from host immune responses or the harsh intracellular setting of a macrophage (111, 114). While the c-di-GMP signaling network is important for the production of either type of biofilm, most of the enzymes responsible for synthesizing c-di-GMP are exclusively expressed and activated in either a CsgD-dependent or -independent manner (44, 110, 191). Further research is necessary to not only identify the host sites and signals that activate the production of cellulose biofilm during infection, but to determine the reasons why cellulose biofilm production is important in host–pathogen interactions. It is possible that the c-di-GMP-specific pathway for cellulose biofilm expression has evolved to reduce the severity of its virulence and increase the possibility for *Salmonella* to successfully transmit to a future host.

In this review, we have touched upon new aspects of *Salmonella* biology that may have important implications for understanding transmission patterns. NTS strains that briefly colonize the host and cause gastroenteritis must contend with survival in both host and non-host environments. On the one hand, they are adapted to colonize the intestines of many different host species and to replicate to high numbers within the inflamed intestinal environment. Perhaps through biofilm formation and the bistable expression of CsgD, the formation of specialized subpopulations of cells (i.e., multicellular aggregates and planktonic cells) represents an evolutionary trade-off for mitigating the unpredictable nature of transmission *via* the fecal–oral route. In contrast, invasive strains of non-typhoidal and typhoidal *Salmonella* have evolved to avoid the immune system in order to persist chronically within the systemic niche of the host. This feature of host adaptation relieves the selection pressure placed on *Salmonella* to persist within an environmental reservoir and correlates with loss of the biofilm phenotype. It could also reflect that loss of biofilm phenotypes relieves selection pressure from the host immune system, which would contribute to their ability to persist *in vivo* (**Figure 4**).

Recent advances in DNA sequencing technology have expanded the availability of pathogen genomic sequences, which has led to unprecedented characterization of both classical and emerging *Salmonella* strains. iNTS strains are an example of how genome sequencing can be complemented by our understanding of *Salmonella* pathogenesis to develop a genetic signature for host adaptation. Similarly, future research efforts are needed to isolate the core genetic processes that govern biofilm formation. We predict that a biofilm gene signature or gene expression profile could be used to predict the transmission properties of *Salmonella*

strains associated with future outbreaks. Understanding common reservoirs and transmission routes is crucial for developing effective public health efforts to reduce the worldwide disease burden of *Salmonella* pathogens.

AUTHOR CONTRIBUTIONS

KM and MP prepared the figures, KM and AW wrote the paper, and MP and WK edited the paper. All authors read and approved the final manuscript.

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Differential Levels of Cecal Colonization by *Salmonella* Enteritidis in Chickens Triggers Distinct Immune Kinome Profiles

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Salmonella enterica serovar Enteritidis are facultative intracellular bacteria that cause disease in numerous species. *Salmonella*-related infections originating from poultry and/or poultry products are a major cause of human foodborne illness with *S.* Enteritidis the leading cause worldwide. Despite the importance of *Salmonella* to human health and chickens being a reservoir, little is known of the response to infection within the chicken gastrointestinal tract. Using chicken-specific kinome immune peptide arrays we compared a detailed kinomic analysis of the chicken jejunal immune response in a single line of birds with high and low *Salmonella* loads. Four-day-old chicks were challenged with *S.* Enteritidis (10^5 cfu) and cecal content and a section of jejunum collected at three times: early [4–7 days post-infection (dpi)], middle (10–17 dpi), and late (24–37 dpi). *Salmonella* colonization was enumerated and birds with the highest ($n = 4$) and lowest ($n = 4$) loads at each time were selected for kinomic analyses. Key biological processes associated with lower loads of *Salmonella* clustered around immune responses, including cell surface receptor signaling pathway, positive regulation of cellular processes, defense response, innate immune response, regulation of immune response, immune system process, and regulation of signaling. Further evaluation showed specific pathways including chemokine, Jak–Stat, mitogen activated protein kinase, and T cell receptor signaling pathways were also associated with increased resistance. Collectively, these findings demonstrate that it is possible to identify key mechanisms and pathways that are associated with increased resistance against *S.* Enteritidis cecal colonization in chickens. Therefore, providing a foundation for future studies to identify specific proteins within these pathways that are associated with resistance, which could provide breeders additional biomarkers to identify birds naturally more resistant to this important foodborne pathogen.

Keywords: chicken, kinome, peptide array, resistance, *Salmonella*

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Abbreviations: BP, biological process; cfu, colony forming units; C_t, threshold cycle; dpi, days post-infection; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; ERK, extracellular signal-regulated kinase; FDR, false discovery rate; GO, gene ontology; IL, interleukin; JNK, c-Jun N-terminal kinase; KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK, mitogen-activated protein kinase; PBS, phosphate buffered saline; PIIKA2, Platform for Intelligent Integrated Kinome Analysis; PLCG1, Phospholipase C gamma 1; Pyk2, protein tyrosine kinase 2; qRT-PCR, quantitative real-time Reverse Transcriptase-PCR; *S.* Enteritidis, *Salmonella enterica* serovar Enteritidis; STRING, Search Tool for the Retrieval of Interacting Genes; USDA, United States Department of Agriculture; XLT-NN, XLT4 agar base plates with XLT4 supplement and nalidixic acid and novobiocin.

INTRODUCTION

Salmonella enterica serovar Enteritidis (*S.* Enteritidis) is the leading cause of bacterial-derived foodborne illness worldwide (1), and *Salmonella*-related infections originating from poultry and/or poultry products are a significant cause of these human illnesses (2). Studies of global gene expression are informative, but many cellular processes are regulated independently of changes in transcription or translation through post-translational modifications of host proteins.

Phosphorylation is the predominant mechanism of post-translational modification for regulation of protein function and has a central role in virtually every cellular event, as well as strong linkages with many diseases (3). Protein kinases are essential components of all cell signaling networks and events and, therefore, regulate fundamental biological processes (BPs) ranging from cellular growth to death and all processes in between (4). Examining the active kinase enzymes responsible for these phosphorylation events can provide key information into numerous host and cellular functions; therefore, there is a considerable interest in defining kinase activities. Active peptides that represent target sites of kinase enzymes can be printed onto array surfaces/slides (5), and are emerging as an important means of characterizing kinome activity (6). Global analysis of the kinome provides information on the abundance, activity, substrate specificity, phosphorylation pattern, and mutational status (4). Our laboratory has designed and developed chicken-specific arrays targeting immune and metabolism kinome activities (7, 8). Kinome analysis using peptide arrays provide site-specific information, display similar biochemical properties to the full protein, and have demonstrated considerable potential as a cost-effective, high-throughput approach for defining phosphorylation-mediated events (9); therefore, potentially making it possible to identify specific biomarkers associated with a desired phenotype.

Previously, our laboratory developed a novel selection method based on identification and selection of chickens with naturally high levels of pro-inflammatory mediators, including interleukin (IL) 6, CXCLi2, and CCLi2 and demonstrated the resultant chickens are more resistant to the foodborne pathogen *S.* Enteritidis (10) and other key foodborne and poultry pathogens (11–13). While our original selection strategy proved effective, an approach utilizing kinome analysis could provide a new molecular-based tool that offers the potential for high-throughput screening and selection of chickens. Identification of specific biomarkers that the poultry industry could use to select individual birds that are more resistant to cecal colonization with *S.* Enteritidis would be beneficial to the industry. This could potentially lead to either fewer *S.* Enteritidis positive birds entering the processing plant or reducing the load of bacteria the birds are carrying and, therefore, fewer positive chicken products reaching the consumer.

Within a single genetic line of birds, one would expect to find individuals that are more or less susceptible to *Salmonella* than some flock mates. The objectives of this study were to (1) identify chickens within a single genetic population with high and low loads of *S.* Enteritidis cecal colonization following an oral challenge, (2) perform innate immune kinome analysis to monitor kinase-mediated signaling activity on jejunal samples from non-challenged, high load *S.* Enteritidis, and low load *S.* Enteritidis birds at three distinct time points, and (3) identify the immunological processes and signaling pathways associated with enhanced resistance to *S.* Enteritidis cecal colonization within a single line of chickens at three times over the 42-day grow-out.

MATERIALS AND METHODS

Experimental Animals

All experiments were conducted according to guidelines established by the United States Department of Agriculture (USDA) animal care and use committee, which operates in accordance with established principles (14). Broiler chickens from a single genetic lineage were obtained from a commercial hatchery. At hatch, straight-run (mixed sexes) chicks were placed in floor pens (4 m × 4 m) containing wood shavings, supplemental heat, water and a balanced, un-medicated corn, and soybean meal-based chick starter diet *ad libitum*. The feed contained 23% protein and 3,200 kcal of metabolizable energy/kg of diet, and all other nutrient levels met or exceeded established requirements (15). The birds were not vaccinated or given any medications during the course of the study.

Bacteria Preparation

A poultry isolate of *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) was obtained from the National Veterinary Services Laboratory (Ames, IA, USA), and was selected for resistance to nalidixic acid and novobiocin and maintained in tryptic soy broth (Difco Laboratories, Sparks, MD, USA) containing antibiotics (20 μg/mL nalidixic acid and 25 μg/mL novobiocin; Sigma Chemical Co., St. Louis, MO, USA). A stock culture was prepared in sterile phosphate buffered saline (PBS) and adjusted to a concentration of 1×10^9 colony forming units (cfu)/mL as previously described (16). The challenges were then diluted from the 1×10^9 cfu/mL stock culture to the desired concentration. The viable cell concentration of the challenge dose for each experiment was determined by colony counts on XLT4 agar base plates with XLT4 supplement (Difco) and nalidixic acid and novobiocin (XLT-NN).

Bacterial Challenge and Recovery

Four-day-old broiler chicks were challenged orally with *S.* Enteritidis (0.5 mL; 4.8×10^5 cfu/chick) while controls were administered sterile PBS; 0.5 mL. Cloacal swabs were collected 3 days post challenge to confirm the controls were not infected and that all birds that were challenged were culture positive for *S.* Enteritidis. Briefly, a sterile cotton swab was gently inserted into the cloaca and a fecal sample was collected. The entire swab and sample for each bird was then placed into a separate tube containing tetrathionate enrichment broth (10 mL, Difco) and incubated overnight at 41°C. Following enrichment, 10 μL were streaked onto XLT-NN plates, incubated 24 h at 41°C, then the plates examined for non-lactose fermenting NN-resistant *Salmonella* colonies. Representative colonies were confirmed positive by plate agglutination using specific Group D_1 antisera (Difco).

Sample Collection and Processing

One-day-old broiler chickens were randomly distributed into two experimental groups: non-infected control and infected ($n = 50$). Early samples were collected between 4 and 7 days post-infection (dpi), middle samples were collected between 10 and 17 dpi, and late samples were collected between 24 and 37 dpi. The experiments were conducted on two separate occasions.

Control and infected chickens ($n = 10$) were euthanized by cervical dislocation and necropsied at three timeframes (early, middle, and late) over the course of a 42-day grow-out. The cecum from each chicken was removed aseptically, and the contents (0.25 g) were serially diluted to 1:100, 1:1,000, or 1:10,000 and spread onto XLT-NN plates to enumerate *S.* Enteritidis. The plates were incubated at 41°C for 24 h, and the number of NN-resistant *S.* Enteritidis cells per g of cecal contents determined. A piece of jejunum (100 mg) was collected and rinsed with PBS to remove content and then placed into a cryovial containing 1.5 mL RNA*later* RNA stabilization reagent (Qiagen, Valencia, CA, USA) and stored at −20°C until tissue homogenization and RNA isolation was performed for quantitative real-time Reverse Transcriptase-PCR (qRT-PCR). Additionally, a section of jejunum (100 mg) was collected from each bird, rinsed with PBS to remove content, and then immediately flash frozen in liquid nitrogen to preserve kinase enzymatic activity for the array. Samples were taken from liquid nitrogen and transferred to a −80°C freezer until further experimental procedures were conducted. Following microbiological analysis of the cecal contents (described previously), the jejunum tissues from four birds with the highest and four birds with the lowest levels of recoverable *S.* Enteritidis (out of the 10 birds per time) were used for the peptide arrays.

Kinome (Peptide) Array

PepStar peptide microarrays were obtained from JPT Peptide Technologies GmbH (Berlin, Germany), and the peptide array protocol was carried out as previously described (6) with the following modifications (8, 17). Jejunum tissue samples were weighed to obtain a consistent 40 mg sample for the array protocol. Samples were homogenized by a hand-held TissueRuptor (Qiagen, Valencia, CA, USA) in 100 µL of lysis buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM Ethylenediaminetetraacetic acid, 1 mM ethylene glycol tetraacetic acid, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mM NaF, 1 µg/mL leupeptin, 1 g/mL aprotinin, and 1 mM Phenylmethylsulphonyl fluoride). All chemicals purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA) unless otherwise indicated.

Antibody Array

The Phospho Explorer Antibody Array kit (catalog PEX100; Full Moon BioSystems, Sunnyvale, CA, USA) consists of over 1,300 antibodies from over 30 signaling pathways and is an alternative approach to procuring phosphor-specific antibodies individually and performing numerous western blot assays. The protocol was carried out as per manufacturer's instructions with the exception that the tissue was homogenized using a hand-held TissueRuptor (Qiagen, Valencia, CA, USA) instead of the bead and vortex method suggested in the kit.

Data Analysis: Kinome and Antibody Arrays

Data normalization and analysis was performed for both the kinome and antibody microarrays as described (17). Images were gridded using GenePix Pro software, and the spot intensity signal was collected as the mean of pixel intensity using local feature background intensity calculation with the default scanner saturation level. The data was then analyzed using the Platform for Intelligent Integrated Kinome Analysis (PIIKA2) peptide array analysis software (http://saphire.usask.ca/saphire/piika/index.html). Briefly, the resulting data points were normalized to eliminate variance due to technical variation, for example, random variation in staining intensity between arrays or between array blocks within an array. Variance stabilization and normalization was performed. Note: as the arrays were printed with triplicate peptide blocks there are three values for each peptide. Using the normalized data set comparisons between treatment and control groups were performed, calculating fold-change and a significance *P*-value. The *P*-value is calculated by conducting a one-sided paired *t*-test between treatment and control values for a given peptide.

This consistent analysis method facilitates a more direct comparison between the two distinct array datasets and allows for a statistically robust analysis of the phosphorylation events being measured. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed by uploading the statistically significant peptide lists to the Search Tool for the Retrieval of Interacting Genes (STRING[1]) (18).

Isolation of Total RNA for qRT-PCR

Tissue homogenization was performed using a BeadBug microtube homogenizer (Benchmark Scientific, Edison, NJ, USA). Briefly, a piece of tissue (30–40 mg) was removed from RNA*later* and placed in a 2 mL prefilled tube containing 1.5 mm high impact zirconium beads (TriplePure M-Bio Grade; Benchmark Scientific). Lysis buffer (350 µL; RNeasy Mini Kit; Qiagen) was added and the sample was homogenized in the BeadBug for 2 min on the maximum speed. Total RNA was then isolated from the homogenized samples according to the manufacturer's instructions, eluted with 50 µL RNase-free water, and stored at −80°C until qRT-PCR analyses performed.

Quantitative Real-time RT-PCR

Interleukin 6 and CXCLi2 mRNA expression was quantified using a well-described method. Primers and probes for cytokines, chemokines, and 28S RNA-specific amplification has been previously described (19–21). The qRT-PCR was performed using the TaqMan one-step RT-PCR master mix reagents (Applied Biosystems, Branchburg, NJ). Amplification and detection of specific products were performed using the Applied Biosystems 7500 Fast Real-Time PCR System with the following cycle profile: one cycle of 48°C for 30 min, 95°C for 20 s, and 40 cycles of 95°C for 3 s and 60°C for 30 s. Quantification was based on the increased fluorescence detected by the 7500 Fast Sequence

[1] www.phosphosite.org.

Detection System due to hydrolysis of the target-specific probes by the 5′ nuclease activity of the *rTth* DNA polymerase during PCR amplification. To correct for differences in RNA levels between samples within the experiment, the correction factor for each sample was calculated by dividing the mean threshold cycle (C_t) value for 28S rRNA-specific product for each sample, by the overall mean C_t value for the 28S rRNA-specific product from all samples. The corrected cytokine mean is calculated: (Average of each replicate × cytokine slope)/28s slope × 28 s correction factor. The data shown are corrected 40-C_t values.

Statistical Analyses

The mean and SEM for each cytokine/chemokine were calculated at each time and statistical analyses performed (Student's *t*-test); comparisons were made between a single timeframe comparing the birds with high and low levels of *S.* Enteritidis cecal colonization. No statistical analysis was performed for the differences in bacterial load. Details for the array analysis are provided in the Data Analysis: Kinome and Antibody Arrays section described above. For all analyses, significance was considered if $P \leq 0.05$.

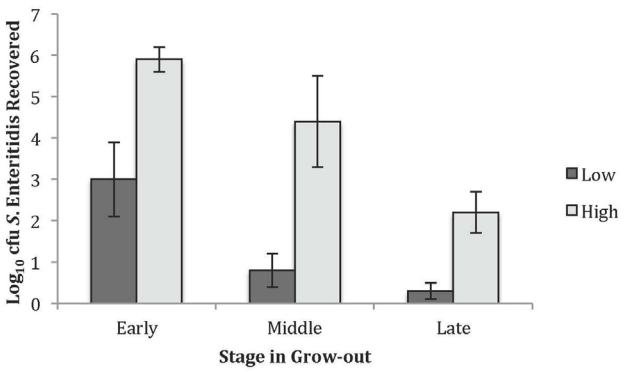

FIGURE 1 | Recovered *Salmonella* Enteritidis in the high and low groups used for the peptide and antibody arrays. The average Log_{10} colony forming units recovered for the low and high *S.* Enteritidis groups at the early, middle, and late times.

RESULTS

S. Enteritidis Colonization

Cloacal swabs were collected on all birds in the study three days post challenge to confirm the controls were not infected and that all challenged birds were culture positive for the challenge strain of *S.* Enteritidis. All birds administered the challenge were positive for *S.* Enteritidis while all of the controls were negative (data not shown).

Within this single line of birds, varying levels of *S.* Enteritidis cecal colonization were observed and, therefore, studying birds with relatively high and low numbers of recoverable *S.* Enteritidis was pursued. The levels of *S.* Enteritidis colonization for the high and low groups at each timeframe are summarized in **Figure 1**. Chickens in the early time point and designated to the low group had 3.0 ± 0.9 Log_{10} cfu of recoverable *S.* Enteritidis while the birds in the high group had 5.9 ± 0.3 Log_{10} cfu. The values for the middle group were 0.8 ± 0.4 and 4.4 ± 1.1 Log_{10} cfu *S.* Enteritidis for the low and high groups, respectively. The recoverable *S.* Enteritidis continued to decline by the late timeframe and were 0.3 ± 0.2 and 2.2 ± 0.5 Log_{10} cfu *S.* Enteritidis for the low and high groups, respectively.

GO BPs

The GO Consortium assigns defined terms representing gene product properties and is broken into three categories: cellular

TABLE 1 | Gene ontology (GO) biological process (BPs) terms identified using the peptide array and the number of differentially phosphorylated peptides associated with high and low loads of *Salmonella* Enteritidis colonization.

GO ID	BP term	Early low SE	Early high SE	Middle low SE	Middle high SE	Late low SE	Late high SE
GO.0050776	Regulation of immune response	60	26	52	40	41	43
GO.0045087	Innate immune response	61	31	55	42	44	43
GO.0002764	Immune response-regulating signaling pathway	48	21	45	34	34	35
GO.0002768	Immune response-regulating cell surface receptor signaling pathway	40	17	38	30	31	28
GO.0002684	Positive regulation of immune system process	45	21	45	29	32	29
GO.0002376	Immune system process	60	28	63	47	46	42
GO.0034097	Response to cytokine	37	16	26	15	24	22
GO.0043549	Regulation of kinase activity	38	17	40	33	25	28
GO.0034142	TLR4 signaling pathway	18	7	17	11	10	9
GO.0000165	Mitogen activated protein kinase cascade	20	8	19	21	12	12
GO.0009617	Response to bacterium	23	6	16	11	14	18
GO.0048522	Positive regulation of cellular process	77	36	66	52	49	54
GO.0006935	Chemotaxis	27	12	22	22	19	7
GO.0006909	Phagocytosis	16	6	11	8	12	12
GO.0006954	Inflammatory response	17	6	9	10	11	11
GO.0001932	Regulation of protein phosphorylation	49	22	48	38	29	37
GO.0006952	Defense response	67	30	58	43	47	43
GO.0007166	Cell surface receptor signaling pathway	81	42	72	58	57	60

SE, S. Enteritidis.

component, molecular function, and BP. The BP terms include operations or sets of molecular events with a defined beginning and end that pertain to the functioning of the integrated living units.[2] Using STRING functionality, GO results for BP were generated for each dataset. The total number of BPs associated with each time and bacterial load were: early low = 1,218; early high = 617; middle low = 1,084; middle high = 1,001; late low = 942; and late high = 1,049 (data not shown). The most significant (based on *P*-value) immunologically relevant GO BP were selected for further analysis. Analysis of the kinome data showed distinct differences in the observed BP between the loads of bacteria, and some of the central differences are provided in **Table 1**. Each term listed had a false discovery rate (FDR) $P \leq 0.01$. The BP with high numbers of peptide phosphorylation events associated with low levels of *S.* Enteritidis colonization regardless

[2]http://geneontology.org/page/ontology-documentation.

TABLE 2 | Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways identified with the peptide array at the early stage infections in chickens with high and low levels of *Salmonella* Enteritidis colonization.

High *S.* Enteritidis	Number of peptides	Low *S.* Enteritidis	Number of peptides
B cell receptor signaling pathway	4	B cell receptor signaling pathway	4
Mitogen activated protein kinase (**MAPK) signaling pathway**	**6**	Chagas disease	4
		Chemokine signaling pathway	**4**
		Epithelial cell signaling pathway in *Helicobacter pylori* infection	2
		ErbB signaling pathway	6
		Fc ε RI signaling pathway	**5**
		Fc-γ receptor-mediated phagocytosis	5
		Focal adhesion	**6**
		GnRH signaling pathway	3
		Insulin signaling pathway	**7**
		Jak–Stat signaling pathway	**7**
		MAPK signaling pathway	**8**
		mTor signaling pathway	2
		Natural killer cell mediated cytotoxicity	6
		Neurotrophin signaling pathway	**6**
		Osteoclast differentiation	4
		Pathways in cancer	**10**
		T cell receptor signaling pathway	**6**
		Toll-like receptor signaling pathway	2
		Toxoplasmosis	3
		Tuberculosis	**3**
		VEGF signaling pathway	5

Pathways listed in bold showed statistically significant changes at all time points in birds with either low or high loads of S. Enteritidis.

TABLE 3 | Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways identified with the peptide array at the middle stage infections in chickens with high and low levels of *Salmonella* Enteritidis colonization.

High *S.* Enteritidis	Number of peptides	Low *S.* Enteritidis	Number of peptides
Bacterial invasion of epithelial cells	5	**Chemokine signaling pathway**	**5**
Chemokine signaling pathway	7	ErbB signaling pathway	4
Chronic myeloid leukemia	4	**Fc ε RI signaling pathway**	**2**
ErbB signaling pathway	7	**Focal adhesion**	**2**
Fc ε RI signaling pathway	1	GnRH signaling pathway	4
Focal adhesion	6	**Insulin signaling pathway**	**3**
Insulin signaling pathway	5	**Jak–Stat signaling pathway**	**4**
Mitogen activated protein kinase (**MAPK) signaling pathway**	**6**	Leukocyte transendothelial migration	2
Natural killer cell mediated cytotoxicity	4	**MAPK signaling pathway**	**4**
Neurotrophin signaling pathway	6	mTor signaling pathway	2
Pathways in cancer	6	**Neurotrophin signaling pathway**	**5**
T cell receptor signaling pathway	4	**Pathways in cancer**	**2**
		Regulation of actin cytoskeleton	1
		T cell receptor signaling pathway	**1**
		Tuberculosis	**3**

Pathways listed in bold showed statistically significant changes at all time points in birds with either low or high loads of S. Enteritidis.

TABLE 4 | Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways identified with the peptide array at the late stage infections in chickens with high and low levels of *Salmonella* Enteritidis colonization.

High *S.* Enteritidis	Number of peptides	Low *S.* Enteritidis	Number of peptides
Endocytosis	2	B cell receptor signaling pathway	3
ErbB signaling pathway	3	**Chemokine signaling pathway**	**5**
Focal adhesion	3	ErbB signaling pathway	3
Jak–Stat signaling pathway	2	**Fc ε RI signaling pathway**	**3**
mitogen activated protein kinase (**MAPK) signaling pathway**	**2**	Fc-γ R-mediated phagocytosis	2
Neurotrophin signaling pathway	2	**Focal adhesion**	**3**
Osteoclast differentiation	1	**Insulin signaling pathway**	**2**
Pathways in cancer	3	**Jak–Stat signaling pathway**	**3**
T cell receptor signaling pathway	1	**MAPK signaling pathway**	**1**
Toll-like receptor signaling pathway	2	Natural killer cell mediated cytotoxicity	2
Toxoplasmosis	2	**Neurotrophin signaling pathway**	**3**
Tuberculosis	3	Osteoclast differentiation	3
VEGF signaling pathway	2	**Pathways in cancer**	**5**
		T cell receptor signaling pathway	**2**
		Toll-like receptor signaling pathway	2
		Toxoplasmosis	2
		Tuberculosis	**2**
		VEGF signaling pathway	3

Pathways listed in bold showed statistically significant changes at all time points in birds with either low or high loads of S. Enteritidis.

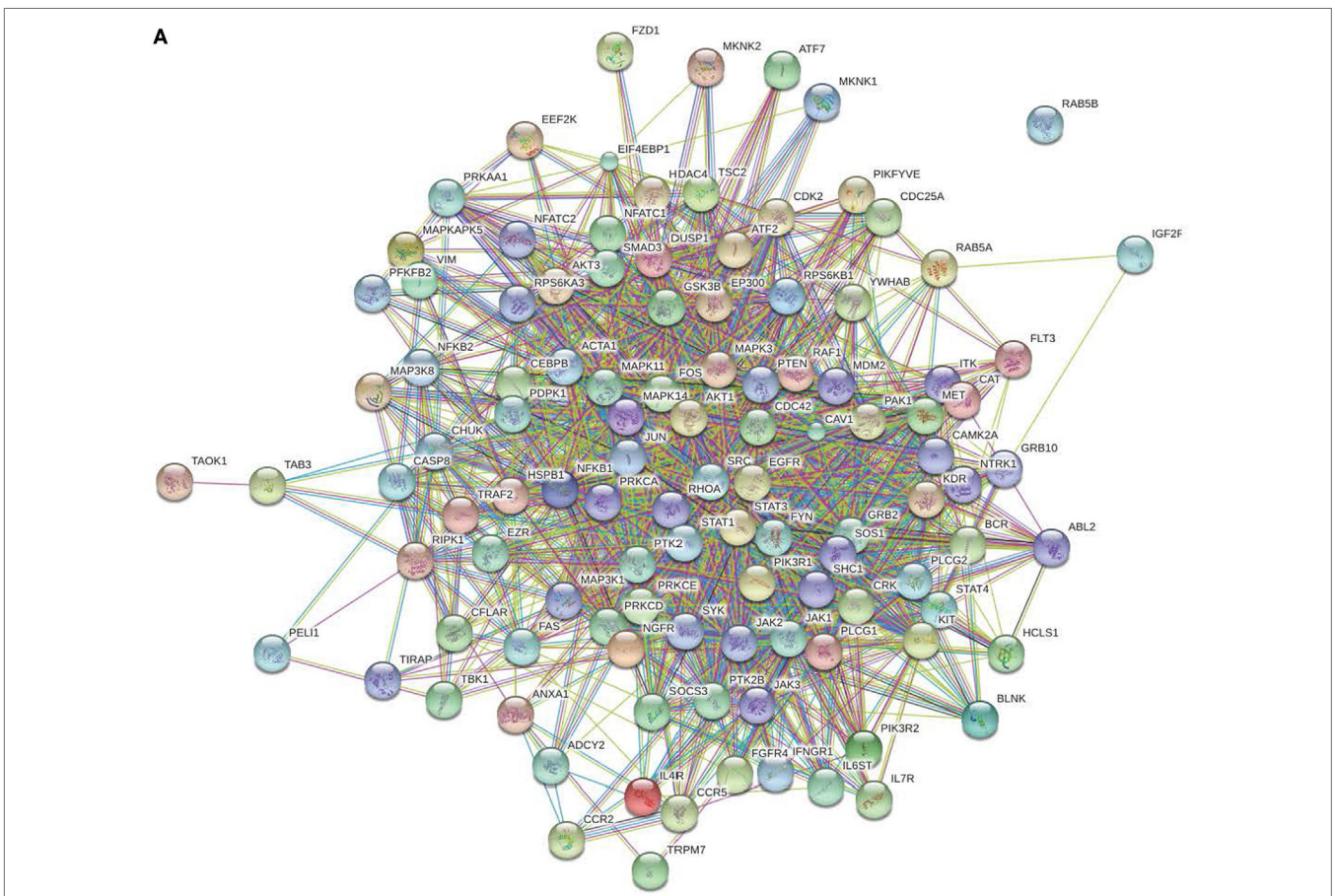

FIGURE 2 | Protein–protein interactions. Predicted protein–protein interactions generated following analysis of significantly different peptides input into the STRING database: **(A)** early low interactions. (Continued)

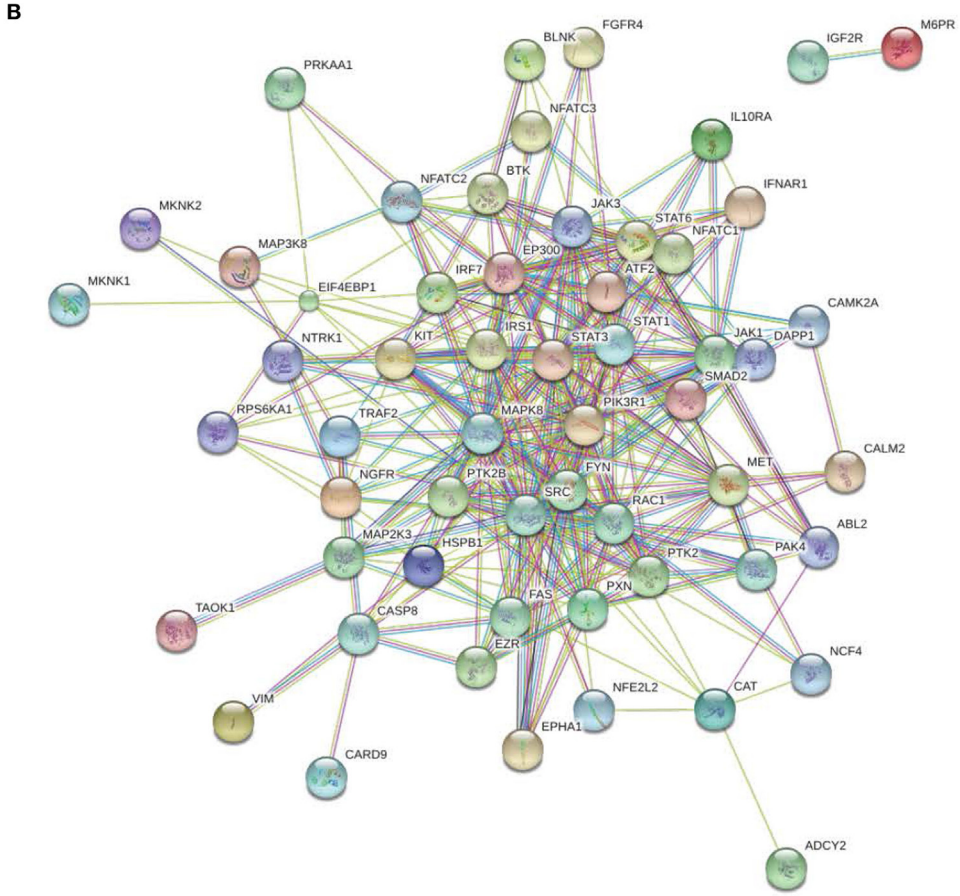

FIGURE 2 | Protein–protein interactions. Predicted protein–protein interactions generated following analysis of significantly different peptides input into the STRING database: **(B)** early high interactions. (Continued)

of time (early, middle, or late) are clustered around immune responses and subsequent signaling pathways and include: cell surface receptor signaling pathway, positive regulation of cellular processes, defense response, innate immune response, regulation of immune response, immune system process, and regulation of signaling. Activation of the BP were observed at the early, middle, and late infection times, but the numbers of differentially phosphorylated peptides between the low/high loads of *S*. Enteritidis were greatest at the early and middle points. By late in the infection, the numbers of peptides was similar between the birds with low and high loads of *S*. Enteritidis as might be expected based on the overall lower numbers of bacteria recovered (**Figure 1**). These data point to the early immune mechanisms that aid in controlling *S*. Enteritidis.

KEGG Pathway Activation

Using STRING functionality, KEGG pathway results were generated for each dataset. To ensure that changes in phosphorylation were a direct result of the infection, the results were corrected using the appropriate age-matched controls. The KEGG pathway results showed numerous pathways that were significantly different between the birds with high and low loads of *S*. Enteritidis at each time of the infection ($P \leq 0.05$ FDR). In order to be included, a pathway had to be significant for each bird within a group and time. Pathways that were not significant for each bird at a specific time and bacterial load were excluded. Additionally, the numbers for a subset of the significantly different peptides within each of the KEGG pathways are also provided. The numbers shown are a small fraction of the total number of significant peptides within a specific pathway; however, as based on our criteria to be included in the dataset, a peptide had to be statistically significantly different from control for every bird in a given group (i.e., every early/low bird, early/high bird, middle/low bird, middle/high bird, late/low bird, or late/high birds).

The significant KEGG pathways observed at the early time are shown in **Table 2**, and the pathways for the middle and late times are provided in **Tables 3** and **4**, respectively. Pathways listed in bold showed statistically significant changes at all time points in birds with either low or high loads of *S*. Enteritidis. The common pathways observed in chickens with low loads of *S*. Enteritidis included: chemokine signaling pathway, Fc ε RI signaling pathway, focal adhesion, insulin signaling pathway, Jak–Stat signaling pathway, mitogen-activated protein kinase (MAPK) signaling pathway, neurotrophin signaling pathway, pathways in cancer, T cell receptor signaling pathway, and Tuberculosis. The specific proteins associated with each of the pathways, including those

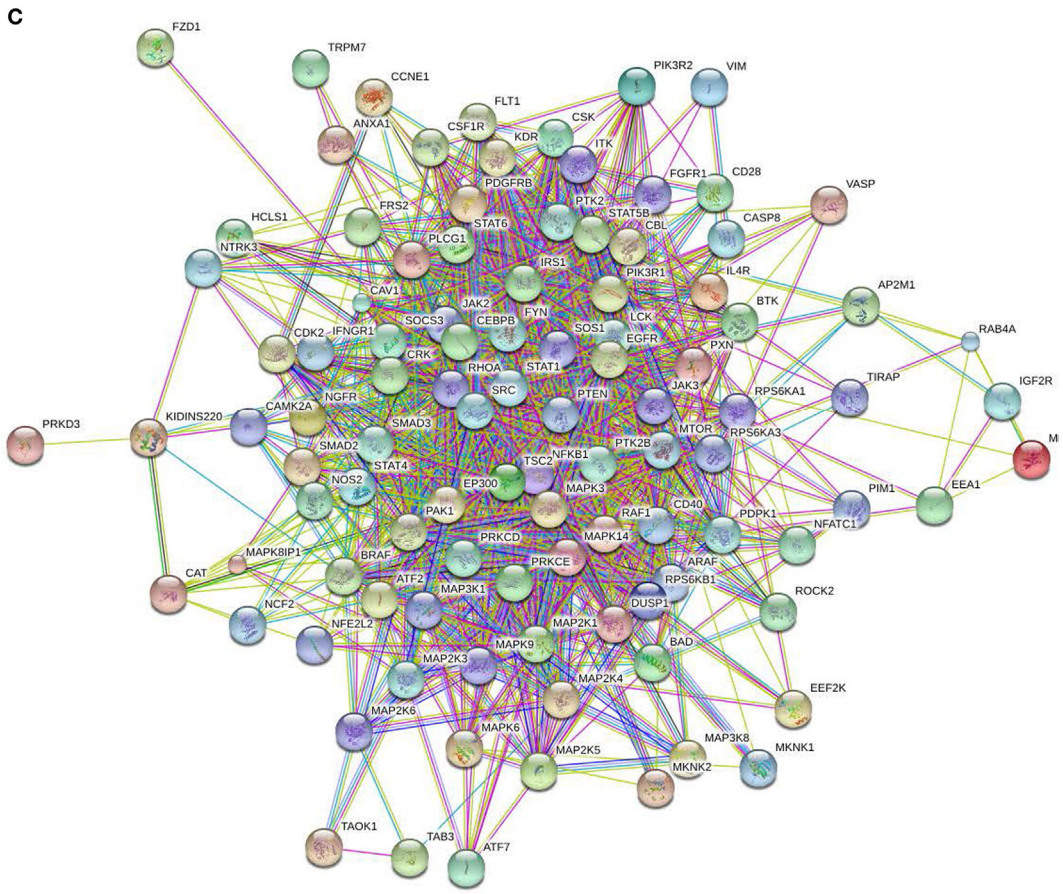

FIGURE 2 | Protein–protein interactions. Predicted protein–protein interactions generated following analysis of significantly different peptides input into the STRING database: **(C)** middle low interactions. (Continued)

specifically related to cancer, would also be pivotal in determining the hosts' immunological response against a challenge and/or disease. The only pathway that was significantly different at each time in birds with high levels of *S*. Enteritidis colonization was the MAPK signaling pathway.

All peptides that were statistically different ($P \leq 0.05$) for each time were input into the STRING database (22) and diagrams depicting the protein–protein interactions were generated (**Figure 2**). The most striking difference in the magnitude of the protein–protein interactions was observed between the birds with low or high loads of *S*. Enteritidis colonization at the early time (**Figures 2A,B**, respectively). The protein–protein interactions for middle low and middle high birds are shown in **Figures 2C,D**, respectively, and the late low and late high interactions are shown in **Figures 2E,F**, respectively.

Validation Using the Antibody Array

To validate the kinome peptide array data, an antibody array containing both pan-specific and phospho-specific antibodies was employed instead of performing individual western blot analyses (8). Analysis of antibody array data *via* the STRING database showed similar GO BP and KEGG pathway activity at the early and middle times as observed with the kinome array; representative samples are shown in **Table 5**. Though outside the scope of this manuscript, but for validation purposes only, a small number of individual peptides associated with KEGG pathways identified with the kinome array (**Tables 2–4**) were selected and evaluated to demonstrate consistency between the kinome and antibody arrays. Phospholipase C gamma 1 (PLCG1), protein tyrosine kinase 2 (Pyk2), Raf1, and SMAD2 shared similar phosphorylation/de-phosphorylation patterns and were, in general, in agreement between the two arrays, thus further validating the kinome array results (**Table 6**). The antibody array was not utilized on the late samples since consistency was demonstrated at the early and middle times. Furthermore, as already described, there were fewer differences between the birds with high and low loads of *S*. Enteritidis at the late time point lessening the applicability of a comparative validation technique.

Quantitative Real-time RT-PCR

The expression of IL6 and CXCLi2 mRNA was quantified (40-C_t) in tissue from birds with high and low levels of *S*. Enteritidis cecal colonization at early, middle, and late times. Birds with lower levels of *S*. Enteritidis at the early time point had

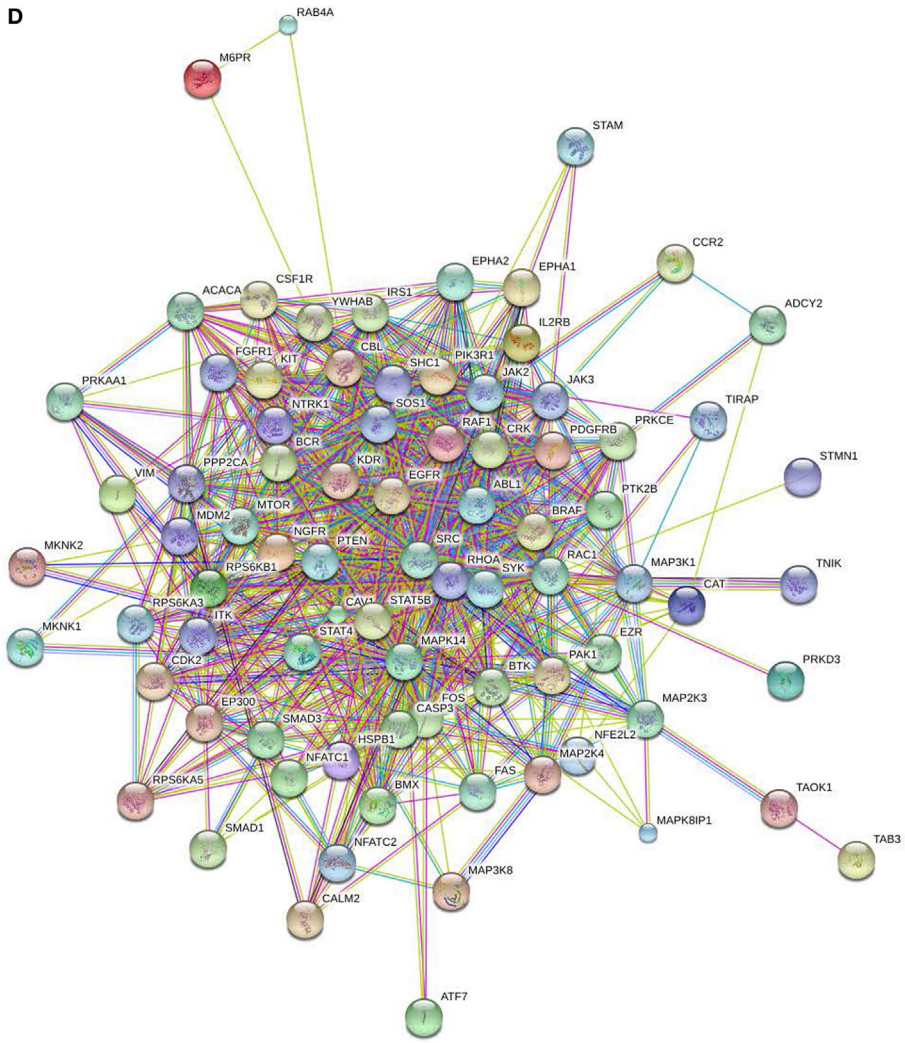

FIGURE 2 | Protein–protein interactions. Predicted protein–protein interactions generated following analysis of significantly different peptides input into the STRING database: **(D)** middle high interactions. (Continued)

significantly ($P \leq 0.05$) higher mRNA expression levels of CXCLi2 than birds with higher loads of *S.* Enteritidis (14.5 and 13.6, respectively). There were no statistical differences in CXCLi2 mRNA expression at the middle and late times. There were no differences in IL6 between birds with high and low loads of *S.* Enteritidis colonization compared to one another or the respective controls at the early, middle, or late times (data not shown).

DISCUSSION

Laboratory challenges using animal models are a vital component for making scientific advances regardless of the field of study. Despite controlling for host genetics, environmental conditions, and challenge preparation and recovery methodologies, investigators accept there will be a certain amount of variability observed between individual animals. Such variability was observed within the line of birds evaluated in the present study, and despite 100% of the challenged chickens being culture positive for *S.* Enteritidis, the actual numbers of recoverable bacteria varied between individuals (**Figure 1**). A recent study suggests the differences in bacterial growth and immune responses seen across genetically identical mice is a result of specific immune elements that facilitate the co-regulation and interconnectedness of the innate and adaptive immune responses (23). The observed differences in cecal colonization could also be due, in part, to intermittent shedding. It is widely recognized that chickens shed varying levels of *S.* Enteritidis over time (24, 25). Observing the different levels of cecal colonization in the study presented herein led us to hypothesize that differences may be detectable by evaluating the host kinome response and, therefore, provide valuable insight into the mechanism(s) that either limits or enables *S.* Enteritidis, one of the most important foodborne bacteria, to colonize the chicken ceca.

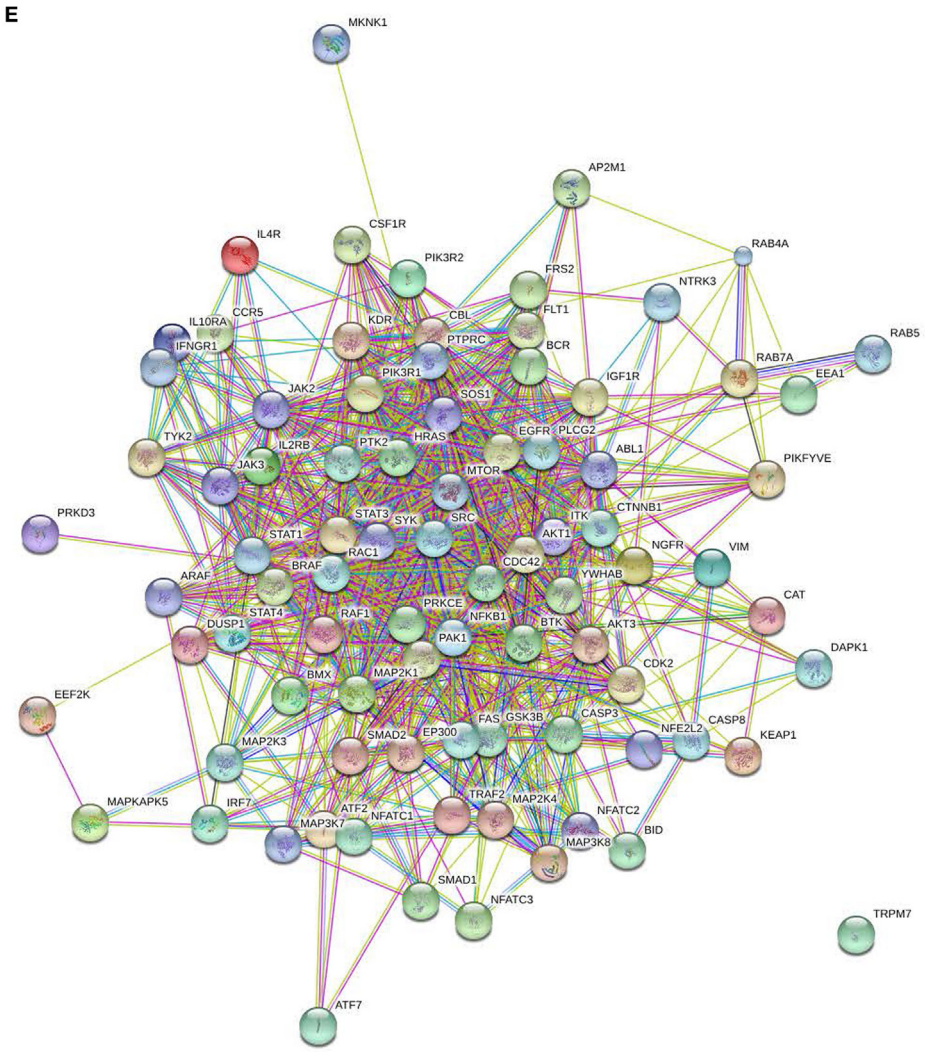

FIGURE 2 | Protein–protein interactions. Predicted protein–protein interactions generated following analysis of significantly different peptides input into the STRING database: **(E)** late low interactions. (Continued)

The results presented herein revealed that birds with high and low levels of cecal colonization with *S.* Enteritidis, at the time of sampling, have distinct kinome profiles (i.e., protein phosphorylation patterns). As such, key BP and immunologically related pathways associated with increased resistance within a single population of birds were identified (**Tables 1–4**). The signaling pathways that differed between birds with high and low loads of *S.* Enteritidis colonization include those associated with chemokine, Jak–Stat, MAPK, and T cell receptor signaling. Differences in these seminal pathways would be anticipated as several studies in poultry show that strong pro-inflammatory cytokine and chemokine responses are associated with increased resistance against disease (26–30). Moreover, differences within individual components of the MAPK signaling pathway [p38, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK)] have been reported in chickens and turkeys. Genes within the MAPK signaling cascade were mapped and shown to be involved in resistance against *Salmonella* in chickens (31). Another study in chickens showed that increased resistance against *S.* Enteritidis organ invasion is associated with elevated production of p38 and decreased production of JNK (32) while increased production of p38, JNK, and ERK are all influential in determining the level of resistance in turkeys (33). Involvement of the MAPK signaling pathway extends beyond mere immunological responses by the host. In fact, the virulence factors encoded by *Salmonella* can promote either activation or deactivation of the MAPK signaling pathway (34–36), so it is possible the observed changes are a direct result of the bacteria and not necessarily the hosts' response to the challenge. Further studies are necessary to dissect this complex host–pathogen interaction.

Additionally, KEGG analysis showed that pathways in cancer were significantly different across all times in birds with lower levels of *S.* Enteritidis cecal colonization. The authors are not

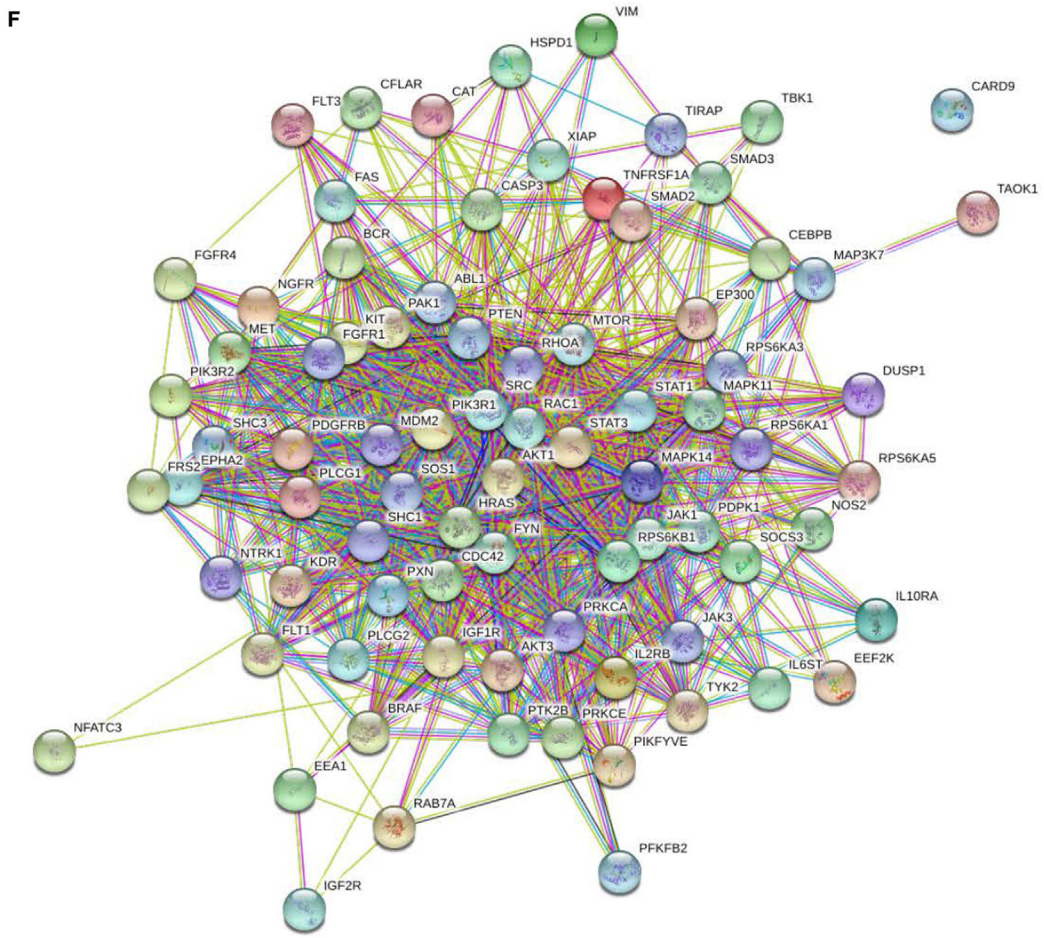

FIGURE 2 | Protein–protein interactions. Predicted protein–protein interactions generated following analysis of significantly different peptides input into the STRING database: **(F)** late high interactions.

TABLE 5 | Gene ontology (GO) biological process (BP) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways identified by the antibody array.

BPs		KEGG pathways	
GO ID	**Term**	**High**	**Low**
GO.0050776	Regulation of immune response	Mitogen activated protein kinase (MAPK) signaling pathway	Pathways in cancer
GO.0045087	Innate immune response		MAPK signaling pathway
GO.0002764	Immune response-regulating signaling pathway		Neurotrophin signaling pathway
GO.0002768	Immune response-regulating cell surface receptor signaling pathway		T cell receptor signaling pathway
GO.0002684	Positive regulation of immune system process		Chemokine signaling pathway
GO.0002376	Immune system process		
GO.0048522	Positive regulation of cellular process		
GO.0006952	Defense response		
GO.0007166	Cell surface receptor signaling pathway		

suggesting the *S.* Enteritidis challenge resulted in cancer in the birds. Of note, studies on the kinome are widely used in cancer research since virtually every cancer displays varying levels of protein and/or lipid kinase dysregulation. Therefore, kinome analysis provides meaningful insight into the pathways and families of kinases involved in specific cancers (37), which would explain why pathways affiliated with cancer are identified in the analysis tools. The specific proteins associated with each

TABLE 6 | Fold-change in specific peptides associated with high and low loads of *Salmonella* Enteritidis using the peptide array and validation with the antibody array.

		Early		Middle	
Peptide	**Accession no.**	**Low peptide (Ab)**	**High peptide (Ab)**	**Low peptide (Ab)**	**High peptide (Ab)**
Phospholipase C gamma 1	P19174	−1.7[a]* (−1.8*)	−1.0 (−1.0)	2.4* (ND)	1.2 (1.5*)
Pyk2	Q14289	ND (−1.5*)	1.7* (1.9*)	ND (1.2*)	1.8* (1.3*)
Raf1	P04049	1.5* (ND)	ND (ND)	1.2 (1.7*)	ND (−2.5*)
SMAD2	Q15796	−2.8* (−5.3*)	1.5* (1.9*)	−1.1 (−5.3*)	1.9 (−7.8*)

(Ab), antibody array; ND, not detected.
[a]*Fold-change from control.*
**P ≤ 0.05.*

of the pathways, including those specifically related to cancer, would also be pivotal in determining the hosts' immunological response against a challenge and/or disease. As previously shown, kinome analysis is beneficial in dissecting pathways involved in animal studies including bovine viral diarrhea virus (38); *in vitro* responses against toll-like receptor agonists (39), Johne's disease in cattle (40), and *Salmonella* in chickens (8, 41) demonstrating the technology is useful in providing valuable information into diverse infections that alter the normal host mechanisms.

Changes in cytokine and chemokine expression in chickens are widely reported following *Salmonella enterica* challenges and/or infections. In the current study, mRNA expression of CXCLi2 was upregulated early in the chickens with lower levels of *S.* Enteritidis. This finding is supported by another study showing CXCLi2 is found in the gut of newly hatched chicks and mRNA expression continues to increase the first week post-hatch (42). More specifically, CXCLi2 mRNA is upregulated in *Salmonella*-resistant chickens (27, 43). CXCLi2 (formerly referred to as IL8) is a potent pro-inflammatory chemokine capable of recruiting immune cells, such as heterophils, to the site of infection (44), and heterophil recruitment is associated with increased resistance against *S.* Enteritidis (16). The role of heterophils was not considered in the current study, but increases in CXCLi2 expression have been reported in various cells and tissue types across diverse breeds of chickens (45–47). Therefore, our study is in agreement and indicates increased CXCLi2 is likely a contributing factor to the lower numbers of bacteria seen at the early time. As might be expected, no differences in CXCLi2 were observed at the middle and late times as the infection had become persistent instead of acute (41). In addition to CXCLi2, IL6 mRNA expression has been shown to increase following infection with *S.* Enteritidis (27, 48). No differences were detected in IL6 mRNA expression, but it is possible the timing of sample collection was not optimized to detect this cytokine. Additional studies are required to understand the role of IL6 and CXCLi2 expression and their impact on influencing the load of cecal colonization of *S.* Enteritidis in broilers over a grow out period.

The current study showed that a single line of birds with high and low levels of cecal colonization with *S.* Enteritidis at the time of collection have distinct kinome profiles. These data support the value of peptide arrays and kinome analysis as a powerful molecular tool to identify key mechanisms and pathways that are associated with increased resistance against *S.* Enteritidis cecal colonization in chickens. These findings provide a foundation for future studies to identify the specific markers associated with lower loads of cecal colonization and will focus on the common pathways identified herein, including chemokine, Jak–Stat, MAPK signaling pathways, or pathways in cancer. Identification of specific biomarkers that the poultry industry could use to select individual birds that are more resistant to cecal colonization with *S.* Enteritidis would be beneficial to the industry. This could potentially lead to either fewer *S.* Enteritidis positive birds entering the processing plant or reducing the load of bacteria the birds are carrying and therefore fewer positive chicken products reaching the consumer.

ETHICS STATEMENT

All experiments were conducted according to guidelines established by the USDA animal care and use committee, which operates in accordance with established principles (14). The protocol was approved by the acting USDA Plains Area animal care and use committee that operates at the location where the experiments were carried out.

AUTHOR CONTRIBUTIONS

CS was the lead investigator and principal author; MK, HH, KG, CJ, and RA were collaborators and coauthors.

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Mention of commercial products is for the sole purpose of providing specific information, not a recommendation or endorsement by the United States Department of Agriculture.

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Impact of Dietary Galacto-Oligosaccharide (GOS) on Chicken's Gut Microbiota, Mucosal Gene Expression, and *Salmonella* Colonization

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Preventing *Salmonella* colonization in young birds is key to reducing contamination of poultry products for human consumption (eggs and meat). While several *Salmonella* vaccines have been developed that are capable of yielding high systemic antibodies, it is not clear how effective these approaches are at controlling or preventing *Salmonella* colonization of the intestinal tract. Effective alternative control strategies are needed to help supplement the bird's ability to prevent *Salmonella* colonization, specifically by making the cecum less hospitable to *Salmonella*. In this study, we investigated the effect of the prebiotic galacto-oligosaccharide (GOS) on the cecal microbiome and ultimately the carriage of *Salmonella*. Day-old pullet chicks were fed control diets or diets supplemented with GOS (1% w/w) and then challenged with a cocktail of *Salmonella* Typhimurium and *Salmonella* Enteritidis. Changes in cecal tonsil gene expression, cecal microbiome, and levels of cecal and extraintestinal *Salmonella* were assessed at 1, 4, 7, 12, and 27 days post infection. While the *Salmonella* counts were generally lower in the GOS-treated birds, the differences were not significantly different at the end of the experiment. However, these data demonstrated that treatment with the prebiotic GOS can modify both cecal tonsil gene expression and the cecal microbiome, suggesting that this type of treatment may be useful as a tool for altering the carriage of *Salmonella* in poultry.

Keywords: chickens, immune, *Salmonella*, prebiotic, galacto-oligosaccharides

INTRODUCTION

Salmonella is a leading cause of foodborne disease in humans with poultry acting as a major source of human infection (1). Controlling *Salmonella* within poultry meat and egg production is critical to increase the safety of these products for human consumption. *Salmonella* infection in poultry is asymptomatic (2), so determining how young birds respond to *Salmonella* is important. Understanding their response will allow for the development of control methods that aid in the removal of *Salmonella* from poultry. While a number of *Salmonella* vaccines have been developed (3, 4), alternative control methods specifically targeting *Salmonella* within the bird's intestinal tract may provide an effective method for reducing intestinal colonization. Prebiotics offer a potential

intestinal *Salmonella* control strategy which can be added to feed and/or water without the need to modify the current production chain.

The reported top *Salmonella* serovars associated with human cases in 2015 were Enteritidis (20%), Newport (12%), and Typhimurium (11%) (5), and salmonellosis from poultry products are one of the top five causes of foodborne disease in the United States (6). Over the past 20 years, a great deal of effort has been spent to try and reduce the role poultry plays in salmonellosis, with some success (7, 8). Most of these efforts have targeted specific serovars, and consequently resulted in increased prevalence of other serovars increase in prevalence (9). Furthermore, different serovars respond differently to changes in the host as well as the host compartments they are associated with (10, 11). This highlights the need to develop methods that can effectively control colonization of multiple serovars.

Salmonella infection in birds is thought to be age dependent. Birds that are infected with *Salmonella* early in life (day 1 of life) carry *Salmonella* for an extended period, and in higher numbers, compared with birds infected at day 8 of life (12). Birds challenged earlier in life appear to clear reinfection slower than birds challenged later in life (3–6 weeks) (13). Exposure to *Salmonella* in the first 4 days post hatch has also been shown to result in detectable changes in the cecum microbiome (14). Modulation of the microbiome through the use of single administration of a *Salmonella* vaccine and or live probiotic (PrimaLac®) at day 1 of life has also been shown to have an effect on the gut microbiome development apparent from day 7 of life (15). However, *Salmonella* exposure at 16 days of life induced fewer changes in cecum microbiota (16). The modification of the microbiome occurring during early infection may allow the establishment of *Salmonella*.

The specialty feed additive market is projected to be worth over US$11 billion globally by 2022 (17). Prebiotics and probiotics represent a major component of this market and provide an alternative strategy, to the use of antibiotics as growth promoters (18, 19). The prebiotic galacto-oligosaccharides (GOS) has been shown to improve the intestinal architecture in the neonatal pig model along with improving the development of the microbiota and stimulating the intestinal defense mechanism (20), indicating that this prebiotic has significant beneficial properties. When fed to chickens, GOS in combination with the enzyme β-galactosidase has been reported to lead to an increase in *Bifidobacteria* and *Lactobacillus* (21). The presence of GOS within the intestinal tract has been shown to reduce the adherence and invasion of *Salmonella* in human enterocytes (22).

Treating birds with prebiotics offers a possible method for reducing *Salmonella* colonization through the modification of both the hosts' immune system and the gut microbiome. The use of oligosaccharides extracts from palm kernels has been shown to improve the health status of broilers and reduce the levels of heterophils and basophils in circulation (23). Indeed, the addition of inulin as a prebiotic or *Lactobacillus lactis* subsp *lactis* 2955 *in ovo* has been shown to result in a reduction in the expression of IL-4, IL-6, IL-8, IL-12p40, and IL-18 in the cecal tonsil, with a reduction seen in the first 35 days after hatch (24). In other studies, *in ovo* administration of prebiotics and probiotics lead to upregulation of cytokine expression in the spleen and decreased expression in the cecal tonsil in birds at 6 weeks of age (25). To understand the effectiveness of prebiotic-supplemented diets on *Salmonella* control, it is important to determine the cecum immune response these treatments induce in poultry and the effect of a subsequent *Salmonella* challenge.

Preventing early infection of chicks is key to reducing the incidence of *Salmonella* in a flock. This study aimed to investigate the response of young birds to treatment with a prebiotic GOS on the carriage of *Salmonella*, cecal tonsil relative gene expression levels of markers of the immune response, and the cecal microbiome. This provides initial information on the effect of GOS on *Salmonella* control and modulation of the bird microbiome and immune response.

MATERIALS AND METHODS

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. All animals were maintained and euthanized according to a protocol no. 15-065-A approved by the Institutional Animal Care and Use Committee (OLAW no. D16-00214). All work was done in an approved biological safety level-2 laboratory or animal facility and appropriate personal protective equipment was used when handling *Salmonella*, a risk group two agent, or *Salmonella* infected birds.

Birds and Experimental Details

Two hundred 1-day-old female Leghorn chickens (Hy-Line; Mansfield, GA, USA) were split into two treatment groups (control and prebiotic). Birds were provided with *ad libitum* food and water with the prebiotic group receiving feed supplemented with 1% functional GOS [1.8% w/w of commercial GOS (Oligomate™ 55NP) (Kanematsu; Somerset, NJ, USA) that contained 55–56% GOS and 44–45% monosaccharides]. The control feed was supplemented with 0.8% glucose to control for the monosaccharides present in the commercial GOS. Birds were housed in 934-1-WP isolators (Federal Designs Inc.; Comer, GA, USA) with regulated temperatures, airflow, 12/12 light/dark cycle, wire flooring, with free access to feed and water. Half of the birds from each treatment group were challenged with100 μL containing a mixture of 5.5×10^8 CFU of rifampicin-resistant (Rif^R) isolate of *S.* Typhimurium FNR-HA—kanamycin-resistant (Kan^R) ATCC 14028s (26) and 6.6×10^8 CFU *S.* Enteritidis ATCC 31194 modified to express FNR-HA—chloramphenicol-resistant (Cm^R) and Rif^R (unpublished) in 100 μL of phosphate-buffered saline (PBS) per bird by oral gavage at day 3 of life; non-challenged birds were given 100 μL of PBS. Challenged birds were housed separately from their non-challenged counterparts. At 1, 4, 7, 12, and 27 days post infection (dpi), eight birds per treatment group were randomly selected, euthanized, and samples of cecum contents, and liver were collected for microbiological analysis, and cecal tonsil collected to assess the effect of diet and *Salmonella* infection on host gene expression.

Salmonella Preparation for Gavage

Overnight cultures of *S.* Typhimurium ATCC 14028s—Kan^R Rif^R (ST) and *S.* Enteritidis ATCC 31194—Cm^R Rif^R (SE) were prepared individually from glycerol stocks in LB (Luria-Bertani) broth (Thermo Fisher Scientific; Waltham, MA, USA) incubated at 37°C without shaking. Antibiotics were added at the following concentrations: Kan, 50 μg/mL; Cm, 20 μg/mL; and Rif, 100 μg/mL. Cells were centrifuged at 8,000× *g* for 15 min and washed three times in PBS with 2-mM magnesium sulfate. Optical density (OD) was determined at 600 nm using a BioRad Smartspec 3000 (BioRad; Hercules, CA, USA) with a 1-cm light path, and adjusted to an OD600 of 10 for ST and 20 for SE (equivalent to ~1,010 CFU/mL of each serovar, according to a standard predetermined relationship between OD600 and viable cell counts). ST and SE cultures were mixed 1:1 just prior to inoculating the chicks. The actual concentration of the bacteria in the gavage mixture was determined by plating a serial dilution on XLT4-Agar (Xylose-Lysine-Tergitol4—Neogen; Lansing, MI, USA) containing the appropriate antibiotics. Challenged birds were given 100 μL of *Salmonella* solution by oral gavage using gavage needles (Thermo Fisher Scientific); control birds were given 100 μL of PBS by oral gavage.

Sample Collection and Preparation

Cecal tonsil tissue, a visible nodule of lymphoid tissue at the proximal end of chicken ceca, was removed and immediately snap frozen in liquid nitrogen and stored at −80°C until processed. Cecum contents were collected from each bird for microbiome and bacteriological analyses. Samples collected for bacteriological analysis were weighed individually and resuspended in 500 μL PBS + 25% glycerol 2-mM $MgSO_4$, and stored at −80°C. Content was serially diluted and plated on XLT4 + 100-mM MOPS (pH 7.4) with relevant antibiotics (ST = Kan, SE = Cm) to determine *Salmonella* levels. The CFU/g of cecum content was determined based on the weight of sample and the volume of PBS. Up to 0.5 g of liver were removed and individually placed in 1 ml PBS + 25% glycerol 2-mM $MgSO_4$ Liver samples were weighed, resuspended to 100 mg/ml and homogenized using a Bio-Gen Pro-200 homogenizer (Pro Scientific Inc.; Oxford, CT, USA). For liver samples that were *Salmonella* negative on XLT4 plates, a volume of a homogenized sample equivalent to 500 mg of liver was enriched in Rappaport—Vassiliadis media (Difco) for 24 h and streaked on XLT4 + 100-mM MOPS (pH 7.4) supplemented with the relevant antibiotics for the detection of SE and ST as stated above. *Salmonella* colonies were confirmed by their resistance to the appropriate antibiotic markers, formation of black colonies on XLY4 plates, and biochemical tests using API- 20E system (Biomerieux; Durham, NC, USA).

RNA Extraction

RNA was extracted from 30 to 50 mg of snap frozen cecal tonsil tissue from each bird in each treatment group and time point. Tissue was homogenized using 2.8-mm ceramic beads (Qiagen; Valencia, CA, USA) and RNA extracted using Nucleospin® RNA kit (Macherey-Nagel; Bethlehem, PA, USA) following the kit protocol with the exception that the RNA was eluted in 50 μL of H_2O. RNA was quantified using a NanoDrop 2000 spectrophotometer (NanoDrop, Wilmington, DE, USA) and then stored at −80°C until needed.

cDNA and Real-Time Polymerase Chain Reaction for Individual Birds

cDNA was made using the High-Capacity cDNA Reverse Transcriptase Kit (Thermo Fisher Scientific) from 1 μg of extracted RNA from each of five birds from each treatment group and time point. Real-time polymerase chain reaction (RT-PCR) was carried out on in triplicate wells for each sample using the ABI Taqman PCR assays listed in **Table 1**. All FAM-labeled gene expression assays for target genes, TaqMan Universal Master mix II, and VIC-labeled Euk 18S rRNA endogenous housekeeping control assay were purchased from Thermo Fisher Scientific. For each sample and each gene of interest, the cDNA was diluted 1:40 in ultrapure water and added to the reaction mix containing 1 μL of gene of interest primer/probe and 1 μL of endogenous control primer/probe; all reactions were carried out in triplicate. RT-PCR was run on an ABI Step-One Plus (Thermo Fisher Scientific) using the standard program (Holding stage: 50°C for 2 min, 95°C for 10 min, Cycle stage: (40 cycles) 95°C for 15 s, 60°C for 1 min). Thresholds were set for both housekeeping and gene of interest set the same within each plate. Each gene was assayed on one plate per time point for all conditions. The ΔCT was calculated (CT of gene of interest—CT of housekeeping gene) for each triplicate well, and an average ΔCT calculated for each RNA sample. The non-treated-non-challenged control RNA sample with the median ΔCT from among the five replicate samples was used as the reference sample for each plate. The ΔΔCT was then determined for all samples [ΔΔCT = ΔCT (average ΔCT of untreated-non-challenged control) − ΔCT (each sample)], such that positive ΔΔCT denotes increased expression and negative ΔΔCT denotes decreased expression. Results are plotted as ΔΔCT which is equal to the $\log_2 2^{\Delta\Delta CT}$. Statistical analysis of the resulting data was carried out using Prism 7.0c (GraphPad Software, Inc.; La Jolla, CA, USA). Differences in *Salmonella* $\log_{10}$ colony forming units, or the ΔΔCT between treatment groups was assessed using a two-way analysis of variance (ANOVA) followed by a Tukey's multiple comparisons test.

TABLE 1 | Assays used in real-time PCR.

Gene	ABI assay ID	Label
MAPK1	Gg03363520_m1	FAM
MAPK14	Gg03323838_m1	FAM
JUN	Gg03356263_s1	FAM
FASLG	Gg03353844_m1	FAM
TLR4	Gg03354643_m1	FAM
MYD88	Gg03355572_m1	FAM
IRF7	Gg03339761_g1	FAM
INFB	Gg03344129_s1	FAM
18S	431913E	VIC

All MGB probes were obtained at 20× working concentration from Thermo Fisher Scientific.
PCR, polymerase chain reaction.

Analysis of Network Determination and Interaction

The pathway network of the eight genes focused on in this study and their connecting genes was determined using Pathway Commons[1] and using neighborhood as the interaction.

Microbiome Analysis

Cecum contents were collected from each bird into duplicate tubes, snap frozen in liquid nitrogen, and stored at −80°C until processed. Isolation of total genomic DNA and microbiome sequencing was performed by Microbiome Core Facility at UNC[2]. Briefly DNA was isolated using a Qiagen BioRobot Universal instrument (Qiagen) and the E.Z.N.A. Stool DNA Kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's specifications. DNA was quantified using a NanoDrop 2000 spectrophotometer (NanoDrop). Amplicons generated from regions V1–V2 of the 16S rRNA gene were sequenced on the Ion Torrent PGM sequencing platform from Life Sciences.

Processing and analysis of sequencing data was done using the Qiagen CLC Genomics Workbench Version 10.1 and the Microbial Genomics Module. Briefly, raw Fastq files were demultiplexed and reads were trimmed to 98 bp. Operational taxonomic units (OTUs) were clustered using the reference Greengenes v13_5 99% database. Chimeras and OTUs with low abundance (less than 10 reads) were removed. Bray–Curtis dissimilarity analysis and permutational multivariate analysis of variance (PERMANOVA) were performed. Statistical analysis of the changes in microbiome was carried out using Prism 7.0c (GraphPad Software, Inc.). Differences in microbiome between control- and prebiotic-treated groups at each time point were assessed using two-way ANOVA using Sidak's multiple comparison test to determine significant difference at the family level. Data presented are the average of eight animals per treatment and time point.

[1]http://www.pathwaycommons.org/about.
[2]https://www.med.unc.edu/microbiome.

RESULTS

Salmonella Typhimurium More Persistent at Invading and Colonizing the Chicken than *Salmonella* Enteritidis

At 1, 4, 7, 12, and 27 dpi, animals were sacrificed and samples collected from the liver and cecum to assess the level of *Salmonella* invasion of systemic tissues, and colonization of the cecum. The results demonstrated that only ST invaded the liver, but only in a few animals, and only between days 4 and 12 post infection (**Table 2**). There was no significant difference ($\alpha = 0.05$) in the numbers of ST positive liver samples between the birds that received the control diet and those fed the prebiotic.

The observation that ST was better able to survive within the chicken's liver was also observed in the cecum (**Figure 1**). While SE and ST were detected in the cecum of both control and prebiotic, there was a difference in the number of positive birds between treatment groups; this was more apparent within the SE infection levels. SE was detectable in 7/8 prebiotic birds at 1 dpi, 3/8 at 4 dpi, and was undetectable by 7 dpi, whereas in control birds SE was detected in 8/8 birds at 1 dpi, 4/8 at 4 dpi, 2/8 at 7 dpi, and then undetectable by 12 dpi (**Figure 1A**). However, ST was able to persist in the cecum much longer (**Figure 1B**). Birds from both the control and prebiotic treatment groups remained positive at 27 dpi with 6/8 positive birds

TABLE 2 | *Salmonella* invasion of the liver—number of positive birds/total challenged.[a]

dpi	Control		Prebiotic	
	Salmonella Typhimurium	*Salmonella* Enteritidis	*Salmonella* Typhimurium	*Salmonella* Enteritidis
1	0/8	0/8	0/8	0/8
4	2/8	0/8	1/8	0/8
7	3/8	0/8	2/8	0/8
12	1/8	0/8	1/8	0/8
27	0/8	0/8	0/8	0/8

[a]*At the specified time points (dpi), eight birds from each treatment were euthanized; liver samples were collected, homogenized, and tested for the presence of ST and SE as described in Section "Materials and Methods."*

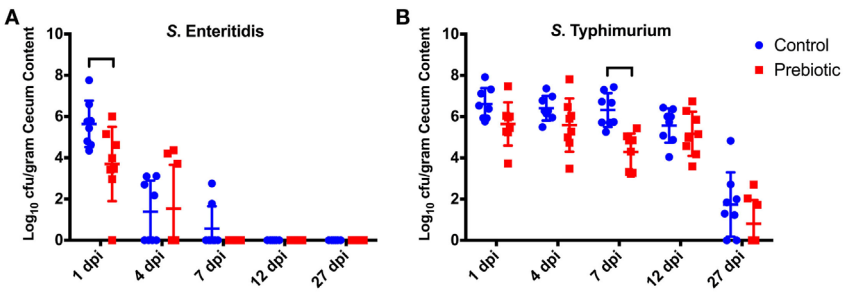


FIGURE 1 | Effect of prebiotic diet on *Salmonella* colonization of the cecum. Birds were fed a conventional diet (Control) or fed a diet containing 1% GOS (Prebiotic). Birds were then infected with 6.6×10^8 CFU SE and 5.5×10^8 CFU of ST by oral gavage at 3 days of age. The levels of SE **(A)** and ST **(B)** were assessed in the cecum at 1–27 days post infection (dpi). The CFU/g of cecal contents is shown for each individual bird sampled at each time point, mean represented by lines. Brackets denote time points where the means were significantly different ($p < 0.05$). GOS, galacto-oligosaccharide.

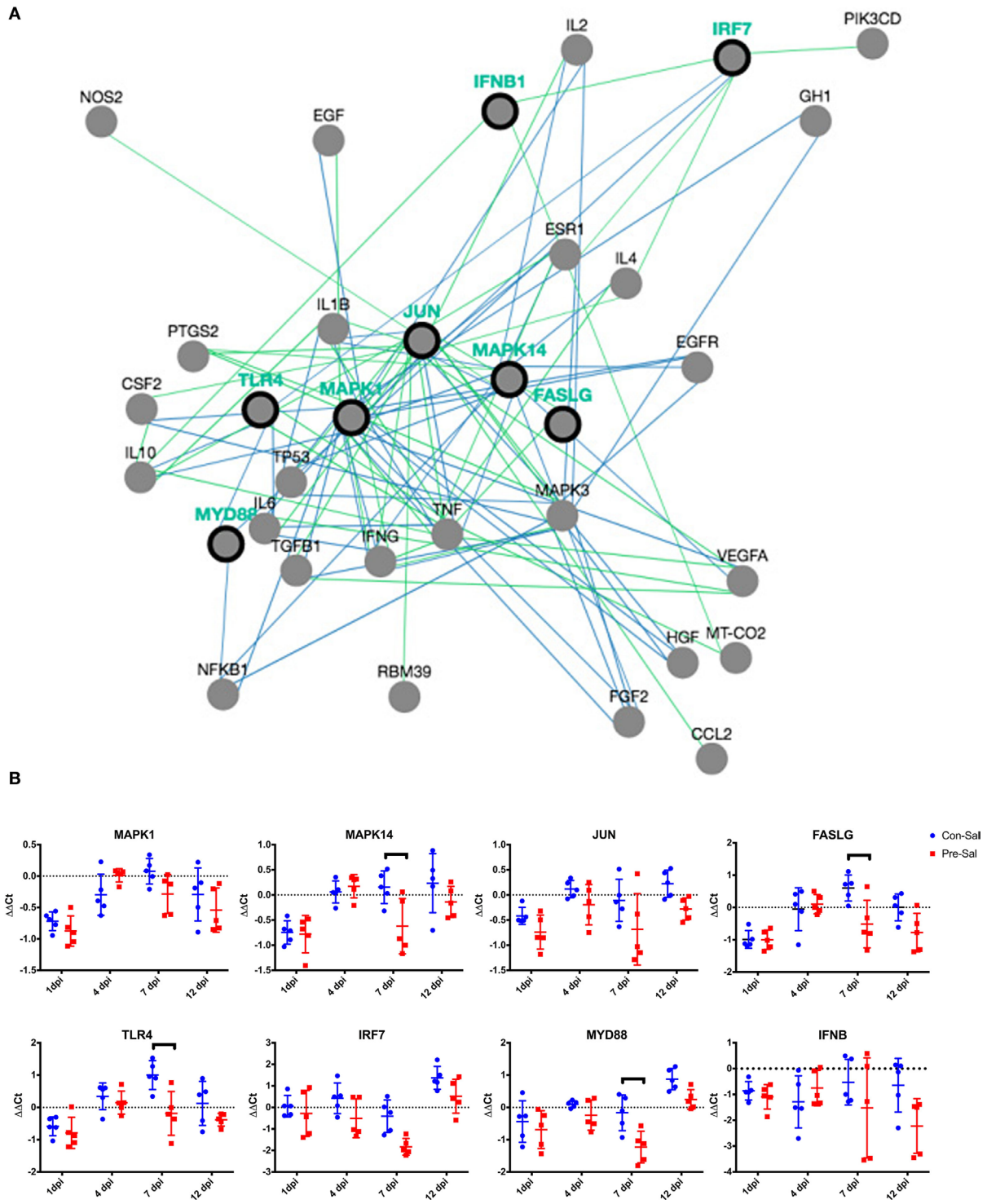


FIGURE 2 | Difference in relative gene expression following *Salmonella* infection between birds fed control or prebiotic diets. **(A)** Gene pathway network showing the eight genes assayed (MAPK1, MAPK14, JUN, FASLG, TLR4, IRF7, MYD88, IFNB, represented by black ringed dots) and 18 nearest neighbors (represented by gray dots). Blue lines denote regulation of protein state. Green lines denote regulation of expression. Network was produced using PCViz (http://www.pathwaycommons.org/). **(B)** Birds were either fed a standard diet (Con) or fed a diet containing GOS (Pre). Birds were infected with SE and ST by oral gavage at 3 days of age (Sal). The ΔΔCt for each individual bird sampled at each time point is represented by blue circles (Con–Sal) or red squares (Pre–Sal). The sample mean is represented by the horizontal line, with upper and lower error bars denoting 1 SD among the five replicate samples. The dashed line denotes no change relative to the control reference sample. Data points above the dashed line represent increased expression, while points below denote decreased expression. Black brackets denote time points where the means were significantly different ($p < 0.05$). GOS, galacto-oligosaccharide.

in the control group and compared with 3/8 in the prebiotic group. In the case of SE the prebiotic-treated birds had significantly lower CFU/g cecum content at 1 dpi compared with the control birds. The prebiotic birds also had significantly lower ST CFU/g cecum content at 7 dpi. No other time points showed significant differences in the carriage of *Salmonella* between treatment groups (**Figure 1**).

Host Response to *Salmonella* Infection

RNA was collected from the cecal tonsils from chicks at 1, 4, 7, and 12 dpi and analyzed for changes in gene expression of MAPK1, MAPK14, JUN, FASLG, TLR4, IRF7, MYD88, and IFNB (**Figure 2A**). Initially samples were compared with identify differences in gene expression between prebiotic- and control-fed animals following *Salmonella* infection. This comparison demonstrated significant ($p < 0.05$) decreases in expression of MAPK14, FASLG, TLR4, and MYD88 at 7 dpi in the prebiotic-treated group (**Figure 2B**). A decrease in the expression was also seen in JUN ($p = 0.051$) and interferon regulatory factor 7 (IRF7) ($p = 0.067$) at 7 dpi in the prebiotic-treated birds.

To understand the effect of GOS treatment on *Salmonella* challenge, the fold change in gene expression between the uninfected and infected animals within in each diet group was determined. This analysis demonstrated that in the control-fed animals, *Salmonella* infection led to a transient downregulation of MAPK1, MAPK14, and FASLG at 1 dpi ($p < 0.05$, **Figure 3A**).

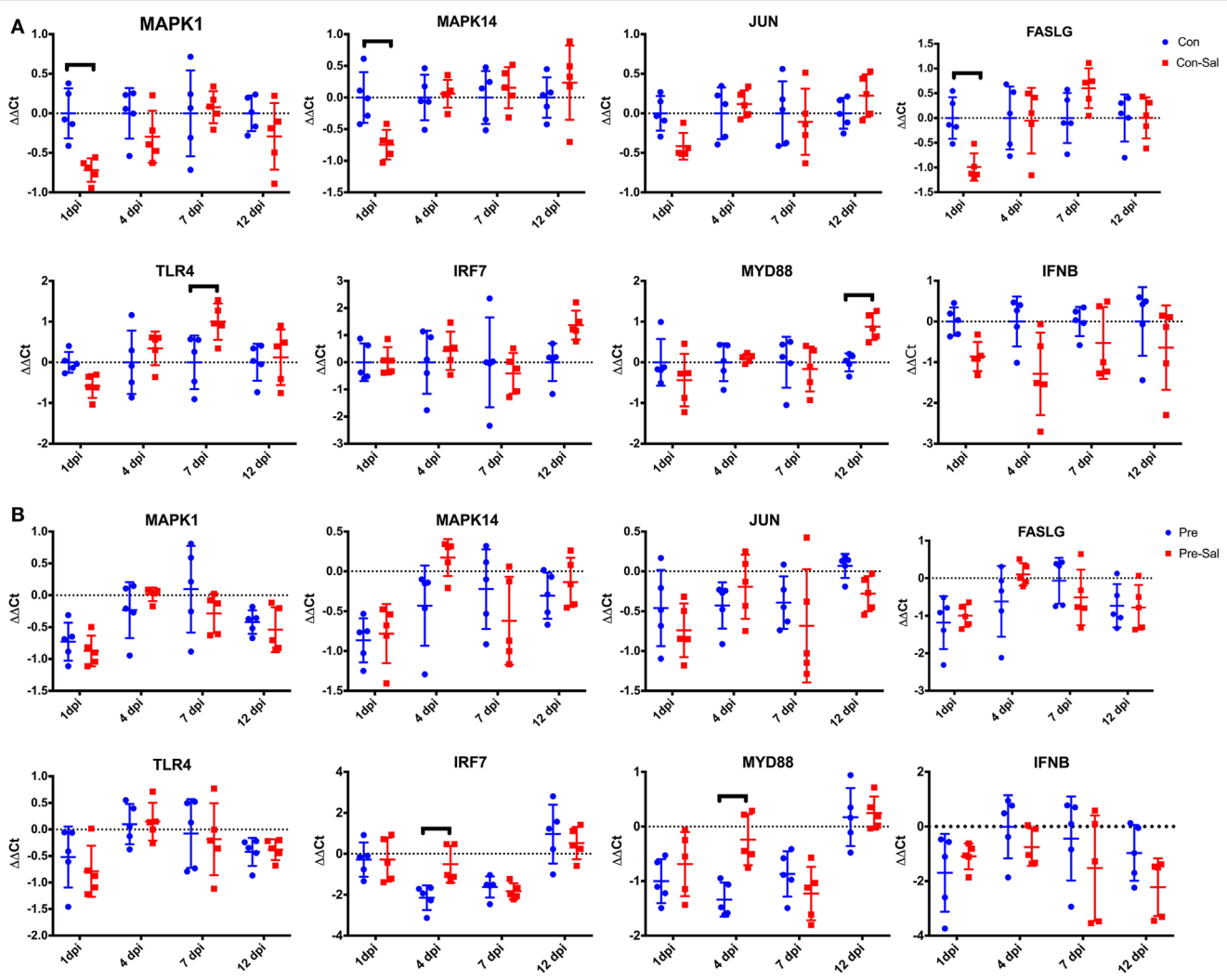


FIGURE 3 | Changes in cecal tonsil gene expression in birds following infection with SE and ST. Birds were fed a conventional diet (**A**, Con) or a prebiotic diet containing GOS (**B**, Pre). At 3 days of age, birds were infected with SE and ST (Sal) or mock infected with PBS. At 1, 4, 7, and 12 dpi, the cecal tonsils were collected from five birds bird treatment group and assayed for relative changes in gene expression. The ΔΔCt for each individual bird sampled at each time point is represented by blue circles (mock infected) or red squares (*Salmonella* infected). The sample mean is represented by the horizontal line, with upper and lower error bars denoting 1 SD among the five replicate samples. The dashed line denotes no change relative to the control reference sample. Data points above the dashed line represent increased expression while points below denote decreased expression. Black brackets denote time points where the means were significantly different ($p < 0.05$). GOS, galacto-oligosaccharide; PBS, phosphate-buffered saline.

This is followed by an increase in expression ($p < 0.05$) of TLR4 at 7 dpi and MYD88 at 12 dpi in the control non-challenged group (**Figure 3A**). The expression of IRF7 increases at 12 dpi but this is not significant ($p = 0.083$). Comparatively, there are fewer changes in gene expression between the uninfected and infected animals fed the prebiotic diet (**Figure 3B**). There was a significant ($p < 0.05$) increase in the expression of IRF7 and MYD88 in the infected animals at 4 dpi. The expression levels of MAPK14 also increase at 7 dpi; however, this is not significant ($p = 0.073$) (**Figure 3B**).

To further assess the effect of the prebiotic on the host immune response, the difference in expression of these genes between the uninfected control-fed and prebiotic-fed animals was assessed (**Figure 4**). This comparison shows a significant ($p < 0.05$) decrease in expression of MAPK1, MAPK14, FASLG, and MYD88 at 1 dpi. At 4 dpi IRF7 and at 4 and 7 dpi, MYD88 showed a significant decrease in expression (note these were not infected, but age-matched birds **Figure 4**).

GOS and *Salmonella* Affecting Microbiome Structure

The analysis of the diversity of the cecal microbiome demonstrated that the prebiotic treatment results in a significantly ($p < 0.004$) diverse population as compared with the control-fed animals (**Figure 5**; **Table 3**). The microbiomes of the uninfected control and uninfected prebiotic groups were found to be significantly diverse ($p < 0.004$) at each time point examined. Interestingly, the uninfected control group is the only group whose microbiome is significantly distinct ($p < 0.02$) from all other groups for the duration of the experiment. Conversely, the microbiomes of the uninfected prebiotic group and the infected prebiotic group were found to be significantly diverged ($p < 0.02$) at 4, 7 and 12 dpi, but by 27 dpi there was no significant difference (**Figure 5**; **Table 4**), in spite of the fact that ST was found in the cecum of the infected birds in both control and GOS diet groups (**Figure 1**). Interestingly, the diversity of the microbiome of infected control and infected prebiotic birds was only significant at 4 dpi ($p < 0.003$).

Analysis of the effect of diet treatment and *Salmonella* challenge on the family level taxonomic changes of the microbiota in each of the treatment groups demonstrated that by 27 dpi there is between 30- and 50-fold more *Lactobacillaceae* in the prebiotic, prebiotic-challenge, and control-challenged groups as compared with the control group (**Figure 6A**). Conversely, the control group contains more members of the order *Clostridiales* than the other three groups (**Figure 6A**).

A further in-depth analysis focusing on the differences in microbiome composition between the prebiotic and control-treated and non-infected age-matched animals at each time point indicated statistically significant differences between the two treatments at all time points investigated. In age-matched GOS-treated birds, there was a general decrease in *Clostridiales*, *Lachnospiraceae*, and *Ruminococcaceae* and increase in *Lactobacillaceae* over time as compared with the control birds over time. Specifically, at 4 dpi there was a significant decrease in *Clostridia Clostridiales*, and *Clostridiales Ruminococcaceae* and an increase in *Clostridiales Lachnospiraceae* in the GOS-treated birds compared with the control-treated birds (**Table 4**; **Figure 6B**). At 7 dpi, the levels of *Lactobacillales Lactobacillaceae* were significantly decreased and *Clostridiales Lachnospiraceae* was significantly increased in the

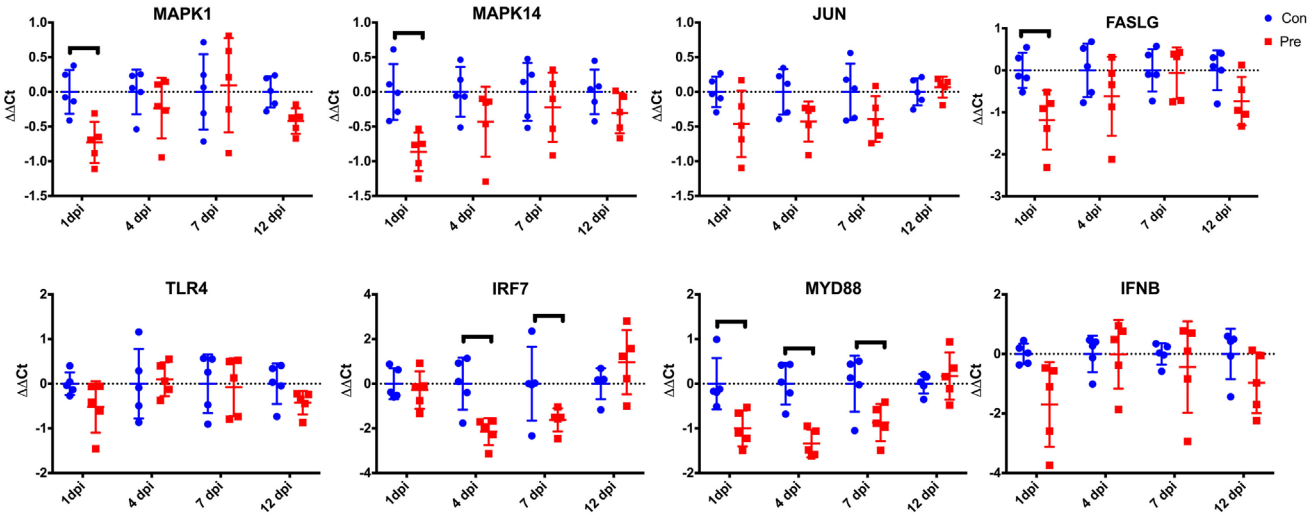


FIGURE 4 | Effect of prebiotic diet on cecal tonsil gene expression in uninfected birds. Birds were fed a conventional diet (Con) or a prebiotic diet containing GOS (Pre). At 3 days of age, birds were mock infected with PBS and the cecal tonsils were collected from five birds bird per treatment group at 1, 4, 7, and 12 dpi. Samples were assayed for relative changes in gene expression. The ΔΔCt for each individual bird sampled at each time point is represented by blue circles (Con) or red squares (Pre). The sample mean is represented by the horizontal line, with upper and lower error bars denoting 1 SD among the five replicate samples. The dashed line denotes no change relative to the control reference sample. Data points above the dashed line represent increased expression, while points below denote decreased expression. Black brackets denote time points where the means were significantly different ($p < 0.05$). GOS, galacto-oligosaccharide; PBS, phosphate-buffered saline.

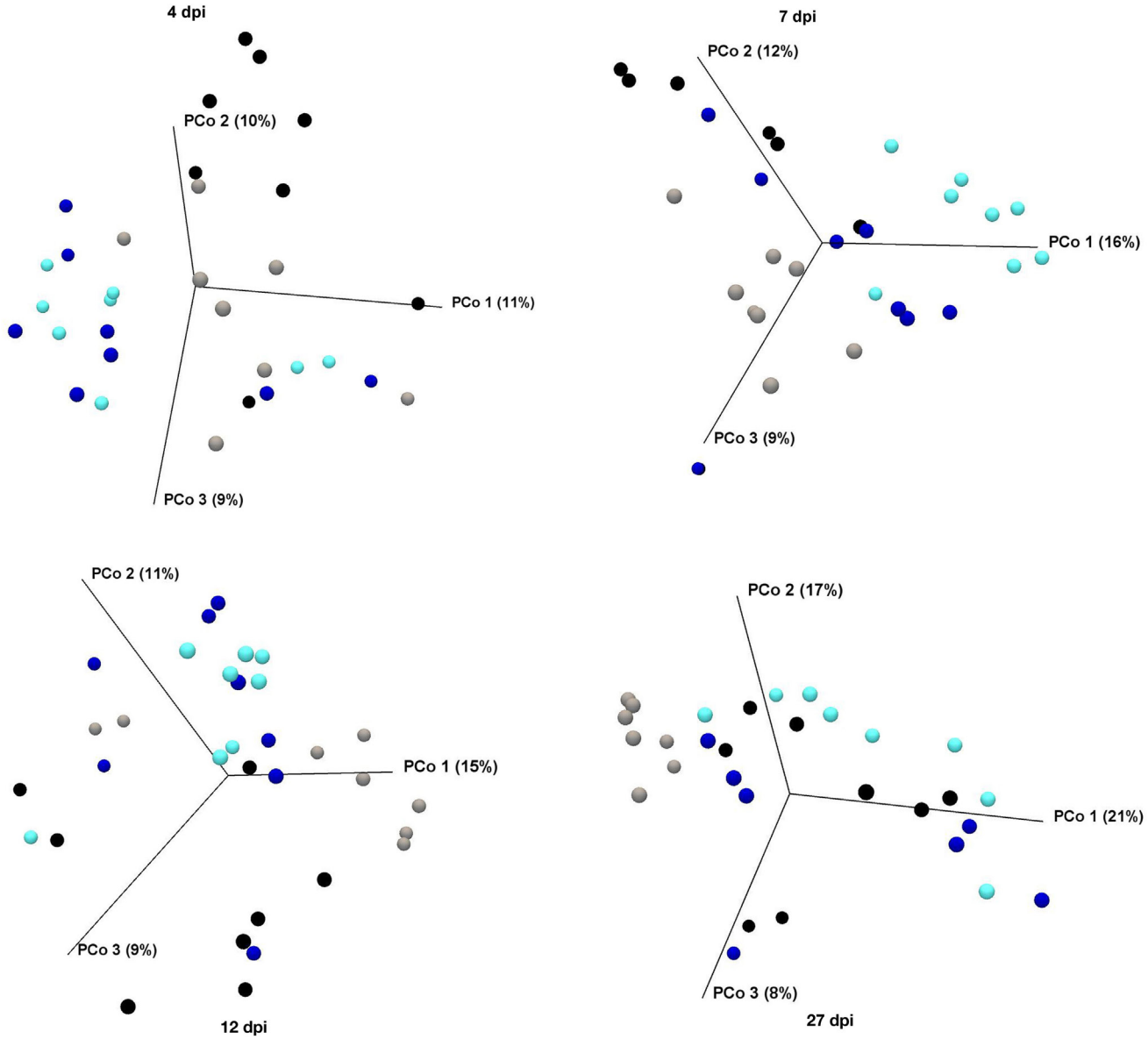


FIGURE 5 | PCoA using OTU-level Bray–Curtis index of the cecal microbiome showing beta diversity following *Salmonella* infection between birds fed control or prebiotic diets. Birds were either untreated (Con, gray dots) or fed a diet containing GOS (Pre, teal dots). At 3 days of age, half of the birds in each diet group were infected with SE and ST by oral gavage (Con-Sal, black dots or Pre-Sal, blue dots). At 4, 7, 12, and 27 dpi, cecal contents were collected for microbiome analysis. GOS, galacto-oligosaccharide; PCoA, principal component analysis; OUT, operational taxonomic units.

GOS-treated birds compared with the control-non-treated birds (**Table 4**; **Figure 6B**). At 12 dpi, there was a significant decrease in the levels of *Clostridiales Ruminococcaceae* and a significant increase in the levels of *Lactobacillales Lactobacillaceae*, *Clostridia Clostridiales*, and *Clostridiales Lachnospiraceae* in GOS-treated birds compared with control-treated birds (**Table 4**; **Figure 6B**). At 27 dpi, the levels of *Clostridiales Lachnospiraceae* and *Clostridiales Ruminococcaceae* were significantly decreased and the levels of *Lactobacillales Lactobacillaceae* were significantly increased in GOS-treated birds as compared with control-treated birds (**Table 4**; **Figure 6B**).

DISCUSSION

Data from this study indicated that there is a difference in the colonization capabilities of the ST and SE strains used. ST demonstrated the greatest degree of persistence within the cecum of control birds; at 27 dpi it was still detectable in six out of eight control birds, whereas SE was undetectable by 12 dpi. The difference in persistence and cecum colonization between ST and SE seen in this study has also been noted by other researchers using a coinfection with SE and ST in 1-day-old chicks (26). Similar differences in competitive fitness have also been reported

between *S. Kentucky* and ST in coinfected chickens *S. Kentucky* and ST/SE (27, 28). Furthermore, challenging birds prior to molt with a non-SE serovar before exposure to SE has been shown to reduce SE-associated problems during molt (29), indicating that delayed coinfection in older birds also results in out competition of SE. The reasons for the differences in persistence between the serovars is unclear; it is possible that the intestinal environmental conditions (microbiota, immune response) within the bird plays a significant role in determining which serovar is able to establish within the cecum and remain detectable for 27 dpi. The addition of GOS to the chicken feed resulted in a minimal change in the rate of clearance of ST and SE, with SE being undetectable at day 7 in prebiotic treated but day 12 in control birds. An increase in the rate of clearance of *Salmonella* has also been reported in birds treated with the prebiotics mannan-oligosaccharides (MOS) or xylooligosaccharides (XOS) (30). Clearly, understanding the competitive fitness of different serovars, and the role prebiotics such as GOS play in modulating the cecal environment (microbiome, pH, and host factors), will enable a better determination of conditions needed to reduce *Salmonella* colonization in young birds.

Vaccination of birds against *Salmonella* has been the dominant control strategy. Commercial vaccines have been shown to provide protection against a *Salmonella* challenge by inducing an IgG response (4). While there is evidence that vaccines can induce a serum IgG response and an intestinal IgA response, the level of this response is dependent on the vaccine schedule with a combination of live and killed vaccines leading to a higher level of IgA response (31). Such a response would be required to protect the intestine from *Salmonella* colonization. However, the IgG response has been shown to be serovar specific with an SE vaccine failing to provide protection against an ST or *S. Heidelberg* challenge (32). It is reasonable to hypothesize that the IgA response induced by the vaccine would also be serovar specific. The use of serovar specific vaccines has also been shown to allow a switch in the dominant serovar present in the chickens; thus, the eradication of *S. gallinarum* through the use of vaccines leads to an increase in colonization by SE (33). The other switches in serovar dominance have also been seen in chickens and in pigs (34). Modulating the chicken intestinal microbiology through *Salmonella* serovar specific exclusion appears to be a method of modulating the serovar of *Salmonella* carriage, and further methods of modulation accompanied by the use of vaccines may allow more robust removal of *Salmonella* from the chicken.

Taking into account the transient presence of ST in the liver (**Table 2**) liver samples from *Salmonella* infected chicks demonstrated that ST was able to transiently infect the liver, indicating that it was able to cross the intestinal barrier whereas SE was not able to do so. The infection of ST in the liver was short lived and

TABLE 3 | PERMANOVA analysis (Bray–Curtis) showing statistical differences in the beta diversity measures between the different treatments.[a]

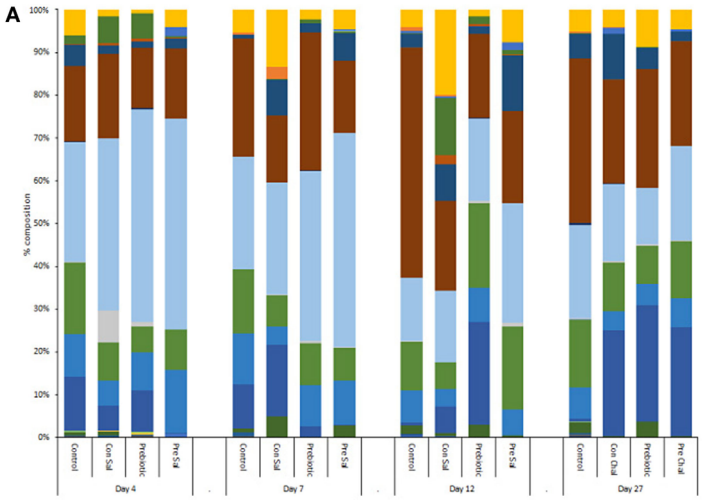
		p-Value (Bonferroni)			
		4 dpi	**7 dpi**	**12 dpi**	**27 dpi**
Control	Control–Challenge	0.00653	0.00559	0.00280	0.00839
Control	Prebiotic	0.00373	0.00093	0.00093	0.00093
Control–Challenge	Prebiotic	0.00466	0.00186	0.00932	0.09604
Control	Prebiotic–Challenge	0.00373	0.00746	0.01399	0.00373
Control–Challenge	Prebiotic–Challenge	0.00280	0.35897	0.05594	0.62378
Prebiotic	Prebiotic–Challenge	0.00373	0.00093	0.01678	0.55664

[a]*p ≤ 0.05 are significant and p ≤ 0.005 are highly significant.*

TABLE 4 | Taxonomic analysis of the cecum micobiome composition of prebiotic and control-treated non-challenged birds.

Taxonomy	**Day 4**		**Day 7**		**Day 12**		**Day 27**	
	Control	**Prebiotic**	**Control**	**Prebiotic**	**Control**	**Prebiotic**	**Control**	**Prebiotic**
Bacteria	0.0000	0.0000	0.0015	0.0000	0.0052	0.0001	0.0014	0.0004
Cyanobacteria, Chloroplast, Streptophyta	0.0027	0.0046	0.0012	0.0001	0.0000	0.0004	0.0037	0.0000
Firmicutes	0.0014	0.0016	0.0081	0.0007	0.0026	0.0004	0.0035	0.0001
Firmicutes, Bacilli, Bacillales, Bacillaceae	0.0078	0.0000	0.0088	0.0001	0.0188	0.0290	0.0260	0.0360
Firmicutes, Bacilli, Lactobacillales, Lactobacillaceae	0.1260	0.0975	0.1026	0.0250****a	0.0086	0.2403****b	0.0070	0.2708****b
Firmicutes, Clostridia, Clostridiales	0.1002	0.0888	0.1201	0.0958	0.0746	0.0791	0.0728	0.0509
Firmicutes, Clostridia, Clostridiales	0.1673	0.0602****a	0.1493	0.0978	0.1128	0.1985****b	0.1599	0.0886
Firmicutes, Clostridia, Clostridiales, Clostridiaceae	0.0009	0.0105	0.0005	0.0056	0.0011	0.0045	0.0030	0.0032
Firmicutes, Clostridia, Clostridiales, Lachnospiraceae	0.2798	0.4958****b	0.2631	0.3974****b	0.1488	0.1930*b	0.2170	0.1315****a
Firmicutes, Clostridia, Clostridiales, Peptostreptococcaceae	0.0008	0.0048	0.0000	0.0020	0.0000	0.0004	0.0043	0.0001
Firmicutes, Clostridia, Clostridiales, Ruminococcaceae	0.1764	0.1394***a	0.2749	0.3221	0.5381	0.1962****a	0.3849	0.2785***b
Firmicutes, Erysipelotrichi, Erysipelotrichales, Erysipelotrichaceae	0.0501	0.0148	0.0082	0.0207	0.0303	0.0171	0.0556	0.0500
Proteobacteria, Gammaproteobacteria	0.0016	0.0082	0.0001	0.0007	0.0002	0.0059	0.0002	0.0000
Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae	0.0187	0.0570	0.0007	0.0087	0.0008	0.0174	0.0018	0.0004
Tenericutes, Mollicutes, Anaeroplasmatales, Anaeroplasmataceae	0.0000	0.0000	0.0002	0.0000	0.0063	0.0000	0.0012	0.0005
Tenericutes, Mollicutes, RF39	0.0001	0.0000	0.0041	0.0000	0.0078	0.0000	0.0033	0.0000
N/A	0.0609	0.0095	0.0542	0.0229	0.0414	0.0167	0.0510	0.0873

*Statistical differences between control and prebiotic non-challenged age-matched birds using two-way analysis of variance (ANOVA) using Sidak's multiple comparison test. ***p < 0.0001, ***p < 0.006, **p < 0.01, and *p < 0.05. A decrease in abundance in the prebiotic group is indicated by "a" and an increase is indicated by "b."*



N/A
p__Verrucomicrobia, c__Verrucomicrobiae, o__Verrucomicrobiales, f__Verrucomicrobiaceae
p__Tenericutes, c__Mollicutes, o__RF39, f__
p__Tenericutes, c__Mollicutes, o__Anaeroplasmatales, f__Anaeroplasmataceae
p__Proteobacteria, c__Gammaproteobacteria, o__Xanthomonadales, f__Xanthomonadaceae
p__Proteobacteria, c__Gammaproteobacteria, o__Thiotrichales, f__Piscirickettsiaceae
p__Proteobacteria, c__Gammaproteobacteria, o__Pseudomonadales, f__Pseudomonadaceae
p__Proteobacteria, c__Gammaproteobacteria, o__Pseudomonadales, f__Moraxellaceae
p__Proteobacteria, c__Gammaproteobacteria, o__Pasteurellales, f__Pasteurellaceae
p__Proteobacteria, c__Gammaproteobacteria, o__Oceanospirillales, f__Oceanospirillaceae
p__Proteobacteria, c__Gammaproteobacteria, o__Enterobacteriales, f__Enterobacteriaceae
p__Proteobacteria, c__Gammaproteobacteria, o__Alteromonadales, f__Psychromonadaceae
p__Proteobacteria, c__Gammaproteobacteria, o__Alteromonadales, f__Alteromonadaceae
p__Proteobacteria, c__Gammaproteobacteria, o__Alteromonadales, f__[Chromatiaceae]
p__Proteobacteria, c__Gammaproteobacteria, ,
p__Proteobacteria, c__Betaproteobacteria, o__MND1, f__
p__Proteobacteria, c__Betaproteobacteria, o__Burkholderiales, f__Oxalobacteraceae
p__Proteobacteria, c__Betaproteobacteria, o__Burkholderiales, f__Comamonadaceae
p__Proteobacteria, c__Betaproteobacteria, o__Burkholderiales, f__Burkholderiaceae
p__Proteobacteria, c__Alphaproteobacteria, o__Rhodobacterales, f__Rhodobacteraceae
p__Proteobacteria, c__Alphaproteobacteria, o__Rhizobiales, f__Methylobacteriaceae
p__Proteobacteria, c__Alphaproteobacteria, o__Rhizobiales, f__
p__Proteobacteria, , ,
p__Firmicutes, c__Erysipelotrichi, o__Erysipelotrichales, f__Erysipelotrichaceae
p__Firmicutes, c__Clostridia, o__Thermoanaerobacterales, f__Thermoanaerobacteraceae
p__Firmicutes, c__Clostridia, o__Clostridiales, f__Veillonellaceae
p__Firmicutes, c__Clostridia, o__Clostridiales, f__Ruminococcaceae
p__Firmicutes, c__Clostridia, o__Clostridiales, f__Peptostreptococcaceae
p__Firmicutes, c__Clostridia, o__Clostridiales, f__Peptococcaceae
p__Firmicutes, c__Clostridia, o__Clostridiales, f__Lachnospiraceae
p__Firmicutes, c__Clostridia, o__Clostridiales, f__Eubacteriaceae
p__Firmicutes, c__Clostridia, o__Clostridiales, f__Clostridiaceae
p__Firmicutes, c__Clostridia, o__Clostridiales, f__Christensenellaceae
p__Firmicutes, c__Clostridia, o__Clostridiales, f__[Mogibacteriaceae]
p__Firmicutes, c__Clostridia, o__Clostridiales, f__
p__Firmicutes, c__Clostridia, o__Clostridiales,
p__Firmicutes, c__Bacilli, o__Turicibacterales, f__Turicibacteraceae
p__Firmicutes, c__Bacilli, o__Lactobacillales, f__Streptococcaceae
p__Firmicutes, c__Bacilli, o__Lactobacillales, f__Leuconostocaceae
p__Firmicutes, c__Bacilli, o__Lactobacillales, f__Lactobacillaceae
p__Firmicutes, c__Bacilli, o__Lactobacillales, f__Enterococcaceae
p__Firmicutes, c__Bacilli, o__Lactobacillales, f__Carnobacteriaceae
p__Firmicutes, c__Bacilli, o__Lactobacillales,
p__Firmicutes, c__Bacilli, o__Haloplasmatales, f__Haloplasmataceae
p__Firmicutes, c__Bacilli, o__Bacillales, f__Staphylococcaceae
p__Firmicutes, c__Bacilli, o__Bacillales, f__Paenibacillaceae
p__Firmicutes, c__Bacilli, o__Bacillales, f__Bacillaceae
p__Firmicutes, , ,
p__Cyanobacteria, c__Oscillatoriophycideae, o__Chroococcales, f__Xenococcaceae
p__Cyanobacteria, c__Chloroplast, o__Streptophyta, f__
p__Chloroflexi, c__Anaerolineae, o__SBR1031, f__oc28
p__Actinobacteria, c__Coriobacteriia, o__Coriobacteriales, f__Coriobacteriaceae
p__Actinobacteria, c__Actinobacteria, o__Actinomycetales, f__Propionibacteriaceae
p__Actinobacteria, c__Actinobacteria, o__Actinomycetales, f__Nocardiopsaceae
p__Actinobacteria, c__Actinobacteria, o__Actinomycetales, f__Microbacteriaceae
p__Actinobacteria, c__Actinobacteria, o__Actinomycetales, f__Dietziaceae
p__Actinobacteria, c__Actinobacteria, o__Actinomycetales, f__Corynebacteriaceae
k__Bacteria, , , ,

FIGURE 6 | Continued

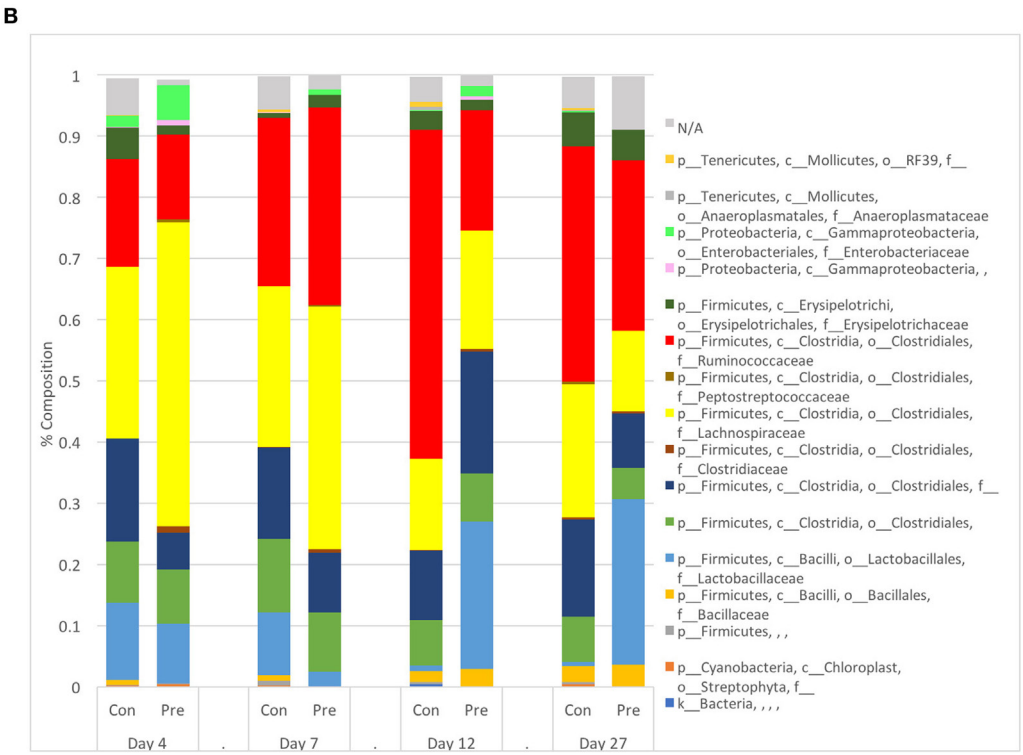


FIGURE 6 | Average relative abundance of the microbiota at the family level in ceca as **(A)** function of time following *Salmonella* infection in birds fed control or prebiotic diets or **(B)** function of time and diet composition. Birds were either untreated (Con) or fed a diet containing GOS (Pre). At 3 days of age, half of the birds in each diet group were infected with SE and ST by oral gavage (Sal). At 4, 7, 12, and 27 dpi, cecal contents were collected for microbiome analysis as stated in Section "Materials and Methods." GOS, galacto-oligosaccharide.

only detectable between 4 and 12 dpi (**Table 2**) and long with the drop in SE cecum levels seen between 1 and 7 dpi (**Figure 1A**). Coupled with the lack of detectable IgG response (data not shown) suggests a role for both the chicks innate immune system and microbiota in reducing the systemic infection.

The prebiotic MOS has been reported to reduce the expression of TNFα and IFNγ in the cecal tonsil of young birds challenged with *Salmonella* (30); and Fructo-oligosaccharides (FOS) inulin has been shown to reduce the expression of IL-1β in the chicken macrophage HD11 cell line when *Salmonella* challenged (35). The genes analyzed in this study were selected from RT-PCR gene expression array panels containing genes related to the antibacterial immune response and inflammation. The eight target genes (MAPK1, MAPK14, JUN, FASLG, TLR4, IFR7, MYD88, and IFNB) represent genes from a cross-section of receptors, ligands, and intracellular signaling factors related the antibacterial response. These genes were selected due to their differential expression in cecum tissues in the presence and absence of *Salmonella* (data not shown) and therefore allow the detection of bird immune response under the conditions reported in this study. Challenged birds treated with prebiotic (GOS) had a significantly lower expression (4/8 genes at 7 dpi **Figure 2B**) than control challenged birds. This is consistent with other studies using prebiotics in both young birds and cell culture (30, 35). At 7 dpi, there was a decrease in the levels of MAPK14 and FASLG in the prebiotic-treated birds along with TLR4 and MYD88, suggesting that both these pathways are involved in the response. This marked difference in gene expression between the two treatment groups may indicate a switch in response pathway induced through changes in the microbiome or changes in the host immune cells repertoire presented in the cecum lumen and cecal tonsil.

The response within each treatment group between challenged and non-challenged birds (**Figures 3A,B**) indicates that there is a greater degree of gene changes within the control group, with a reduction in gene expression seen at 1 dpi with 3/8 genes and an increase seen in one different gene at both 7 and 12 dpi. The downregulation of genes at 1 dpi occurring in the MAPK–FASLG pathways prior to an increase in TLR4 (7 dpi) and MYD88 (12 dpi) indicates that initially the response to *Salmonella* in the control birds is modulated through a downregulation of the MAPK-FASLG pathway (**Figure 2A**). While the prebiotic challenge group underwent an increase in the expression of IRF7 and MYD88 at 4 dpi, this is a different mechanism of response compared with the control challenged birds. The mechanism behind this altered immune response is currently unclear, and further studies are needed to understand the signals that led to this change in response, along with determining the response of the other genes contained within these two pathways in response to *Salmonella* in the presence and absence of prebiotic.

Interestingly, age-matched non-challenged prebiotic birds had a significant reduction in 4/8 genes at 1 dpi, 2/8 at 4 dpi, and 1/8 at 7 dpi, indicating a downregulation of genes in the MYD88-IRF7 pathway occurring at 4 and the FASLG-MAPK occurring at the time point equivalent to 1 dpi. A similar response was seen in cecal tonsil cytokine expression when the prebiotic inulin was given *in ovo* to chickens (24). These data suggest that feeding GOS can modulate the bird's immune response and in turn potentially change the cecum environment (microbiome, pH, host factors) within the bird.

Myeloid differentiation primary response protein 88 (MYD88) has been shown to interact with IRF7 leading to the induction of INF? and INF inducible genes in heterophils exposed to *Salmonella* LPS (36). The downregulation of the gene encoding for these proteins in the prebiotic and prebiotic challenged birds suggest that this pathway is not activated in prebiotic-treated birds regardless of *Salmonella* challenge. The difference in basal expression of these genes suggests a different level of stimulation by the intestinal contents, and that the difference in *Salmonella* load in the cecum could be due to changes in the microbiome.

The addition of GOS to the diet led to a significant difference in microbiome compared with the control birds at all-time points investigated. Control birds remained distinct from the other groups suggesting that both *Salmonella* challenge and prebiotic or + infected-prebiotic modified the chicken cecum microbiome. The control challenge and prebiotic challenge groups were not significantly different starting from 7 dpi, suggesting that while the microbiome was initially different challenging the birds with *Salmonella* lead to a similar microbiome regardless of bird treatment background. Changes in cecum microbiome have been reported in birds given candidate *Salmonella* vaccine strains (37), and infection with *Salmonella* has also been shown to modify the natural development of the chicken microbiota (38). The control un-challenged birds remain distinct from the other treatment groups throughout.

Statistical analysis of the composition of the non-infected age-matched birds showed significant differences in the composition over all time points investigated. In our study, there was a decrease in the level of *Clostridiales Ruminococcaceae* significantly decreased in the prebiotic-treated birds at 4, 7, and 27 dpi; a decrease in the level of these bacteria was also reported by Videnska et al. (16) and Mon et al. (38) in the cecum of after *Salmonella* challenge. *Clostridiales Lachnospiraceae* was significantly increased at 4, 7, and 12 dpi and significantly reduced at 27 dpi in prebiotic-treated birds compared with control-treated birds. *Clostridiales* showed a significant decrease at 4 dpi and a significant increase at 12 dpi in prebiotic-treated birds compared with control-treated birds. *Lactobacillus* was significantly lower at 7 dpi and significantly increased at 12 and 27 dpi. *Lactobacilli* isolated from chickens have been shown to be inhibitory to the growth of *Salmonella* (39), and the increased presence of *Lactobacillius* in the chicken cecum seen from 12 dpi may play a role in the reduction in *Salmonella* CFU/g cecum content seen in the challenged birds. In this study, the development of the microbiome plays a role in the cecum immune response during a *Salmonella* challenge; the rate of the development of the microbiome is also influenced by the addition of GOS and *Salmonella* challenge. The changes in gene expression seen during treatment with GOS and/or *Salmonella* challenge along with the difference in clearance rates between the two *Salmonella* serovars may be driven by the changes in the microbiome. Further analysis of the changes in the microbiome will be the focus of future studies.

Taken together, these changes in gene expression indicate that there is an underlying effect on the birds with the addition of GOS and these changes result in a reduction in the level of immune response when birds are challenged with *Salmonella* at day 3 of life. While these changes result in a reduction in gene expression they correspond with changes in the microbiome, specifically an increase in the level of *Lactobacillales* and a decrease in *Clostridiales* suggest that these changes may affect *Salmonella*'s ability to colonize birds. The data presented here demonstrate that GOS can be used to cause subtle changes the gene expression in both the TLR4 and MYD88 pathways, and more substantive changes in the microbiome. This is consistent with other studies where the addition of GOS leads to an increase in *Bifidobacteria* and *B. lactis* in chickens (21), and suggests that GOS along with other prebiotics can be used to modify the intestinal ecosystem, and in turn the host immune response. The specific changes within the microbiome induced by GOS are beyond the scope of this current study. Our lab is currently investigating the specific microbiome and metabolomic changes induced by GOS to better decipher the mechanisms of the changes in gene expression, and microbiome changes seen in this current study.

ETHICS STATEMENT

This study was carried out in accordance to the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) (Protocol no. OLAW D16-00214) protocol (Protocol no. 15–065-A).

AUTHOR CONTRIBUTIONS

R-AH contributed to the sample collection, real-time PCR data collection, data analysis, and writing of the manuscript. RA contributed to the sample collection, data analysis, and writing of the manuscript. MM contributed to the sample collection, microbiology data collection, microbiology data analysis, microbiome data analysis, and writing of the manuscript. HH and MK contributed to the experimental design of the study and writing of the manuscript.

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Effect of Feeding *Bacillus subtilis* Spores to Broilers Challenged with *Salmonella enterica* serovar Heidelberg Brazilian Strain UFPR1 on Performance, Immune Response, and Gut Health

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Salmonellosis is a poultry industry and public health concern worldwide. Recently, *Salmonella enterica* serovar Heidelberg (SH) has been reported in broilers in Brazil. The effect of feeding a blend of three strains of *Bacillus subtilis* (PRO) was studied in broilers orally challenged (10^7 CFU/chick) or not with a SH isolated in south of Brazil (UFPR1 strain). Twelve male Cobb 500 broilers per pen were randomly assigned to six treatments in a 3 × 2 factorial experiment where PRO was added at 0, 250, or 500 g/ton of broiler feed and fed to either SH-challenged (SH Control, SH + PRO 250, and SH + PRO 500) or non-challenged birds (Control, PRO 250, and PRO 500). Broiler performance, histologic alterations in intestinal morphology, *Salmonella* quantification and immune cells counts in liver (macrophages, T CD4+ and T CD8+) were analyzed. Changes in the intestinal microbiota of broilers were also studied by metagenomics for Control, SH Control, SH + PRO 250, and SH + PRO 500 only. Feeding PRO at 250 or 500 g/ton reduced SH counts and incidence in liver and cecum at 21 days of age. It was observed that PRO groups increased the macrophage mobilization to the liver in SH-challenged birds ($P < 0.05$) but reduced these cells in the liver of non-challenged birds, showing an interesting immune cell dynamics effect. PRO at 250 g/ton did not affect gut histology, but improved animal performance ($P < 0.05$) while PRO at 500/ton did not affect animal performance but increased histologic alteration related to activation of the defense response in the ileum in SH challenged birds compared to control birds ($P < 0.05$). SH + PRO 500 group presented a more diverse cecal microbiota (Shannon–Wiener index; $P < 0.05$) compared to Control and SH Control groups; while SH + PRO 250 had greater ileal richness (JackkNife index) compared to Control ($P < 0.05$). PRO

was effective in reducing *Salmonella* colonization in liver and cecum when fed at 250 or 500 g/ton to broilers inoculated with SH strain UFPR1. PRO promotes positive alterations in performance (at 250 g/ton), immune modulatory effect in the gastrointestinal tract, SH reduction, and intestinal microbiota modulation.

Keywords: 16S, gut health, gut microbiome, immunity, poultry, probiotic, salmonellosis

INTRODUCTION

Despite advances in the treatment of infectious diseases, pathogenic microorganisms such as *Salmonella* are an important threat to both human and animal health worldwide (1). *Salmonella* is a pathogen but it also has the ability to live in animals and poultry as a transient member of the intestinal microbial population without causing disease. Colonization of most types of *Salmonella enterica* does not often affect poultry performance and consequently asymptomatic infections may increase the likelihood of zoonotic transmission to humans through the food chain (2). *S. enterica* serovar Heidelberg (SH) ranks among the top three serovars isolated from patients with salmonellosis in North America, higher than in other regions of the world (3), provoking more invasive infections (e.g., myocarditis and bacteremia) than others non-typhoidal *Salmonella* (4). The Brazilian SH strain used in this trial (UFPR1) had its complete genome described recently, showing high resistance to short-chain organic acids and intermediate resistance to some antibiotics (5).

Oral administration of probiotics may reduce the intestinal colonization of *Salmonella* (6, 7), along with the inflammation caused by this bacterium, in broiler chickens (8). Probiotics are live microorganisms that offer an advantage to their hosts by enhancing the hosts' beneficial microbiota (9, 10). Studies have demonstrated that *Bacillus* spp. and *Bacillus subtilis* spores may be successful competitive exclusion agents (11). *B. subtilis* modulates the intestinal microbiota and favors the growth of lactic acid bacteria with recognized health-conferring properties (12). A spore monoculture has the advantage of being readily produced, having a long shelf life, and, in the case of *B. subtilis*, being avirulent (11). *B. subtilis* has been studied and used as a feed additive to improve broiler performance (13, 14), modulate immune response (15, 16) and act as a prophylactic agent against bacterial diseases, by balancing gut microbiota (17, 18).

Some probiotics may be able to decrease the invasiveness of pathogens, which use inflammation to enhance their own colonization, by decreasing innate inflammatory responses, including macrophage activation phenotypes. Probiotics are also well documented to increase modulation of adaptive immunity (19). These findings suggest a specific immune interaction of each probiotic strain used, and its abilities to improve protection against certain pathogens, maintaining health and homeostasis through intestinal and systemic immunomodulation, in order to enhance animal performance and health.

The objective of this trial was to evaluate the ability of a probiotic composed of three different *B. subtilis* strains to reduce the invasiveness and gut colonization of the Brazilian SH UFPR1 strain, and its effects on performance, intestinal mucosa morphology, immune cells dynamics (macrophages, CD4+ and CD8+ cells) in liver, and gut microbiota in broiler chickens.

MATERIALS AND METHODS

Animals and Experimental Design

The experiment was conducted at Center of Immune Response in Poultry at Federal University of Parana, Curitiba, Brazil, and was approved by the Ethical Committee of Agricultural Sector of Federal University of Parana under approval number: 037/2016.

Six, previously disinfected, BSL-2 rearing rooms were used. Each room contained four battery cages (replications) stacked vertically with sterilized litter, nipple drinkers, automatic temperature and lighting controls, all under a negative pressured air system.

A total of 288 one-day-old male Cobb 500® broilers were distributed in a completely randomized block design (each block is a room) with six treatments of four replicates and 12 birds each where PRO was fed at 0, 250, or 500 g/ton of feed in either SH-inoculated or non-inoculated birds, as shown in **Table 1**. At the initiation of the trial, birds were allocated at in such a way that equal average initial body weight per cage was obtained. The trial was carried out from 1 to 21 days of age.

Aiming at minimizing the possibility of unexpected *Salmonella* contamination, the chickens used in this trial corresponded to the male line of a grandparent stock farm not vaccinated against any type of *Salmonella*.

Product and Dosage

The probiotic (PRO) used in this trial is a feed additive manufactured with three isolated live spores of *B. subtilis* strains (NP122, B2 and AM0904; Sporulin®, Novus International Inc.). PRO was provided at three different levels: 0 g/ton (Control and SH Control groups), 250 g/ton (PRO 250 and SH + PRO 250), or 500 g/ton (PRO 500 and SH + PRO 500; **Table 1**). The recommended dosage

TABLE 1 | Treatments description.

Treatments	*Salmonella* Heidelberg Challenge	Probiotic[a] added (g/ton of feed)
Control	No	0
PRO 250	No	250
PRO 500	No	500
SH Control	Yes	0
SH + PRO 250	Yes	250
SH + PRO 500	Yes	500

[a]*Live spores of Bacillus subtilis (PRO) strains (NP122, B2, and AM0904; Sporulin®, Novus International Inc.).*

by the manufacturer is 250 g/ton, which provides 10^6 spores per g of feed.

Feed Formulation and Mix

A balanced basal diet was offered in mash form and was formulated to provide nutrients at or above requirement levels (20). Corn and soybean meal were used as main ingredients and no antibiotics or growth promoters were added. The diet was designed for a unique feeding phase (Starter) and it was offered to broilers *ad libitum* from 1 to 21 days of age for all treatments.

The basal diet was sterilized by autoclave at 120°C for 15 min. After this process, PRO, amino acids, vitamin and mineral premix were added according to each treatment, and mixed for 10 min using a 50 kg "V" mixer. Batches were mixed in such an order to avoid interference among treatments. The PRO supplemented diets were mixed at last. The mixer was cleaned after each batch.

S. enterica serovar Heidelberg

Salmonella enterica serovar Heidelberg (SH), strain UFPR1 sequences were submitted to the database NCBI/biosample identified as SAMN06560104, GenBank: CP020101. This pathogen was isolated from commercial broiler carcasses obtained from a broiler farm located in the south of Brazil. Samples from 20 livers and ceca were collected randomly from one-day-old chicks and tested negative for *Salmonella*. At 3 days of age, chicks from the SH Control, SH + PRO 250, and the SH + PRO 500 groups were orally challenged with 10^7 CFU of SH per chick. At 7 and 21 days of age, 12 birds from the SH Control, SH + PRO 250, and the SH + PRO 500 cages were subjected to necropsy, while *Salmonella* sp. counts were quantified in liver and cecum samples. A pool of four ceca and four livers per treatment (Control, PRO 250, and PRO 500 birds) were also collected to evaluate the presence or absence of *Salmonella* sp. (qualitative analysis). In order to quantify typical colonies of *Salmonella* sp. (quantitative analysis), samples were processed using the modified methodology of Cox et al. (21). The abundance of *Salmonella* in ileum and cecum was also measured using metagenomic analysis.

Performance

All chicks and feed were weighed weekly to evaluate feed intake (FI), body weight (BW), body weight gain (BWG), and feed conversion ratio (FC). All birds used for tissue sampling were weighed individually to estimate FC corrected for mortality. Mortality due to other causes rather than sampling procedures was not observed in this trial.

Macrophages, CD4+ and CD8+ Cells Quantification by Immunohistochemistry

At 7 and 21 days of age, 12 birds per treatment (3 birds per replicate) were euthanized and the accessory lobe of their livers were collected. Immunohistochemistry was performed to obtain macrophage, CD4+ and CD8+ lymphocyte counts according to Lourenço et al. (22) using the rabbit macrophage clone RAM-11 Dako. The labeled cells were counted in an optical microscope (Nikon Eclipse E200, Sao Paulo, Brazil) with a 100× magnification objective. Five fields per bird, totalizing 25 microscopic fields per treatment of liver, were measured using only the hepatic parenchyma aiming at avoiding lymphoid aggregates.

Evaluation of Intestinal Health—Histology by ISI (I See Inside Methodology)

At 7 and 21 days of age, 12 birds per treatment (3 birds per replicate) were euthanized, liver and ileum samples collected and further subjected to microscopic evaluation using the ISI Methodology ("I See Inside"; Pat. INPI-BR1020150036019) (23) as published by Kraieski et al. (24). Shortly, this methodology was developed based on a numeric score of histological alterations. For each alteration observed during microscopic analysis, an impact factor (IF) is defined according to its importance in affecting organ functional capacity based on previous knowledge of literature and background research (e.g., necrosis has the highest IF because the functional capacity of affected cells is totally lost). The IF ranges from 1 to 3, where 3 represents an IF of the greatest significance in terms of the organ function. In addition, the extent of each alteration (intensity or observed frequency compared to non-affected tissue) is evaluated per field (liver) or per villi (intestine) and scored ranging from 0 to 3. To reach the final ISI value, the IF of each alteration is multiplied by the respective score number, and the results of all alterations are summed.

Genomic DNA Purification of Luminal Gut Microbiota and DNA Sequencing

The ileal (distal) and cecal luminal contents from 12 birds (3 birds per replicate) of the Control, SH Control, SH + PRO 250, and the SH + PRO 500 treatments were collected, frozen in liquid nitrogen and stored at −80°C until further analysis. Genomic DNA from each sample was purified using QIAamp Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer, and then DNA quantification and quality were evaluated using the NanoVue Plus spectrophotometer (GE Healthcare, Marlborough, USA). Samples were diluted at 50 ng/μL and pooled using the same volume for each one (three samples were used to form one pool, resulting in four replicates per treatment). The pooled samples from ileum and cecum were used to amplify approximately 460 bp of the 16S ribosomal RNA by PCR using specific primers V3 and V4. The PCR products were used to build the metagenomics library for sequencing using MiSeq Reagent kit v3 (600 cycle) (Illumina Inc.). The sequencing of partial 16S ribosomal RNA was performed by next-generation sequencing method using Illumina MiSeq platform that produced thousands of 300 bp paired-end reads (2 × 300 bp) for each library. The full-length primer sequences to follow the protocol targeting this region are: 16S Amplicon PCR Forward Primer = 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTA CGGGNGGCWGCAG and 16S Amplicon PCR Reverse Primer = 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGA CTACHVGGGTATCTAATCC.

Processing of the Reads and Phylogenetic Analysis

The sequencing data were analyzed in the Bioinformatics Lab of the UNICAMP (www.lge.ibi.unicamp.br). The paired-end reads

from each treatment were submitted to quality filtering and adapter trimming using Trim Galore software (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore). The trimmed paired-end reads were merged into single reads using PEAR software (25). The single reads were then submitted to phylogenetic analysis and taxonomic assignments of the V3-V4 portion of the 16S rRNA gene using QIIME package (26) configured for constructing Operational Taxonomic Units (OTUs) with 97% of identity and assign taxonomy based on the Greengenes reference database (currently version 13_8). The full data sequence has been registered at NCBI BioProject and the information should be available at the following link: http://www.ncbi.nlm.nih.gov/bioproject/413291. The rarefaction curves were conducted to evaluate the coverage of OTUs.

Diversity Analysis and Comparison among Treatments

Only taxonomic groups with abundance higher than 1% at the deepest level identified were submitted to cluster analysis. The clustering of different treatments was done using the Multiple Experiment Viewer software (27). Ecological indexes, such as diversity $H' = -\sum_{i=1}^{s} p_i \ln p_i$; where p_i is the proportion of characters belonging to the i*th* type of letter in the string of interest (28), richness and equitability $J = \frac{H'}{H_{\max''}}$; where $H_{\max'} = \log_b S$, were calculated using the program R. For all ecologic indexes, all OTUs obtained were used except those that appeared only once.

Statistical Analysis

Data were analyzed using the statistical software Statistix 9®. The microbiological data were evaluated by the Shapiro–Wilk normality test. The parametric data were subjected to analysis of variance (ANOVA) and Tukey's test to establish differences among treatment means. The nonparametric data were submitted to the Kruskal–Wallis test at a 5% probability value. When presence or absence of *Salmonella* was assayed, the chi-square test was used to establish statistical differences. For performance, immunohistochemistry, and histology analysis, data were submitted to ANOVA using a 2 × 3 factorial design, once no difference for block were observed. Changes in the populations of individual bacteria were analyzed by ANOVA and Tukey's test accordingly. For heat maps, only bacteria with abundance higher than 1% were used. A complete list of microorganisms identified are showed in Table S1 in Supplementary Material for ileum and Table S2 in Supplementary Material for cecum.

RESULTS

There was no interaction between SH and PRO birds for live performance and SH did not affect these parameters at any age period. The addition of PRO at 250 g/ton increased ($P < 0.05$) FI and BWG from 1 to 21 days compared to Control (**Table 2**).

As expected, the non-challenged Control, PRO 250, and PRO 500 groups tested negative for *Salmonella* therefore data were analyzed using the SH challenged treatments only as a completely randomized design. In liver, the SH + PRO

TABLE 2 | Mean ± SD of feed intake (FI) (g), body weight gain (BWG) (g), and feed conversion (FC) during the periods 1 to 7, 1 to 14, and 1 to 21 days of age.

	FI 1–7 (g)	FI 1–14 (g)	FI 1–21 (g)	BWG 1–7 (g)	BWG 1–14 (g)	BWG 1–21 (g)	FC 1–7	FC 1–14	FC 1–21
Challenge					**Main effects**				
Control	122.99 ± 8.54	468.06 ± 35.08	1079.7 ± 83.76	110.53 ± 2.49	390.83 ± 12.09	886.97 ± 27.03	1.112 ± 0.01	1.202 ± 0.02	1.220 ± 0.01
SH	116.99 ± 13.03	463.20 ± 35.56	1093.0 ± 62.82	107.20 ± 2.98	408.63 ± 14.76	916.90 ± 32.57	1.091 ± 0.01	1.142 ± 0.02	1.202 ± 0.03
Probiotic									
Control	114.79 ± 14.63	447.58 ± 49.59	**1035.2 ± 83.95^{b}**	103.25 ± 3.86	374.65 ± 19.95	**839.11 ± 38.50^{b}**	1.110 ± 0.02	1.204 ± 0.03	1.243 ± 0.03
250	127.45 ± 5.88	482.56 ± 18.51	**1131.3 ± 48.07^{a}**	114.24 ± 1.85	427.26 ± 13.61	**965.98 ± 31.46^{a}**	1.116 ± 0.01	1.137 ± 0.03	1.176 ± 0.02
500	116.98 ± 7.53	466.56 ± 17.51	**1094.5 ± 43.77^{a,b}**	108.89 ± 3.13	398.22 ± 8.29	**902.68 ± 23.25^{a,b}**	1.075 ± 0.02	1.173 ± 0.01	1.214 ± 0.01
Probabilities									
Challenge (P_1)	0.204	0.763	0.659	0.374	0.350	0.483	0.496	0.098	0.655
Probiotic (P_2)	0.058	0.163	**0.031**	0.071	0.062	**0.040**	0.411	0.229	0.278
Interaction ($P_1 \times P_2$)	0.639	0.755	0.865	0.743	0.259	0.410	0.787	0.061	0.336

^{a,b}Different letters in the same column indicate significant differences at $P < 0.05$ at Tukey's test.
Shading was used to distinguish columns from FI, BWG,FC.
Bold values were used to distinguish statistical differences.

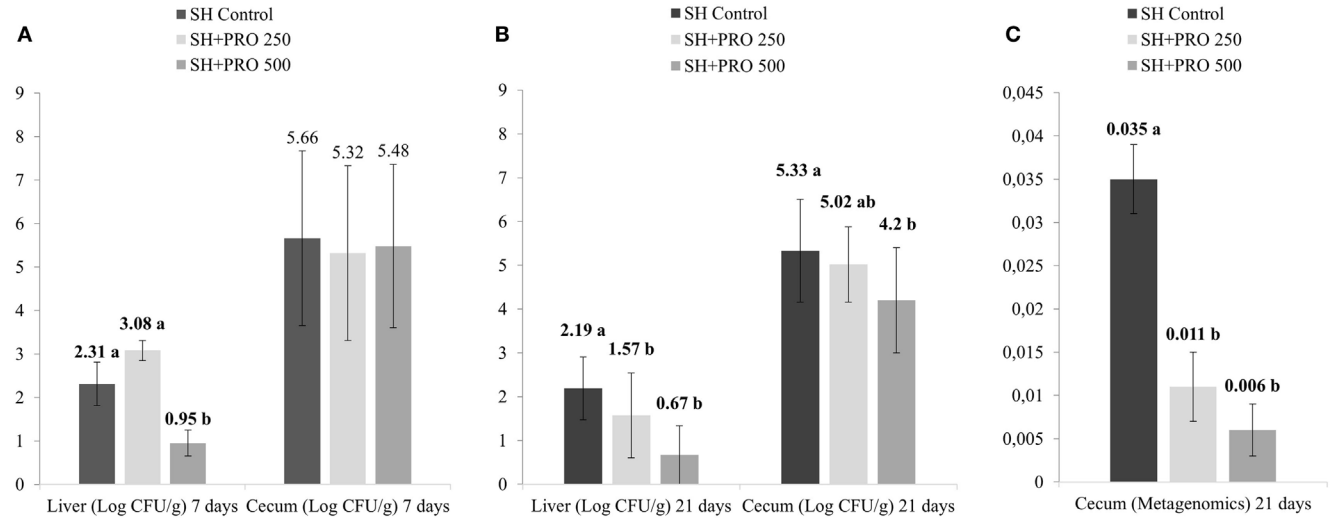

FIGURE 1 | *Salmonella* sp. quantification. **(A)** *Salmonella* sp counts (Log CFU/g) in liver and cecum at 7 days of age (4 days after inoculation) in treatments SH Control, SH + PRO 250, and SH + PRO 500 according to adapted methodology by Cox et al. (21). **(B)** *Salmonella* sp. counts (Log CFU/g) in liver and cecum at 21 days of age in treatments SH Control, SH + PRO 250, and SH + PRO 500 according to adapted methodology by Cox et al. (21). **(C)** Relative abundance using metagenomics analysis in ceca at 21 days of age in treatments SH Control, SH + PRO 250, and SH + PRO 500. Non-challenged groups (Control, PRO 250, and PRO 500) were negative for *Salmonella* in both methodologies. Different letters indicate significant differences at $P < 0.05$ at Kruskal–Wallis.

500 chicks had reduced SH counts ($P < 0.01$) compared to the SH Control birds at 7 days (**Figure 1A**), whereas both the SH + PRO 250 and the 500 birds had reduced SH counts at 21 days ($P < 0.01$) compared to the SH Control group (**Figure 1B**). In ceca, only the SH + PRO 500 group had reduced ($P < 0.05$) *Salmonella* counts (**Figure 1B**) using the bacteriological quantification (21). However, the PRO when fed at either dose significantly reduced *Salmonella* frequencies in cecum according to the more refined metagenomic analysis (**Figure 1C**) at 21 days of age.

Liver histologic alterations by ISI and immunohistochemistry analysis are summarized in **Tables 3** and **4**, respectively. No differences in ISI scores in liver were found among treatments in non-challenged birds at 7 days. Still, immunohistochemistry analysis revealed that the PRO fed at 500 g/ton reduced macrophages and CD4+ cells recruitment in the liver of those chickens compared to the Controls ($P < 0.05$).

The challenged birds fed the PRO had livers with lower histological alteration scores compared to the SH Control group ($P < 0.01$) at 7 days of age. A reduction on hydropic degeneration and necrosis of liver parenchyma were associated with those observations. In addition, higher macrophage counts in liver were found in both the SH + PRO 250 and the 500 groups compared to the SH Control (**Table 4**). This could be related to the SH reduction in this organ (at least for the PRO when fed at 500 g/ton). The opposite was observed in non-challenged birds when the PRO 500 chicks exhibited reduced ($P < 0.01$) macrophages and CD4+ cells in liver parenchyma.

At 21 days of age, the PRO 500 birds had increased ISI liver scores compared to the Control and the PRO 250 groups in non-challenged birds (**Table 3**). No differences were found in the SH-challenged broilers on this parameter. Still, increased CD4+ cells counts were observed in both the SH + PRO 250 and the SH + PRO 500 groups compared to the SH Control birds (**Table 4**). The macrophage counts were higher in liver at 21 days of age regardless of the SH challenge.

TABLE 3 | Mean ± SD of histological alterations (ISI) in liver (score per field) and ileum (score per villi) at 7 and 21 days of age.

	Liver		Ileum	
	7 days	**21 days**	**7 days**	**21 days**
Challenge				
Control	**23.49 ± 6.53^{a}**	**12.26 ± 5.78^{b}**	5.29 ± 4.39	9.99 ± 4.55
SH	**20.25 ± 7.13^{b}**	**20.63 ± 6.61^{a}**	4.56 ± 4.54	10.42 ± 3.54
Probiotic				
Control	**24.09 ± 5.49^{a}**	**16.84 ± 6.74^{b}**	**4.36 ± 4.32^{b}**	**9.11 ± 4.16^{b}**
250	**20.82 ± 7.63^{b}**	**17.48 ± 7.41^{b}**	**4.42 ± 4.24^{b}**	**11.52 ± 3.95^{a}**
500	**19.08 ± 7.09^{b}**	**19.19 ± 7.06^{a}**	**5.71 ± 4.84^{a}**	**10.21 ± 3.20^{b}**
Interaction				
Control	**23.70 ± 5.82^{a}**	**9.93 ± 5.25^{c}**	5.01 ± 0.57	**10.50 ± 5.22^{a,b}**
PRO 250	**23.10 ± 7.27^{a}**	**11.40 ± 5.28^{c}**	4.11 ± 0.57	**10.47 ± 4.94^{a,b}**
PRO 500	**23.67 ± 6.52^{a}**	**15.45 ± 5.42^{b}**	6.75 ± 0.57	**9.00 ± 3.13^{b,c}**
SH Control	**24.28 ± 5.34^{a}**	**20.31 ± 6.39^{a}**	4.03 ± 0.40	**8.42 ± 3.33^{c}**
SH + PRO 250	**19.67 ± 7.58^{b}**	**20.52 ± 6.38^{a}**	4.57 ± 0.40	**12.04 ± 3.24^{a}**
SH + PRO 500	**16.79 ± 6.23^{c}**	**21.06 ± 7.06^{a}**	5.13 ± 0.42	**10.79 ± 3.08^{a}**
Probabilities				
Challenge (P_1)	**<0.001**	**<0.001**	0.081	0.204
Probiotic (P_2)	**<0.001**	**<0.001**	**0.002**	**<0.001**
Interaction ($P_1 \times P_2$)	**<0.001**	**<0.001**	0.106	**<0.001**

^{a,b,c}Different letters in the same column indicate significant differences at $P < 0.05$ at Kruskal–Wallis test.
Bold values were used to distinguish statistical differences.

TABLE 4 | Mean ± standard error of macrophages, CD4+ and CD8+ cells quantification by immunohistochemistry in liver (cells per field at 100× of magnification) at 7 and 21 days of age.

	Macrophages		CD4+		CD8+	
	7 days	**21 days**	**7 days**	**21 days**	**7 days**	**21 days**
Challenge						
Control	19.25 ± 1.09	10.88 ± 0.66	3.48 ± 0.26	4.35 ± 0.28	**4.63 ± 0.31^{b}**	4.50 ± 0.29
SH	23.96 ± 0.33	11.78 ± 0.61	3.53 ± 0.10	4.12 ± 0.25	**5.30 ± 0.13^{a}**	4.78 ± 0.25
Probiotic						
Control	**20.97 ± 0.95^{b}**	**8.32 ± 0.65^{b}**	**4.00 ± 0.22^{a}**	3.50 ± 0.28	**5.53 ± 0.33^{a}**	3.92 ± 0.28
250	**25.35 ± 0.47^{a}**	**13.21 ± 0.79^{a}**	**3.63 ± 0.16^{a}**	4.85 ± 0.35	**5.05 ± 0.15^{a,b}**	5.76 ± 0.34
500	**20.87 ± 1.01^{c}**	**12.91 ± 0.80^{a}**	**2.92 ± 0.17^{b}**	4.25 ± 0.35	**4.67 ± 0.20^{b}**	4.38 ± 0.33
Interaction			**Interactions**			
Control	**20.15 ± 1.35^{b}**	7.70 ± 1.30	**4.40 ± 0.57^{a}**	**5.15 ± 0.56^{a}**	**4.65 ± 0.79^{a,b}**	4.1 ± 0.56
PRO 250	**26.80 ± 1.11^{a}**	11.4 ± 1.3	**4.15 ± 0.24^{a}**	**4.25 ± 0.43^{a,b}**	**5.30 ± 0.30^{a,b}**	5.50 ± 0.56
PRO 500	**10.80 ± 1.13^{c}**	13.55 ± 1.3	**1.90 ± 0.22^{b}**	**3.65 ± 0.41^{a,b}**	**3.95 ± 0.40^{b}**	3.90 ± 0.56
SH Control	**21.37 ± 0.72^{b}**	8.62 ± 0.92	**3.80 ± 0.16^{a}**	**2.67 ± 0.22^{b}**	**5.97 ± 0.28^{a}**	3.82 ± 0.39
SH + PRO 250	**24.62 ± 0.41^{a}**	14.12 ± 0.92	**3.37 ± 0.20^{a}**	**5.15 ± 0.47^{a}**	**4.92 ± 0.17^{a,b}**	5.90 ± 0.39
SH + PRO 500	**25.90 ± 0.26^{a}**	12.6 ± 0.92	**3.42 ± 0.18^{a}**	**4.55 ± 0.49^{a}**	**5.02 ± 0.21^{a,b}**	4.65 ± 0.39
Probabilities						
Challenge (P_1)	**<0.001**	0.332	0.817	0.567	**0.020**	0.475
Probiotic (P_2)	**<0.001**	**<0.001**	**<0.001**	0.233	**0.050**	**0.001**
Interaction ($P_1 \times P_2$)	**<0.001**	0.271	**<0.001**	**0.001**	**0.035**	0.576

^{a,b,c}*Different letters in the same column indicate significant differences at $P < 0.05$ at Kruskal–Wallis test.*
Bold values were used to distinguish statistical differences.

Birds fed PRO at 500 g/ton had higher ISI scores in ileum at 7 days of age (**Table 3**). The main alterations observed in challenged birds were an increase in lamina propria thickness, epithelial thickness and proliferation of goblet cells ($P < 0.05$). At 21 days of age, a significant interaction for ileal ISI scores was found, where both the SH + PRO 250 and the SH + PRO 500 groups presented higher ISI scores than the SH Control, while no significant differences were observed in non-challenged birds (**Table 3**). The main histologic alterations found in the PRO 500 g/ton group at that age were also observed at 7 days (**Figures 2C,D**).

The metagenomic analysis of gut microbiota revealed an average of 411.360 and 157.658 reads per sample of cecum and ileum, respectively. Based on 97% species similarity, an average of 9.330 and 1.942 operational taxonomic units (OTUs) were obtained in cecum and ileum, respectively. The rarefaction curves suggested that in all treatments enough sequence reads per sample were collected, showing that sampling has been exhaustively sequenced and was enough to uncover major OTUs (**Figure 3**). The diversity index by Shannon–Wiener revealed that cecal microbial composition of the SH + PRO 500 group was significantly more diverse compared to the Control and the SH Control groups. The SH + PRO 250 birds had significant ($P < 0.05$) higher richness (JackkNife test) in ileal microbiota compared to the Control group, while evenness test (Hill) revealed that the SH + PRO 500 birds have lower species evenness in the cecum compared to the SH Control group (**Figure 4**).

The family profiles of the corresponding ileal microbial populations are shown in **Figure 5A**. As expected, the data on microbiota presented high coefficients of variation addressing the difficulties in establishing statistical differences. The Clostridiaceae family (mostly represented by *Clostridium perfringens*) presented numerically lower abundance in the SH + PRO 500 chickens. *C. perfringens* were detected in high quantity in ileum because the samples were collected in the distal section. The unidentified members of Clostridiales order (group 1) revealed higher numerical abundance in the SH + PRO 500 broilers as opposed to other groups. The unidentified members of *Enterococcus* genus (phylum Firmicutes) and members of Peptostreptococcaceae family (group 1; class Clostridia) were significantly higher ($P < 0.05$) in the SH + PRO 250 chickens compared to the Control ones (**Figure 6A**). Another significant difference in ileum ($P < 0.05$) is related to unidentified members of Streptophyta order, within the Cyanobacteria phylum. This bacterium was more abundant in the SH + PRO 250 group compared to the Control and the SH + PRO 500 treatments (**Figure 6A**).

In cecal microbiota, the majority of Clostridiales detected fall primarily into Ruminococcaceae, Lachnospiraceae and Clostridiaceae families (**Figure 5B**). An unidentified member of RF39 order (phylum Tenericutes, class Mollicutes) presented a statistical difference ($P = 0.041$) between the Control and the SH Control cages (**Figure 6B**). The abundance of *Salmonella sp* in ceca was lower than 1% (i.e., up to 0.035%) been significantly lower in broilers fed PRO at both dosages comparing to the Control and the SH Control birds (**Figure 1C**; $P < 0.05$).

DISCUSSION

No loss in performance resulted from challenging birds with SH at any time. This agrees with previous studies in our laboratory which showed that not all *Salmonella* influence the performance of broilers (29). As the current trial was not primarily designed to test performance, the experimental layout had low statistical power to detect differences in parameters such as intake and weight gain. Still, a significant improvement in performance

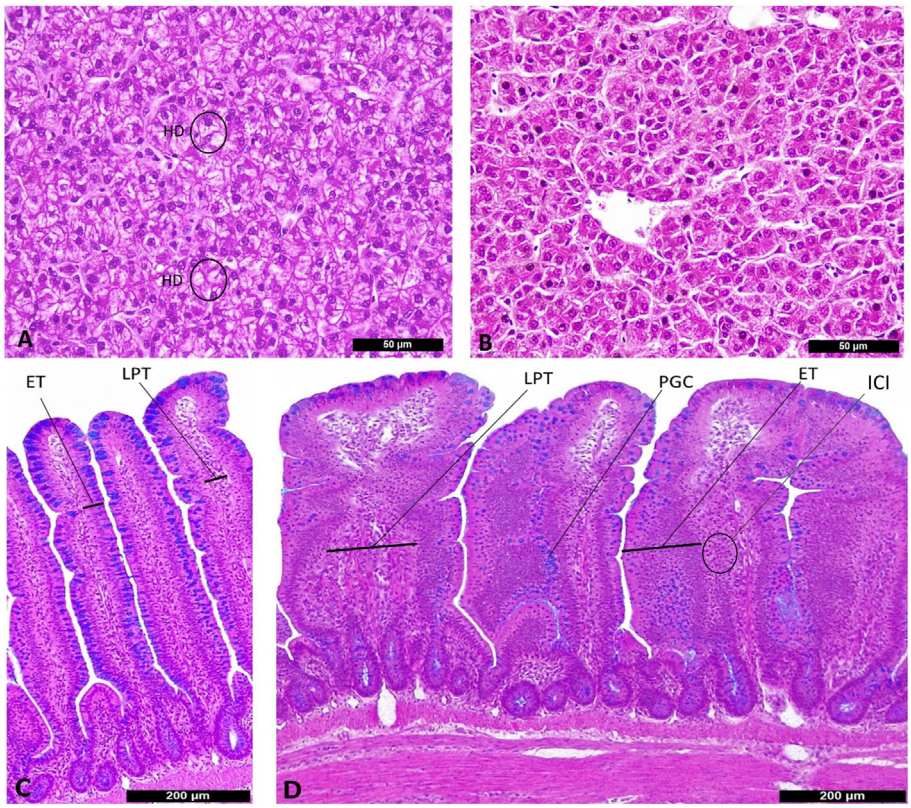

FIGURE 2 | Histological alterations in liver **(A,B)** and ileum **(C,D)** according to I See Inside (ISI) scoring methodology (100×). **(A)** Liver from SH Control, presenting score 3 of hydropic degeneration (HD) at 7 days of age. **(B)** Liver from SH + PRO 500, normal hepatocytes at 7 days of age. **(C)** Ileum from SH Control, villi with scores zero for epithelial thickness (ET) and lamina propria thickness (LPT) at 21 days of age. **(D)** Ileum from SH + PRO 250 with score 2 for epithelial thickness (ET), score 2 for proliferation of goblet cells (PGCs) and score 2 for LPT with inflammatory cells infiltration (ICI) at 21 days of age.

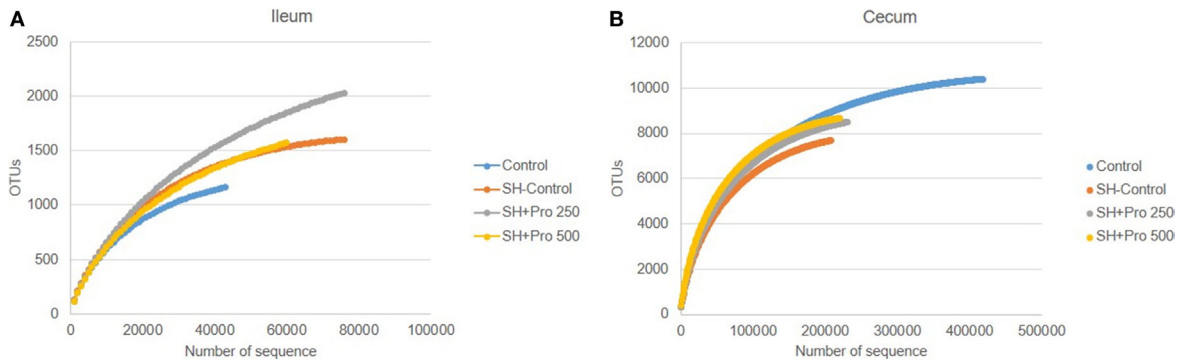

FIGURE 3 | Rarefaction plot from ileal **(A)** and cecal **(B)** microbiota of groups Control, SH Control, SH + PRO 250, and SH + PRO 500. *$P < 0.05$. **$P = 0.08$. Rarefaction analysis suggested that the number of sequences from all experimental samples were enough to uncover major Operational Taxonomic Units (OTUs).

resulted from feeding PRO at 250 g/ton. This has also been observed by other workers when feeding some *B. subtilis* strains to broilers (13, 14, 30).

It is worth noticing that the resulting abundance of *Salmonella* in cecum was relatively low (up to 0.035% for the SH Control group) compared to other bacterial groups (**Figures 5** and **6**); and that it was not detected in the ileum of chickens even in those orally challenged with SH, confirming the low affinity of *Salmonella* for that organ. Still, Feeding PRO at 500 g/ton reduced *Salmonella* counts in both liver and cecum by the end of the trial. In the latter organ, metagenomics showed that both dosages were equally effective in reducing *Salmonella* abundance.

Other studies (17) have also shown that adding *B. subtilis* spores in the diet could reduce SH colonization at 42 days of age

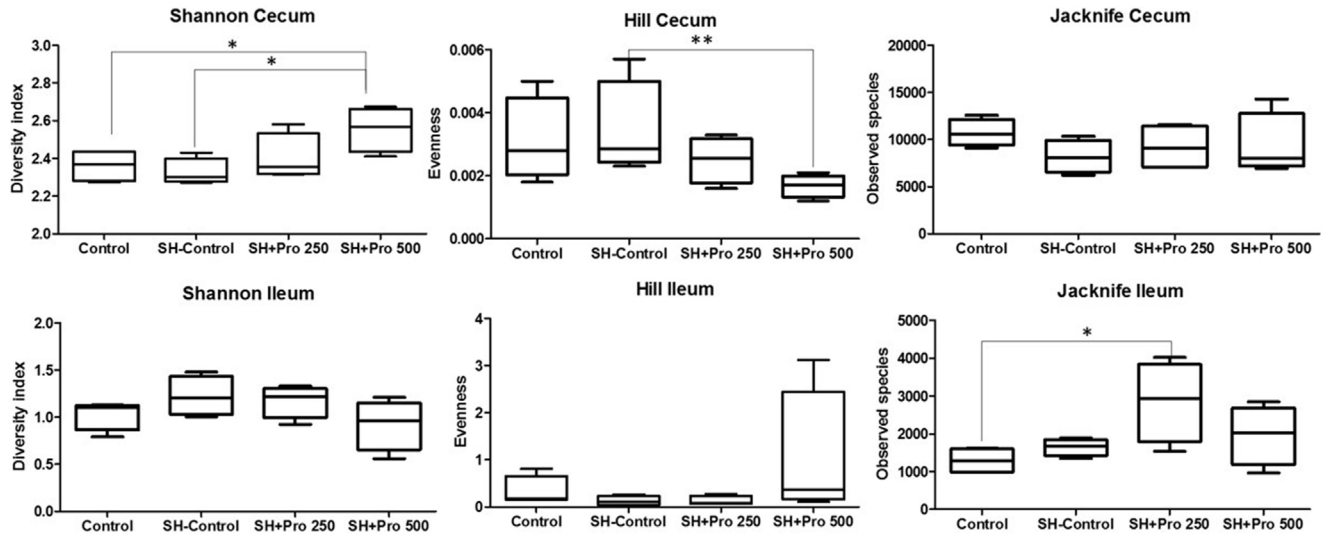

FIGURE 4 | Ileal and cecal diversity (Shannon–Wiener), evenness (Hill), and richness (JackkNife) index of groups Control, SH Control, SH + PRO 250, and SH + PRO 500. *$p < 0.05$. **$p = 0.08$.

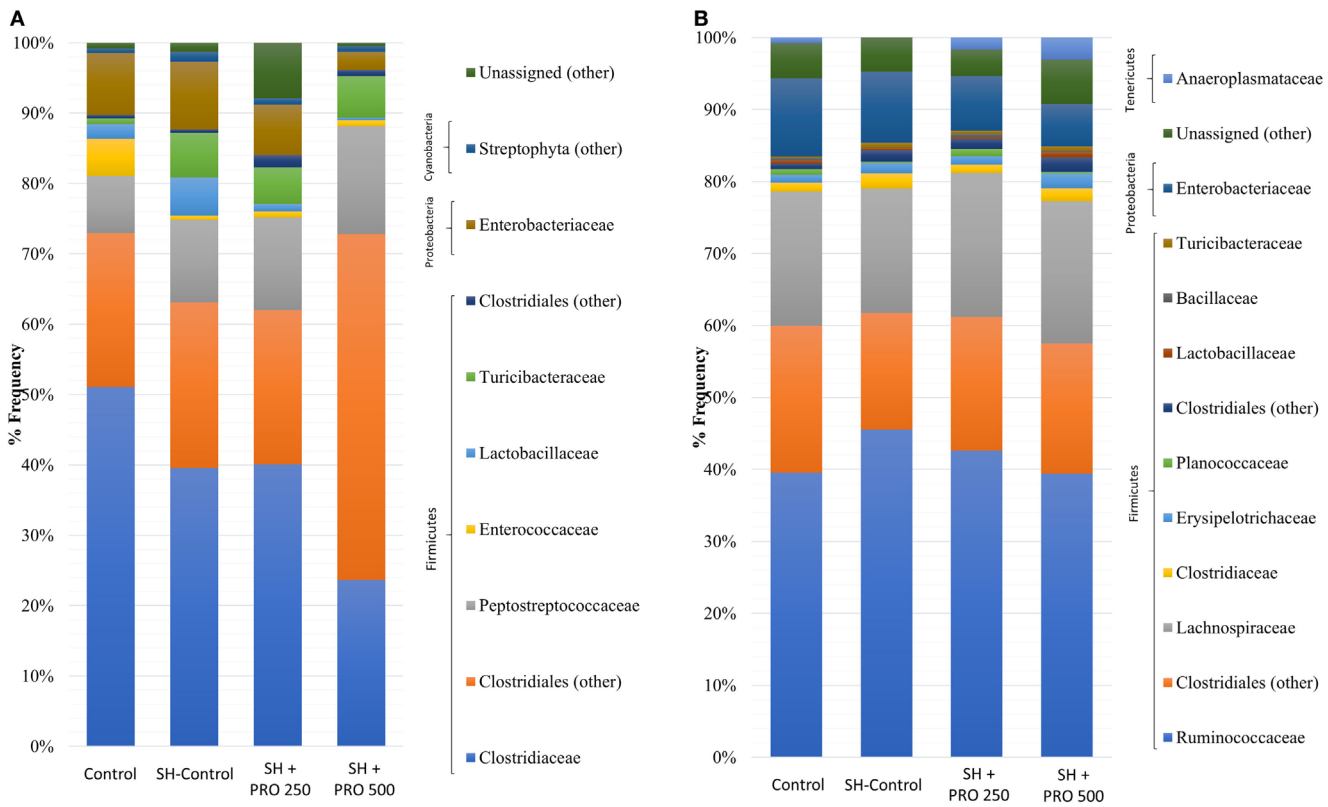

FIGURE 5 | Relative abundance of bacteria population in ileal microbiota **(A)** and cecal microbiota **(B)** of groups Control, SH Control, SH + PRO 250, and SH + PRO 500 at 21 days of age, analyzed by sequencing using Illumina MiSeq System.

by up to 58%. The most commented mechanism been competitive exclusion by which *B. subtilis* bacteria occupy adhesion locations of the membranes of enterocytes, goblet and enteroendocrine cells regularly used by *Salmonella*, therefore preventing it from establishing itself in the gut (31). An agonist effect caused by the secretion of substances by *B. subtilis*, such as bacteriocins,

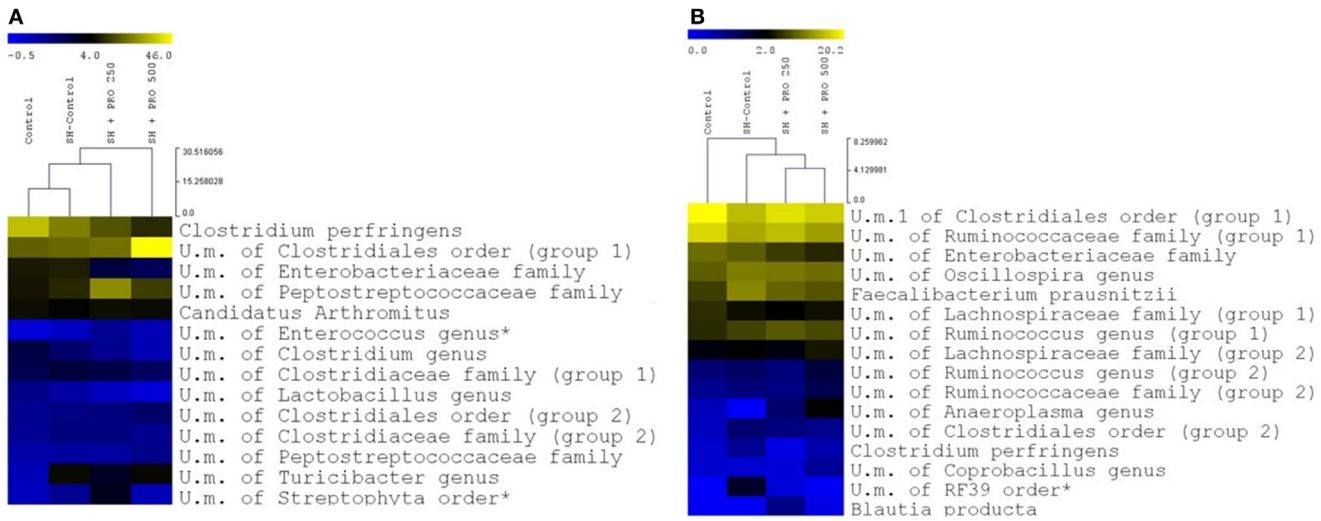

FIGURE 6 | Relative abundance of distinct groups at the deepest level identified in ileum **(A)** and cecum **(B)** of groups Control, SH Control, SH + PRO 250, and SH + PRO 500. A yellower color depicts a greater bacterial abundance to up to 46.2% in ileum **(A)** abundance and up to 20.2% in cecum **(B)** abundance. Groups with abundance less than 1% were not considered. U.m., unidentified members. * Indicate significant differences at $P < 0.05$.

organic acids, and hydrogen peroxide, can also inhibit the growth and development of pathogenic bacteria. Likewise, some strains of *B. subtilis* are known to favor the growth of lactic acid-producing bacteria (12) with a subsequent acidification of the intestinal environment (32). These effects could modulate the host's microbial populations and the intestinal immune response potentially reducing the frequency of *Salmonella* in the gut and its capacity to migrate from the intestinal lumen into other organs. These are in agreement with the observations in the current trial.

Feeding PRO may help to reduce some deleterious alterations in liver parenchyma caused by SH. Hydropic degeneration is an intracytoplasmic fluid accumulation, secondary to disturbance of cell membrane integrity causing vacuolation of hepatocytes (**Figures 2A,B**). One of the causes is bacterial infections with differing lobular localization and may be a precursor to hepatocyte necrosis (33). Also, the interesting transport of immune cells of PRO in liver was reported by other study (34) where probiotic bacteria reduced monocyte and macrophage recruitment to the intestines and spleen compared to control animals. Probiotics may ameliorate proinflammatory immune cell recruitment to systemic lymphoid tissues such as liver and other organs. This could save metabolic energy and have positive effect on performance, which in the present trial was observed in broilers fed PRO at 250 g/ton of feed. This performance improvement was not observed when feeding PRO at the highest dose, however, this group of birds showed a significant reduction in *Salmonella* infection when challenged with SH, recruited macrophages to eliminate bacteria by phagocytosis, secreted cytokines to modulate immunity and presented antigens to helper T cells (35).

The relationship between chicken macrophages and *Salmonella,* as well as intracellular survival of *Salmonella* in chicken macrophages, remains poorly understood. According to Van Immerseel et al. (36), the encounter between specialized epithelial cells and microorganisms quickly stimulates the release of proinflammatory chemokines that attract innate immune cells (i.e., granulocytes and macrophages), which are able to trigger a wide range of new immune responses such as the emergence of T helper lymphocytes (CD4+ cells). An early increase in CD4+ and CD8+ cells has been reported in chickens fed probiotics (37–39). In some cases, *Salmonella* cells invade and multiply within the macrophages (40–42) and widely distribute themselves in the lymphoid and nonlymphoid tissues, facilitating their spreading to various organs of the host.

In this study, histology observations in ileum seemed atypical as reported in other *Salmonella* trials (37, 38) suggesting a considerable variation on ileal morphology when *Salmonella* is present. This variation in ileum histology could be associated with the fact that *Salmonella* has the cecum as target tissue.

Some alterations were observed on ileum histology due to PRO activity such that lamina propria and epithelial thickness increased along with goblet cells proliferation. Probiotics exert a range of effects on mucosal barrier function and on responses of the underlying immune tissue of the gut associated with lymphoid tissue (19). This barrier function is enforced by the ability of probiotics to influence mucin expression and mucus secretion of goblet cells. It is likely that the probiotic-mediated modulation of mucin expression is a host's strategy to allow beneficial microbes to colonize the gut (43). Furthermore, mucins may exert prebiotic-type effects as carbohydrate content can account for 90% of their weight (44). Muniz et al. (37) observed similar effects when four different probiotics increased the proliferation of goblet cells in ileum. The association of probiotics with epithelial cells might be sufficient to trigger signaling cascades at epithelium level and activate underlying

immune cells in lamina propria (45). Probiotics may increase epithelial and lamina propria thickness, characterized by cell proliferation and inflammatory cells infiltration, respectively (**Figure 2D**), describing a mucosal wound repair (46). In a recent publication, Kraieski et al. (24) observed a positive correlation between ileal epithelial thickness and goblet cells proliferation with BWG, and a negative correlation with FC at 21 days of age. In the present experiment, PRO fed at 250/ton improved BWG while the SH + PRO 250 group presented higher ileal ISI than the SH Control birds at 21 days along with increased epithelial thickness, goblet cells proliferation and lamina propria thickness.

The metagenomics analysis also showed a significant increase in *Bacillus* genus abundance in the ileum of birds fed PRO going from 0.004 ± 0.002% for the Control group to 0.019 ± 0.004% for the SH + PRO 500 animals (Table S1 in Supplementary Material). That could be due to the presence of *Bacilli* from PRO in that organ itself or, could have been the result of gut microbial changes in *Bacilli* populations not necessarily of PRO origin, since the *Bacillus* genus is commonly found in the ileal microbiota of broilers.

The diversity index by Shannon–Wiener revealed that cecal microbial composition of the SH + PRO 500 group was significantly more diverse compared to the Control and the SH Control groups (**Figure 4**). Pereira (47) detected less diversity in chickens fed with *B. subtilis* spores. However, it has been reported that the use of probiotics can increase the intestinal microbiota diversity in different organisms (48, 49). Diversity is a combination of richness and evenness. Increasing the diversity tends to suggest more stable ecosystems with more connections within them, even though statistical differences in performance were not observed in the SH + PRO 500 treatment.

In general, the most abundant phylum in the chicken intestinal microbiota is Firmicutes followed by two minor phyla, Proteobacteria and Bacteroidetes. In addition, members of phylum Actinobacteria, Tenericutes (50), Cyanobacteria, and Fusobacteria (51) can be found in very low abundance. In the present study, Firmicutes was the most predominant phylum found in ileum and cecum in all groups. Proteobacteria, Cyanobacteria (ileum), and Tenericutes (cecum) were also observed but showing lower abundance (**Figures 5A,B**).

Enterococcus (phylum Firmicutes) is a large genus of lactic acid bacteria, commensals of animal and human gut (52). In ileum, this genus was significantly higher ($P < 0.05$) in the SH + PRO 250 rather than in the Control group (**Figure 6A**). Many enterococci species such as *E. faecium* produce bacteriocins which have been associated with growth inhibition of food-borne pathogens in the gut (53). It might be possible that increases in the relative abundance of above mentioned commensals in probiotic treated chickens reduced *Salmonella* colonization or simply contributed to intestinal health. Members of Peptostreptococcaceae family (class Clostridia) seemed to be more abundant in the SH + PRO 250 broilers compared to the Control group (p=0.06). The Peptostreptococcaceae was isolated from various environments including clinical human and animal samples, manure, soil, marine and terrestrial sediments, and deep-sea hydrothermal vents. High percentage of Peptostreptococcaceae was found in ileal samples from conventional broiler chickens at 7 and 41 days of age, assuming that this family might be considered a commensal bacteria group (54). Another significant difference in ileum ($P < 0.05$) is related to unidentified members of Streptophyta order, within the Cyanobacteria phylum, that could be attributed to chloroplasts, non-photosynthetic bacteria commonly found in the animal gut (55). This bacterium was more abundant in the SH + PRO 250 group compared to the Control and the SH + PRO 500 treatments (**Figure 6A**).

An unidentified member of RF39 order (phylum Tenericutes, class Mollicutes) was more abundant than in Control when SH was present while feeding PRO could reduce it numerically in cecal microbiota (**Figure 6B**). In past studies, it was reported that Mollicutes were enriched in birds affected by necrotic enteritis disease and this could possibly be associated with intestinal disorders for chickens (56). However, Perez-Brocal et al. (57) observed that humans with Crohn's disease (inflammatory bowel disease) showed lower abundance of bacteria from RF39 order compared to the Control group. Goodrich et al. (58) observed an increase of RF39 order in lean body mass adults, compared to obese individuals. Besides the lack of information in literature, it is not possible to assume correlations with those data once the genus from RF39 order was unidentified in the current experiment.

CONCLUSION

A probiotic composed by three strains of *B. subtilis* improved animal performance when fed at 250 g/ton and reduced *Salmonella* colonization in liver and cecum at 250 and 500 g/ton when birds were orally challenged with SH strain UFPR1. The mobilization of immune cells in liver can be a relevant mode of action of PRO in birds challenged with SH. PRO can promote important histologic alterations related to activation of defense response and gut absorption. In addition, the supplementation of PRO increased the diversity of cecal microbiota, which suggests a more stable ecosystem, and increased some commensal bacterial groups in ileum, some of which are lactic-acid producing organisms.

ETHICS STATEMENT

The experiment was approved by the Ethical Committee of Agricultural Sector of Federal University of Parana under approval number: 037/2016.

AUTHOR CONTRIBUTIONS

RH: *in vivo* trial, analysis of the data, microbiology, immunohistochemistry, and performance analysis; drafting and revising it critically and final approval of the version. ML: *in vivo* trial, microbiology analysis. AK: *in vivo* trial, statistical analysis. RA: microbiome analysis orientation, development of *B. subtilis*. RG-E: statistical analysis, development of *B. subtilis*. EL: microbiome

analysis and interpretation of results. AC: microbiome analysis and interpretation of results. MC: microbiome analysis and interpretation of results. PM: microbiome analysis and interpretation of results. ES: orientation of the all experiment; drafting and revising it critically and final approval of the version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fvets.2018.00013/full#supplementary-material.

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REVIEW
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The Potential Link between Thermal Resistance and Virulence in *Salmonella:* A Review

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In some animals, the typical body temperature can be higher than humans, for example, 42°C in poultry and 40°C in rabbits which can be a potential thermal stress challenge for pathogens. Even in animals with lower body temperatures, when infection occurs, the immune system may increase body temperature to reduce the chance of survival for pathogens. However, some pathogens can still easily overcome higher body temperatures and/or rise in body temperatures through expression of stress response mechanisms. *Salmonella* is the causative agent of one of the most prevalent foodborne illnesses, salmonellosis, and can readily survive over a wide range of temperatures due to the efficient expression of the heat (thermal) stress response. Therefore, thermal resistance mechanisms can provide cross protection against other stresses including the non-specific host defenses found within the human body thus increasing pathogenic potential. Understanding the molecular mechanisms associated with thermal responses in *Salmonella* is crucial in designing and developing more effective or new treatments for reducing and eliminating infection caused by *Salmonella* that have survived heat stress. In this review, *Salmonella* thermal resistance is assessed followed by an overview of the thermal stress responses with a focus on gene regulation by sigma factors, heat shock proteins, along with the corresponding thermosensors and their association with virulence expression including a focus on a potential link between heat resistance and potential for infection.

Keywords: *Salmonella*, thermal stress response, heat shock proteins, sigma factor, virulence

INTRODUCTION

Salmonella is a Gram-negative foodborne pathogen that is a major concern for the food industry and public health authorities because of its capability to cause both widespread contamination and infection within the United States (US) and worldwide (1–5). An estimated one million cases of *Salmonella*-related illnesses occur annually within the US. For example, in 2014, *Salmonella* was responsible for 10 multistate outbreaks with approximately 1,000 reported outbreak cases (3, 6). Numerous strategies have been implemented to reduce *Salmonella* transmission, contamination, and infection. *Salmonella* infections are most commonly acquired through ingestion of contaminated foods such as eggs and poultry meat (7). *Salmonella* can colonize the small intestines of

poultry birds, along with the cecum, without demonstrating any symptoms related to *Salmonella* infections. Therefore, poultry serves as an efficient vector of transmission for multiple serovars of *Salmonella* to humans through consumption of contaminated food products.

In order for *Salmonella* to survive and colonize the human body, it must overcome multiple non-specific host defenses encountered within the host such as low pH, limited nutrient availability and in poultry birds, a high body temperature (42°C). Due to the wide temperature range that *Salmonella* may grow in, it must possess specific mechanisms that can overcome thermal stress to proliferate and survive. However, prior to ingestion, *Salmonella* is already preexposed to a higher core body temperature in poultry compared to humans (37°C). During infection, one of the primary defenses of the innate immunity is an increase in body temperature through pyrogens (antigens that stimulate fever) such as lipopolysaccharide found in the cell wall of Gram-negative bacteria (8). This preexposure could increase the potential of *Salmonella* to establish infection of the host due to adaption to higher temperatures. Therefore, the aim of this review is to provide an overview of phenotypic and molecular responses to temperature changes as it relates to poultry, thermal stress regulation, and how this increases pathogenic potential of *Salmonella*.

THERMAL AND NON-THERMAL STRESSES

With over 2,500 serovars of *Salmonella*, several have developed the ability to overcome high temperatures allowing for survival through thermal processing; however, this is strain specific (9–11). O'Bryan et al. (12) reviewed the thermal resistance of *Salmonella* species and other foodborne pathogens associated with meat and poultry. They concluded that a variety of factors and parameters are involved in the thermal resistance and inactivation of those pathogens and spoilage microorganisms such as various temperature exposures, growth phase, and the intrinsic conditions of the food product. Strains of the same microbial species were found to be capable of responding differently to the same treatments possibly due to specific variations in gene composition for each respective strain. Likewise, the stages of growth, the age of the culture, and the conditions of bacterial growth have yielded various outcomes regarding heat inactivation or destruction of microorganisms, which could contribute to determining the best methods to reduce microbial growth and contamination within these products (12).

There are several factors that allow *Salmonella* strains to survive the food processing environment and overcome thermal treatment. For example, preexposure to stress and growth conditions prior to thermal treatment could increase survival capability during processing. Specifically, *S.* Senftenberg was found to survive in broiler litter for up to 24 h at 80°C (13). Microorganisms tested against heat are known to elicit different responses in regard to prior growth conditions with stationary phase cells being more resistant to heat than log phase cells (14–16). In addition, stressed cells such as those exposed to temperatures slightly above an organism's optimal growth range (heat shocked cells), those grown on limited carbon sources, those experiencing desiccation, and those undergoing starvation stress prior to heat treatments have been shown to exhibit more thermal tolerance (17–21).

Exposure to non-thermal stress may also have an impact on the capability of *Salmonella* to respond to thermal threats. For example, Milillo and colleagues concluded that combining organic acids with heat can effectively reduce *Salmonella* over a short period of time (22, 23). They applied mild thermal treatments and organic acids with a 1-min exposure time. Sodium propionate in combination with heating was demonstrated to be the most significantly effective for reducing viable *Salmonella* (22). In a follow-up study, Milillo et al. (23) conducted microarray experiments to explore the specific response of *S.* Typhimurium to organic acids in combination with heat. Exposure to sodium acetate with heat (55°C) and sodium propionate with heat (55°C) led to differentially 288 (124 upregulated and 168 downregulated genes) and 319 (131 upregulated and 181 downregulated genes) gene expression level changes, respectively. Numerous heat shock genes including *dnaK*, *hptJ*, *dnaJ*, *grpE*, *clpP*, and *hscAB* were repressed by both treatments. They concluded that this synergism may be attributed to damage in the synthesis of heat shock genes of *S.* Typhimurium due to membrane damage. Given the potential for such synergism among otherwise unrelated interventions, there may be opportunities for optimizing hurdle technologies in the food industry and demonstrating the utility of using genomic screening to develop application approaches for these technologies.

THERMOSENSORS

In order for *Salmonella* to overcome and adapt to an ever-changing environment it must overcome stressors encountered during its travel through the host; therefore, adaptation through sensory mechanisms is imperative. Thermosensors are considered the cell's "thermometer" by utilizing various types of biological systems to detect temperature fluctuations within the cell. There are four different groups of thermosensors including proteins, lipids and membrane fluidity, RNA's that are temperature responsive, and DNA structure and topology. Thermosensors play a major role in temperature detection and are found within the 5′ UTR region, which can regulate gene expression to produce adaptive heat stress responses. When temperature decreases or increases to harmful levels, stress responses (cold and heat shock) are needed to protect the bacterial cell and are thoroughly dependent on bacterial signal transduction mechanisms (24). Genes involved in these mechanisms are regulated at different genetic stages beginning from transcription through translation and into posttranslational levels (25, 26).

As a protective reaction, misfolded and unfolded proteins are present in considerable numbers in the cytoplasmic membrane and the outer membrane during exposure to higher than optimal temperatures which, in turn, initiates the expression of heat shock proteins (HSPs) through the regulation of the heat shock factor σ^H (27–31). Proteins involved in heat shock are summarized in **Table 1**. Induction of HSP formation is accomplished through the production of chaperones, proteases, and small heat

TABLE 1 | Proteins involved in heat shock and their function are described.

Summary of proteins involved in heat shock

Protein	Function	Reference
DnaK	DNA replication under heat shock; chaperone protein	(23)
DnaJ	Prevents aggregation of denatured proteins under hyperosmotic and heat shock	(23)
GrpE	Nucleotide exchange factor for DnaK; thermosensor	(23)
ClpP	Protease that degrades regulatory proteins	(23)
HscAB	Chaperone; maturation of iron–sulfur clusters during heat shock	(23)
σ^H and σ^{32}	Regulators heat shock response; controls envelope stress response to heat shock, acid stress	(27, 38–40)
FourU	Thermosensor; temperature-responsive RNA element	(40–46)
TlpA	Unknown but suggested to be a transcriptional regulator	(25, 47)
HtrA	Thermosensor endopeptidase; chaperone in the outer membrane and degrades misfolded proteins	(48–54)
RpoS[a]	General stress response sigma factor; DNA repair under stress	(55, 56)
FkpA	Involved in intracellular survival of macrophages	(57, 58)
SurA[a]	Outer membrane protein development and assembly; folding of proteins involved in transportation channels	(59–64)
H-NS	Virulence factor regulator under thermal changes	(65–70)

[a]*Those involved in both heat shock and virulence.*

shock proteins (s-HSPs). These function in protection, refolding salvaged proteins, removing damaged proteins, and repairing degrading protein aggregation (32–37).

FourU is a family of thermosensors located at the untranslated region (5′-UTR). This temperature-responsive RNA element contains a stretch of four uridine nucleotides within the ribosomal binding site. It pairs with a sequence of AGGA and was initially discovered in *S.* Typhimurium as the small heat shock gene *agsA*, aggregation suppression A (40–46). Afterward, a similar RNA thermometer was also confirmed to be associated with *Yersinia* virulence through the induction of the transcriptional activator *lcrF* (44, 71).

TlpA, TIR-like protein A, is considered one of the first reported proteins with thermosensory gene regulation activity to the high temperature response (HTR) encoded by *Salmonella* enteric virulence plasmid, pSLT (25, 47). It is a robust homolog to a eukaryotic protein family known as tropomyosin, and the structure of TlpA is in a dimer form with an unusually long alpha-helical coiled coil structure (72). It consists of an N-terminal DNA binding domain and exhibits transcriptional autoregulatory repression activity. At temperatures below 30°C, the transcription of *tlpA* is low and the TlpA repression activity is high. The TlpA exists in two forms, as a dimeric α-helical (folded) coiled coil oligomer at low temperature (28°C) and an unfolded (non-functional) monomer at high temperature (37°C) that leads to increased transcription (25, 73, 74). Although the function of this protein is still unidentified, it was demonstrated that this transcriptional regulator was not essential for virulence of *Salmonella* using a mouse infection model (75).

Another thermosensing gene known as *htrA*, high temperature requirement A, is a member of the serine proteases group within the endoproteases family and is regulated by sigma factor E (48–52). It is a highly conserved gene in numerous microorganisms and was first discovered in *Escherichia coli* as *degP*. At low temperature, the protein HtrA (DegP) functions as a chaperone in the outer membrane; however, at high temperatures, it acts as a protease to degrade misfolded proteins with ATP-independent activity and other cofactors (53, 54). An earlier study also linked the activity of this gene to its sensitivity to thermal stress (76). A strain with a mutation in this gene exhibited an inability to grow at high temperature characterized by the inability to degrade unfolded proteins in the periplasmic space. *S.* Typhimurium was less affected by the sigma factor E mutation than *E. coli* (77–79).

CELLULAR RESPONSES AND REGULATION TO HEAT STRESS

Salmonella can proliferate either in a planktonic form, floating freely within a liquid medium, or attach and grow while immobilized to a solid medium. A large number of proteins form the family of s-HSP that consists of proteins with up to 50 amino acids, which are considered energy free and universally found in numerous microorganisms with diverse group and variable molecular weights. These proteins possess chaperone-like functions and commonly maintain protein homeostasis. The s-HSPs are active primarily during stress to stabilize cell proteins at diverse cellular activities (metabolism, translation, transcription, and others), binding unfolded proteins and forming a complex that blocks non-specific irreversible aggregation (80–83). With the detection of heat stress, the adaptive regulation of genes is initiated with the expression of sigma factors. Two sigma factors are generally expressed: a cytoplasmic thermal stress response regulated by heat shock sigma factor, σ^H or σ^{32}, and an extracytoplasmic thermal stress response regulated by the extracytoplasmic function sigma factor, σ^E or σ^{24}, also known as extreme heat stress sigma factor (84–88).

Sigma factors comprise a large group of genes expressing proteins with critical mechanisms associated with the RNA polymerase holoenzyme complex that function as guidance for core RNA polymerases to recognize their promoters and initiate transcription. The sigma factors are primarily divided into two categories, sigma factor 70 family (σ^{70}) that coordinates the transcriptional activities in various stress responses, also known as σ^A in *Bacillus subtilis* and other bacterial species (51, 89–91), and a second identified family of sigma factors encoded by *rpo*^{N}, known as sigma factor 54 ($\sigma^{54/N}$) (92–94), identified in *Campylobacter jejuni, Enterococcus faecalis*, *Listeria monocytogenes,* and *Pseudomonas* spp. (29, 95–97).

Heat shock responses are regulated by the alternative sigma factors $\sigma^{32/H}$ and $\sigma^{24/E}$. These two factors make up the third and fourth subgroups of sigma factors encoded by *rpoH* and *rpoE* genes, respectively (98, 99). *rpoH* regulates the transcription of heat shock genes and is itself regulated during translation. When the temperature is at an optimal microbial growth range, the translation of the *rpoH* gene is blocked. The stem III and I of the *rpoH* mRNA secondary structure is liberated with increasing temperatures (42°C), facilitating the ribosomal binding and enhancing the efficiency of translation (27, 38–40). Sigma factors associated with heat stress response have been demonstrated to regulate over a 100 genes. Of those, sigma factor $\sigma^{32/H}$ controls

more than 30 proteins, most of which are associated chaperones and proteases (30, 31, 100–103). A more recent study by Lim et al. (104) made it clear that $\sigma^{32/H}$ is not just localized at the bacterial cytoplasm but is also found in the inner membrane through a direct interaction with the signal recognition particle and its signal receptor.

Proteases expressed by sigma factor $\sigma^{32/H}$ can control and decrease the expression of the membrane HSPs to a level as needed by the cell to withstand environmental stresses. For instance, FtsH is one of the ATP-dependent proteases, which possesses numerous cellular functions and has been demonstrated to be very critical to *E. coli* viability (105–108). In addition, FtsH functions as a protein qualifying protease and has a role in membrane protein degradation activities primarily those with SsrA-tagged cytoplasmic proteins at their carboxy terminal (109, 110). FtsH degrades MgtC, a membranous protein with five transmembrane domains. MgtC, a virulence factor, has been identified as being required for survival inside macrophages (111). Katz and Ron (108) demonstrated a maintenance role of FtsH for lipopolysaccharide biosynthesis with a shielding permeability function (108, 112–114). Although $\sigma^{32/H}$ and $\sigma^{24/E}$ are alternative sigma factors, $\sigma^{32/H}$ regulates HSPs for the cytoplasmic components and $\sigma^{24/E}$ regulates the extracytoplasmic (cell envelope) proteins in response to high temperatures and other envelope stress factors (50, 52, 88, 115–118). An interesting finding is that one of the four promoters of *rpoH* gene expression is regulated by $\sigma^{24/E}$ for additional coordination of thermal responses requiring both cytoplasmic and extracytoplasmic components (119–124).

HEAT SHOCK AND VIRULENCE

The adaptation of *Salmonella* to heat shock can also lead to a range of other effects, including an increase in virulence potential through gene regulatory mechanisms. Exposing *Salmonella* to thermal stress results in protective responses and can induce changes in gene expression levels of virulence genes. Numerous chaperones and proteases regulated by the alternative heat shock factors, $\sigma^{H/32}$, $\sigma^{E/24}$, and others such as σ^{S} (RpoS) are notably involved in bacterial virulence with several studies linking these proteins to *Salmonella* and *E. coli* virulence factors (51, 125–129). Although both $\sigma^{H/32}$ and $\sigma^{E/24}$ are regulators for heat shock stress, their molecular mechanisms for initiation responses are not similar.

Sirsat et al. (130) examined the effect of heat stress on *S.* Typhimurium gene expression using transcriptional profiling. Microarray analysis was applied to identify the thermal stress response of *S.* Typhimurium at a sublethal temperature of 42°C with 144 upregulated and 167 downregulated genes detected. These genes belonged to various functional categories, but primarily to the general stress response sigma factor S (RpoS) and HSPs, and to sigma factors H and E (RpoH and RpoE). The latter protein has been shown to be critical in the virulence of numerous pathogens (131–133). However, RpoS regulates genes responsible for lethality in mice where preadaptation through RpoS by increasing virulence potential of *Salmonella* cells that survive processing as suggested by Dodd and Aldsworth [(55); Ibanez-Ruiz et al. (56)]. Therefore, sigma factors and HSPs may increase pathogenic potential by overcoming various stressors and increasing pathogenic and colonization potential. Interestingly, research has indicated that RpoS can function as a DNA repair protein that is active under stressful conditions. Thermal stress can induce DNA damage suggesting that there is correlation between thermal stress, the general stress response, and virulence of *Salmonella* (56). However, more research is needed to confirm this. Generally, genes associated with stress and energy metabolism represent the first responses of the cells to tolerate heat stress. These genes may possibly give the pathogen cross-resistance to other stresses and result in more virulent cells. The study conducted by Sirsat et al. (130) was considered the first to report that sublethal heat stress-influenced *Salmonella* interaction with Caco-2 cells through the expression of fimbriae-associated genes. Genes of two *Salmonella* pathogenicity islands (SPI-2 and SPI-5) were upregulated, resulting in improved adhesion (SPI-5 only) and survival in the host while genes of SPI-1 were downregulated.

A loss of *rpoE* gene activity has also been shown to cause a defect in cell viability of *E. coli* and increase cell envelope stress (50, 118, 122, 134). In *Salmonella*, *rpoE* mutants were found to be less responsive to heat shock temperatures, exhibiting an intracellular defect in the survivability within a macrophage and becoming avirulent in a mouse infection model (126, 135–137). In addition, the *rpoE* gene has been shown to be essential in response to starvation stress (138), oxidative stress (92), antimicrobial peptide resistance (139), and osmotic and cold stresses (127). Lewis et al. (128) discovered that both functions of *htrA*, are important with the function of the proteases being most critical inside the host. A more recent study verified that HtrA protein activity is critical for *S.* Enteritidis persistence in egg whites at 42°C (129).

FkpA, an FKBP-type periplasmic peptidyl-prolyl cis/trans isomerase (PPIase), is involved in heat tolerance (116). This protein is comparable to proteins known as macrophage infectivity potentiators found in other pathogenic bacteria and improves the survivability and proliferation inside the macrophages and epithelial cells (140). Horne et al. (141) demonstrated that a mutation in *fkpA* causes the corresponding *Salmonella* strain to become avirulent; however, Humphreys et al. (57) argued that a single mutant deletion of *fkpA* was not enough to reach that conclusion. They observed that only when combining that mutation with one of the other σ^{E} regulated genes, *surA* or *htrA*, would the virulence of *S.* Typhimurium be disrupted (57, 58). In a more recent study reported by Weski and Ehrmann (142), they conducted a genetic analysis of chaperones and proteases of *E. coli* associated with the cell envelope, evaluating single and double mutant deletions under different growth conditions. A *fkpA* mutation was examined at 37 and 42°C using rich medium agar plates with and without 0.5 M NaCl with the corresponding mutants found to not exhibit any detectable defects under any of the conditions. However, when combining this strain with another mutation in *dsbA*, disulfide bond formation A, the strain displayed weak growth on the hyperosmolar media when incubated at 37°C, while no sign of growth was observed on the hyperosmolar media

when incubated at 42°C with a minimal growth of *dsbA* single mutants at the latter condition (142, 143).

SurA, survival protein A, is also a PPIase. It is regulated by σ^E and contributes to thermotolerance fitness. This protein participates in outer membrane protein (OMPs) development and assembly and plays a role in the folding of transportation channels, known as porins (59–64). Sklar et al. (62) observed that the *surA* role is associated with the initial phases of OMP biosynthesis. Previously, Tormo et al. (144) had demonstrated that *surA* was critical to *E. coli* for survival during stationary phase. Tamae et al. (145) screened approximately 4,000 single mutant deletions, among them a Δ*surA* that exhibited chemical sensitivity to the drugs and detergents used in the study. It is not clear whether similar functionalities exist with *surA* in *Salmonella* but it does appear to have the same association with virulence in the host. Sydenham et al. (146) found a mutation of *surA* in *S.* Typhimurium that exhibited extensive attenuation when introduced to mice orally or intravenously. It has also been demonstrated in several studies that *surA* is a critical factor for OMP transport and associated with virulence of uro-pathogenic *E. coli* and *Salmonella* (64, 147). Using a high-throughput Tn-seq technique to screen the entire genome, Khatiwara et al. (148) identified numerous genes in *S.* Typhimurium associated with high temperatures with *surA* identified as a gene associated with growth at 42°C.

Numerous studies have associated virulence factors with thermal changes that mediate DNA topology. These modifications include overall DNA helical conformation "supercoiling," the degree of helical twists and coiling (25, 149–152), or alterations in the specific-sequence curvature of chromosomal or plasmid DNA (153–156). Some studies have demonstrated that DNA topology plays a role in *Salmonella* pathogenicity (157, 158). Positive DNA supercoiling after heat exposure causes DNA to be twisted in a right-handed fashion until it generates a knot, as seen mainly in plasmid DNA, and is controlled by DNA gyrase and topoisomerase I. Changes at the level of DNA supercoiling trigger SPI-1 gene expression levels and initiate the subsequent intestinal invasion. Once inside the host cells, the DNA changes its form and as a result, SPI-2 genes are induced (159, 160). For a more detailed discussion of SPI-1 and SPI-2 regulation, please see Ref. (160).

The second mechanism is through a recognized bending DNA sequence "promoter-curvature." Commonly, this bending DNA region is an AT-rich sequence that has been primarily identified in the 5′-end upstream of the promoter region influencing RNA polymerase binding as a silencing factor. Initially, thermal stress induces some alterations in the DNA topology as bends in the AT-rich sequence regions on the transcriptional level. This can influence the interaction between RNA polymerase and the promoter region, altering gene expression (155, 156).

CROSS PROTECTION

The microorganisms' responses to temperature changes (inflammation, fever) vary from one microorganism to another with cell metabolic changes occurring when sensing external environmental shifts resulting in protection from certain stresses and/or cross protection for other additional stresses (130, 161, 162). This can be a major concern within the host by increasing potential for overall pathogenesis. Prior exposure to prevention strategies utilized within industry before human consumption occurs could increase survivability of *Salmonella* and their ability to establish infection once ingested (163). When Nielsen et al. (164) compared two different growth forms of *S.* Typhimurium, immobilized versus planktonic cells, diverse responses were elicited in response to heat shock at 45°C for 30 min. The results revealed that 538 genes were expressed differently with flagellar and virulence genes upregulated in the immobilized heat stressed cells compared to the non-stressed cultures. Greater invasiveness was observed in immobilized HeLa cells after this sublethal treatment compared to decreased invasiveness in the planktonic cells. Based on this study, it would appear that inadequate cooking and heat treatments during food processing could actually increase survival and thermal resistance of *Salmonella* and other foodborne pathogens through cross protection by increasing virulence capability (164–166). Gruzdev et al. (21) found that desiccated *Salmonella* cells in sterile deionized water showed high tolerance to dry heat at 60°C with no significant population change within 1 h, in comparison to a 3-log reduction in the number of non-desiccated cells under identical conditions. A previous study also found that *Salmonella* cells that had previously adapted to desiccation conditions survived substantially longer in aged chicken litter than non-adapted control cells exposed to the same treatment (13).

However, as environmental conditions change, *Salmonella* must be able to rapidly adapt through alterations in gene expression in order to overcome stress efficiently. For example, this can be accomplished through attachment, which results in a phenotypic change allowing *Salmonella* to become more resistant to thermal stress than cells in planktonic form (167–170). Multiple studies have concluded that modifications of the membrane fatty acid composition of *Salmonella* strains were directly associated with their ability to resist thermal treatment where those cells with less membrane fluidity possessed greater thermal resistance (171–174). Similarly, in *E. coli,* the increase in membrane fluidity also leads to increased synthesis of HSPs, thus suggesting that membrane composition is directly related to thermal resistance (175). Under low temperatures, the physiological state of the cell can switch to a reversible, less fluid like lipid bilayer, whereas under high temperatures, the state of the cell switches to a membrane with higher fluidity. This is regulated by thermosensors (175). A review by O'Bryan et al. (12) noted that foodborne pathogens in contaminated food products possessing a high fat content demonstrated increased pathogenic potential.

A well-known gene encodes for nucleotide-associated protein (H-NS), a histone-like nucleotide-structuring protein, which has been associated with virulence factors as a temperature-dependent phenotype (65–70). This protein is considered a common transcriptional regulator that can be induced by thermal changes in *Salmonella*. At low temperature, H-NS binds to an AT-rich sequence and forms a complex. When temperature rises to 37°C (host body temperature), the binding capacity is reduced until dissociation occurs, leading to virulence gene expression. This mechanism was demonstrated in *E. coli* K-12 to control over

60% of the genes regulated by temperature including virulence factors (176). The association of H-NS with virulence has been verified in other pathogens such as *Salmonella* (69, 177–179), *Shigella*, *Yersinia enterocolitica,* and *Yersinia pseudotuberculosis* (180–182). Two studies were conducted to identify the mechanism of H-NS in *Salmonella*. The first study was performed on *S.* Typhimurium LT2, and it was noted that H-NS negatively regulated approximately 254 genes (69). The second study was carried out on *S.* Typhimurium 14028 (183), and it was discovered that 265 unique *Salmonella* genes were negatively associated with H-NS which contained low G + C content (183). In both studies, among the identified genes were those present in SPI-1, 2, 3, and 5 (184–186).

A more recent study by Pesingl et al. (187) demonstrated that protein-L-isoaspartyl methyltransferase (PIMT) is required by *Salmonella* to survive at 42°C and it in turn contributes to virulence capability in poultry (body temperature of 42°C). Proteins were susceptible to damage induced by thermal stress and thus PIMT assisted in prevention and repair of proteins. Under stress, aspartate is converted to iso-aspartate, which can lead to unfolded proteins and modified amino acids residues (188). Pesingl et al. (187) found that PIMT contributes to survival under both thermal and oxidative stress during stationary phase due to its direct role in protection of proteins at elevated temperatures. Therefore, further research is needed in the correlation between the heat shock responses and virulence gene expression and how their respective regulation patterns influence the pathogenic potential of *Salmonella*.

CONCLUSIONS

Salmonella typically encounters various thermal stresses that can be host-specific and can represent a component of the overall immune and physiological response to infection. However, *Salmonella* spp. have developed thermal resistance mechanisms to overcome these changes in host temperature through the induction of stress response mechanisms. In particular, sigma factors play a leading role in thermal stress response. Preexposure to thermal stress can lead to an increase in pathogenic potential through activation and regulation of genes associated with thermal stress. This thermal stress response can influence the activation of genes associated with virulence and the general stress response allowing for *Salmonella* to overcome host defenses and establish infection. The type of host can also play a role on the ability to establish infection. A host with a higher body temperature than humans could activate thermal stress resistance mechanisms allowing for easier colonization and establishment of infection compared to a host with a body temperature at 37°C in which these thermal stress resistance mechanisms are not expressed. An understanding of the *Salmonella* thermal resistance is essential for elucidating survival and infection mechanisms. It could be useful to identify specific targets for prevention and treatment of *Salmonella* infections. Therefore, it is imperative that the proteins involved in regulation and activation of these genes be thoroughly studied in order develop novel strategies to reduce outbreak cases and infection in all types of hosts.

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Phenotypic and Genotypic Features of a *Salmonella* Heidelberg Strain Isolated in Broilers in Brazil and Their Possible Association to Antibiotics and Short-Chain Organic Acids Resistance and Susceptibility

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Salmonella enterica serovar Heidelberg is a human pathogen also found in broilers. A strain (UFPR1) has been associated with field reports of resistance to short-chain organic acids (SCOA) in broilers in the South of Brazil, but was susceptible to a *Bacillus subtilis*-based probiotic added in feed in a related study. This work aimed to (i) report clinical symptoms caused by SH UFPR1 in broilers, (ii) study its susceptibility to some antibiotics *in vitro*, and (iii) SCOA *in vivo*; and (iv) relate these phenotypic observations with its genome characteristics. Two *in vivo* trials used 1-day-old chicks housed for 21 days in 8 sterilized isolated negative pressure rooms with 4 battery cages of 12 birds each. Birds were challenged or not with 10^7 CFU/bird of SH UFPR1 orally and exposed or not to SCOA in a 2 × 2 factorial design. Zootechnical parameters were unaffected ($P > 0.05$), no clinical signs were observed, and few cecal and hepatic histologic and immune-related alterations were seen, in birds challenged with SH. Formic and propionic acids added together in drinking water, fumaric and benzoic acid in feed (Trial 1), and coated calcium butyrate in feed (Trial 2) did not reduce the SH isolation frequencies seen in cecum and liver in broilers after SH challenge ($P > 0.05$). SH UFPR1 was susceptible to amikacin, amoxicillin + clavulanate, ceftiofur, cephalexin, doxycycline and oxytetracycline; and mildly susceptible to ampicillin + sulbactam, cephalothin, ciprofloxacin, enrofloxacin, and gentamycin in an *in vitro* minimum inhibitory concentration model using Mueller–Hinton agar. The whole genome of SH UFPR1 was sequenced and consisted of a circular chromosome, spanning 4,760,321 bp with 52.18% of GC-content encoding 84 tRNA, 22 rRNA, and 4,427 protein-coding genes. The comparison between SH UFPR1 genome and a multidrug-resistant SL476 strain revealed 11 missing genomic fragments and 5 insertions related to *bgt*, *bgr*, and *rpoS* genes. The deleted genes codify proteins associated with cell cycle regulation, virulence, drug resistance, cellular adhesion, and

salt efflux which collectively reveal key aspects of the evolution and adaptation of SH strains such as organic acids resistance and antibiotic sensitivity and provide information relevant to the control of SH in poultry.

Keywords: antibiotic, comparative genomics, organic acids, resistance, UFPR1 strain

INTRODUCTION

Despite recent advances in the treatment of infectious diseases, pathogenic microorganisms, including *Salmonella*, remain an important threat to human and animal health worldwide (1). Non-typhoid *Salmonella* serovars are well-known pathogens but they also silently infect animals, particularly poultry, as transient members of the intestinal microbial population without causing disease (2, 3). Intestinal colonization of several *Salmonella enterica* serovars often have no effect on poultry zootechnical performance (3). Kogut et al. (4) described that the infection in chickens caused by *Salmonella* Enteritidis leads to immune tolerance beginning around 3–4 days post-primary infection. This decreases the host immune responsiveness resulting in the establishment of *Salmonella* and persistent colonization. This asymptomatic infection could increase the probability of transmission to humans *via* contaminated food (5).

Therefore, reducing *Salmonella* colonization and fecal shedding in live chickens, and its subsequent chicken meat contamination, can reduce the burden of salmonellosis in humans. Although many aspects related to their mechanisms of action are unknown, short-chain organic acids (SCOA) have been added to chicken feed, drinking water, and other matrices, as part of several strategies to prevent *Salmonella* colonization in animal tissues and transmission through the food chain with many positives results (6).

In that context, *Salmonella enterica* serovar Heidelberg is one of the most prevalent serovars and is commonly isolated from patients with salmonellosis in North America. That region has the greatest prevalence comparing to other continents (7). More invasive human infections, such as myocarditis and bacteremia, are produced by SH compared to other non-typhoid *Salmonella* (8). Since 1962, SH has been isolated and reported from poultry and their products worldwide (9) including Brazil (10). Voss-Rech et al. (11) reported 20 different *Salmonella* serovars in samples from broilers and SH was prevalent in 7.31% of them. SH UFPR1 strain was isolated from a chicken carcass in the South of Brazil. This strain showed susceptibility and resistance to antibiotics and organic acids, respectively. In a previous trial, our group showed reduction in SH counts in the cecum and liver of broilers fed with a probiotic composed of three strains of *Bacillus subtillis* previously challenged orally with SH UFPR1 strain. No studies comparing the genome of Brazilian UFPR1 to other SH strains have been reported.

Whole-genome sequencing is a tool that allows to investigate the genomic features of any organism. Several genomes from *Salmonella* strains have been decoded using this technique, aiming at improving the understanding of some aspects of their evolutionary biology, distinguishing outbreak-related strains of sporadic infections (12) and comparing genomes of strains with different clinical history and resistance profile (1, 13, 14). Whole-genome sequencing was recently used to study the differences among SH serotypes (15, 16) and resistance to different antibiotics (17, 18).

The objective of this work was to report possible clinical symptoms caused by SH UFPR1 strain in broilers, test the efficacy of SCOA to reduce SH UFPR1 strain proliferation in broilers, study the susceptibility of this strain to some antibiotics *in vitro*, and relate these phenotypic observations with its genome characteristics through comparative genomics.

MATERIALS AND METHODS

In Vivo Experiments

The SH UFPR1 strain used herein was isolated from commercial broiler carcasses in the South of Brazil according to the Brazilian Ministry of Agriculture's procedures [MAPA (19)] and sent to Fiocruz Institute (protocol number 6830/2012) for further serological identification.

Two experiments were conducted at CERIA (Center of Immune Response in Poultry) at the Federal University of Parana, Curitiba, Brazil, to evaluate the effectiveness of several SCOA to control UFPR1 in broilers, and report possible clinical symptoms in orally challenged birds. In Trial 1, an SCOA blend (30% of formic acid and 18% of propionic acid) was offered in drinking water at 0.05% from 1 to 7 and from 15 to 21 days of age, along with 3 kg/ton of feed of a SCOA blend composed of fumaric and benzoic acids at 92% and fed from 1 to 21 days of age. In Trial 2, the effect of adding a product containing 89% of coated calcium butyrate when added at 2 kg/ton in feed from 1 to 21 days was studied.

All experimental procedures were approved by the Ethical Committee of Agricultural Sector of Federal University of Parana under approval number 037/2016 and 014/2016, respectively. All other methods were equal for both trials as described below.

Chicken Housing and Sample Collection

Eight previously disinfected negative-pressured isolation rooms were used. Rooms were equipped with automatic temperature and lighting controls, and each contained four battery cages (replications) stacked vertically with sterilized litter and nipple drinkers. Before the start of each trial, swabs from all walls and cages within the rooms were collected to verify the absence of *Salmonella* by qualitative analysis. One day-old male chicks (Cobb® 500; $n = 192$) were kept from 1 to 21 days of age and distributed using a completely randomized design of four treatments ($n = 48$ birds per treatment, with four replicates per treatment, 12 birds per replication) detailed as follows: T1: non-challenged plus

control diet, T2: non-challenged plus SCOA treatment according to Trial, T3: challenged with SH plus control diet, T4: challenged with SH plus SCOA according to Trial (**Table 1**).

At day 1, 10 chicks were necropsied, and liver and cecum samples were collected to confirm the negativity in both *in vivo* experiments by qualitative analysis and at day 21, feed intake (FI), body weight gain (BWG), and feed conversion (FC) were calculated.

At days 7 and 21 in Trial 1 and days 14 and 21 in Trial 2, 12 birds from each treatment were euthanized by cervical dislocation, necropsied and liver and cecum were collected for *Salmonella* sp. counting procedure. In Trial 2, liver and cecum of five birds per treatment were collected for histology at day 14. At that age, mRNA expression of IL-10 and IL-12 was also evaluated in liver.

Diets

The nutritional value of experimental diets was formulated to supply nutrients at requirements (20). Diets were corn and soybean meal based and were offered in mash form *ad libitum* at all times. Rations were formulated without coccidiostatics or antibiotics and were designed for a unique feeding phase (Starter) from 1 to 21 days of age for all treatments.

A basal diet with all ingredients except amino acids, vitamin, and mineral premix was autoclaved at 120°C/15 min. After this process, SCOA, amino acids, vitamin, and mineral premix were added according to each treatment. All dietary components were mixed for 10 min in a 50 kg blender. Batches were blended in such an order as to avoid interference among treatments.

Salmonella Heidelberg Challenge and Quantification

At 3 or 7 days of age for Trials 1 or 2, respectively, chicks from T3 and T4 were challenged orally with 10^7 CFU/chick of SH UFPR1.

The quantification of typical colonies of *Salmonella* sp. (quantitative analysis) was performed in liver and cecum samples processed according to the modified methodology by Pickler et al. (21). The organs were weighed, mashed and homogenized in 2% buffered peptone water (1:9). Further dilution was conducted by successively placing 1 mL of the solution in a test tube with 9 mL 0.1% peptone water until a 10^{-3} dilution was achieved. Then, 100 µL aliquots of each dilution were transferred to duplicate plates in Brilliant Green Agar (BGA) medium and uniformly spread with a sterile Drigalsky loop. Plates were incubated at 35°C for 24 h before typical colonies were counted. For all samples, pre-enrichment was performed with 2% buffered peptone water at 35°C for 24 h. Samples that did not show typical *Salmonella* colonies during BGA counting, were enriched with 10 mL Rappaport-Vassiliadis broth and incubated at 42°C for 24 h. Thereafter, a drop of the enriched broth was placed on BGA medium. The samples that were negative after direct BGA plate counting, but positive after enrichment were assumed to have 10^1 CFU/g. Samples that were negative after enrichment were assumed to have 0 CFU/g. To verify the *Salmonella* serotype, isolated *Salmonella* samples were sent to the Sector of Enterobacteria of the Oswaldo Cruz Institute, Brazil for serotyping.

TABLE 1 | Experimental treatment design.

Treatment	Challenge[a]	Short-chain organic acids supplementation[b]
T1: non-challenge (NC)	No	No
T2: NC + short-chain organic acids (SCOA)	No	Yes
T3: SH challenge (SHC)	*Salmonella* Heildelberg UFPR1	No
T4: SHC + SCOA	*Salmonella* Heildelberg UFPR1	Yes

[a]*Challenged orally with 10^7 CFU/chick. Trial 1 day 1, Trial 2 day 7.*

[b]*In Trial 1, 0.05% of an organic acids blend (30% of formic acid and 18% of propionic acid) offered in drinking water from 1 to 7 days and from 15 to 21 days of age, associated with a treatment with 3 kg/ton of a product with minimum 92% of fumaric and benzoic acids in feed, from 1 to 21 days of age. In Trial 2, a product constituted with coated 89% of calcium butyrate at 2 kg/ton in feed from 1 to 21 days was evaluated.*

Cecal and Hepatic Histologic Evaluation

For trial 2 only, samples of cecum and liver were processed according to Kraieski et al. (22). Tissues embedded in paraffin were cut in 5 µm sections and both later stained with hematoxylin and eosin, and with Alcian Blue for cecum also. Liver samples were examined using 5 fields per bird with a 10× objective and 100× of magnification. Congestion, hydropic degeneration, cell vacuolation, bile-duct proliferation, immune cells infiltration, pericholangitis, and lymphocytic aggregate were observed. The "I See Inside" (ISI) methodology applied herein was described by Santin et al. (23) where an impact factor (IF) was assigned to each type of alteration according to its potential to reduce organ functionality. The basis for these criteria was previous literature review on the relationship of organ functionality and type of lesion, and background research. The IF ranges from 1 to 3, where 3 defines an alteration that impacts organ functionality the most, and 1 the least. For instance, necrosis has the highest IF because the functional capacity of affected cells is totally lost. In addition, an observer score (OS) is assigned to each lesion based on its observed intensity or frequency compared to non-affected organs, during histologic inspection. This evaluation is performed in each organ/tissue per animal and OS values range from score 0 (absence of lesion or frequency), score 1 (alteration up to 25% of the area or observed frequency), score 2 (alteration ranges from 25 to 50% of the area or observed frequency), and score 3 (alteration extent more than 50% of the area or observed frequency). In order to calculate the final ISI Index, the IF of each alteration is multiplied by the respective score number, and the results of all alterations are summed [$ISI = \Sigma (IF \times OS)$]. The scale ranges from 0 to 39 for the liver.

For cecum samples, 5 fields per bird in 40× objective and 400× of magnification were evaluated, and villus height, villus thickness, presence of erythrocytes, and infiltration of immune cell on lamina propria were measured.

Cytokines mRNA Expression in Liver (Trial 2)

Six birds per treatment were euthanized, their livers removed and immediately stored at −20°C until further analysis. Total RNA from that tissue was isolated using Trizol reagent (15596-018,

Invitrogen, Carlsbad, CA, USA) following the manufacturer's procedures. Turbo-DNAse kit (AM1907, Applied Biosystems, Foster City, CA, USA) was used for the collected samples. RNA concentrations were quantified by NanoDrop Spectrophotometer (ND1000, Thermo Scientific, Bonn, Germany) and RNA integrity determined by Experion Automated Electrophoresis System (700-7000, Bio-Rad, Hercules, CA, USA). RNA samples were reverse transcribed and RT-qPCR analyses performed with a MyiQ System (170-9740, Bio-Rad). One microgram of RNA was converted to cDNA in a 20 µL reaction volume using the iScript™ Reverse Transcription Supermix kit (170-8841, Bio-rad) at 25°C for 1 h, 42°C for 30 min, and then 85°C for 5 min.

The genes analyzed by RT-qPCR were: IL-10 (5′-cgggagctgagggtgaa-3′ and 5′-gtgaagaagcggtgacagc-3′), IL-12 (5′-agactccaatgggcaaatga-3′ and 5′-ctcttcggcaaatggacagt-3′), and GAPDH (5′-ggtggtgctaagcgtgttat-3′ and 5′-acctctgtcatctctccaca-3′). The final 20 µL PCR contained 2 µL reverse transcription product, 2 µL of the forward and reverse gene, and 10 µL of iTAq® Universal SYBR Green Supermix (172-5122, Bio-Rad). PCR cycle conditions of all primer pairs used an initial 60 s denaturation step at 95°C followed by 40 cycles of denaturation (15 s at 95°C), annealing and extension (30 s at 60°C). The melting profile of each sample was analyzed after every PCR run to confirm PCR product specificity; and later determined by heating samples at 65°C for 30 s and then increasing the temperature at a linear rate of 20°C/s to 95°C while continuously monitoring fluorescence. Sample PCR amplification efficiencies were determined in the log-linear phase with the LinRegPCR program (24). Additionally, the delta–delta equation subtracts sample and reference Ct values from an endogenous control. However, the endogenous control (GAPDH) Ct was affected by treatments in this study ($P < 0.05$) and, therefore, was removed from the equation. All data were normalized to the mRNA level of the control group (group non-challenged and without SCOA) and reported as the fold-change from the reference, which was calculated as $E_S^{(40 - Ct\ Sample)}/E_R^{(40 - Ct\ Reference)}$, where E_S and E_R are the sample and reference PCR amplification efficiencies, respectively (25).

Statistical Analysis of *In Vivo* Studies

Data were analyzed using the statistical software Statistix 9. For microbiological analysis, data were evaluated by the Shapiro–Wilk normality test. Parametric data were subjected to analysis of variance (ANOVA) and Tukey's test ($P < 0.05$), while the Kruskal–Wallis test ($P < 0.05$) was used for non-parametric data (quantitative microbiological data and histology data). The chi-square test was used in microbiological results of presence/absence (qualitative) of *Salmonella* in liver for Trial 2. For zootechnical performance, and immunohistochemistry analysis, data were subjected to ANOVA using a factorial 2 × 2 design.

In Vitro Antimicrobial Susceptibility Tests

The susceptibility of SH UFPR1 against a panel of 12 antimicrobials commonly used in human and veterinary clinics in Brazil was determined by the dilution antimicrobial method using Mueller–Hinton agar after incubation at 37°C for 18–24 h. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) results were interpreted in agreement with the interpretative criteria provided by Clinical and Laboratory Standards Institute (26). The 12 antimicrobials tested included amikacin (250 mg/mL), amoxicillin + clavulanate (14 g + 3.5 g/100 mL), ampicillin + sulbactam (1 g + 0.5 g/10 mL), ceftiofur (50 mg/mL), cephalexin (250 mg/5 mL), cephalothin (1 g/10 mL), ciprofloxacin (2 mg/mL), doxycycline (4.6 g/100 mL), enrofloxacin (10 g/100 mL), gentamycin (40 mg/mL), penicillin (6,000,000 UI/15 mL), and tetracycline (20 g/100 mL). The *Escherichia coli* ATCC 25,922 was used as reference strain. The MIC breakpoints were set based on CLSI (26) and FDA (9).

Genomic Analysis and Comparative Analysis

The isolated SH UFPR1 strain was cultured overnight in liquid LB medium, its genomic DNA extracted using the QIAamp DNA Mini Kit (Qiagen) and quantified using the NanoVue spectrophotomer (GE Healthcare). A total of 70 µg of DNA was sent to the High-Throughput Sequencing Facility at University of North Carolina. The library was prepared using the PacBio's 20 Kb template prep protocol (PN_100-286-000-06) and it was size-selected by using a range setting of 8,000 bp to 50,000 bp. *De Novo* assembly was performed using PacBio native pipeline (27). Comparative genomic analysis was independently performed with MAUVE v.20150225 (28) and Mummer v.3.23 (29) programs, using the annotated genome of SH strain SL476 as reference (GenBank assembly accession: GCA_000020705.1) (30). The shared genomic fragments between UFPR1 and SL476 were identified with Mummer while the regions with no match between them were identified with a Perl script (available in https://github.com/CaioFreire/Scripts). PROKKA v.1.12 software was used for genome annotation (31) and the circular map was drawn using DNAPlotter v.10.2 (32). The fully sequenced SH UFPR1 genome was deposited at the NCBI genome database under the number CP020101. In addition, missing fragments between each other were found using Megablast (https://blast.ncbi.nlm.nih.gov) and verifying if the gene sequences in these missing fragments were present in other parts of the genome.

RESULTS

At the *in vivo* trails, SCOA were offered to the birds during the early days of life to allow the gut to adapt to the treatment before the SH challenge. No effect in zootechnical parameters was observed at any time in both trials ($P > 0.05$) either from the adding SCOA in drinking water and/or feed, from challenging birds with SH UFPR1 strain, or from the interaction between these factors (**Table 2**). It should be mentioned that the primary objective of this work was not measuring performance and that more replicates would be needed to appropriately test possible effects on these parameters.

As expected, all non-challenged groups tested negative for *Salmonella* while all challenged groups tested positive. Therefore, data on *Salmonella* counts were statistically evaluated in a completely randomized design using only challenged groups. Still, the use of SCOA did not influence the percentage of SH positive in cecum or liver (**Figure 1**; Trial 1). Similar findings were observed

in Trial 2 in cecum (**Figure 2**) where SH counts were only performed in that organ. In that experiment, liver microbiological results were qualitative only and showed that, at 14 days of age, 100 and 42% of the samples were SH positive in challenged and challenged + SCOA birds, respectively, while, at 21 days of age, 25 and 58% tested SH positive for those two groups of broilers ($P < 0.05$). When comparing results from both trials, it seems that challenging birds with SH UFPR1 later in life (7 days in Trial 2 vs. 3 days in Trial 1) reduced the recovery of SH in liver.

Liver histology revealed that challenging birds with SH increased the ISI score at 14 days indicating greater histological alterations (**Table 3**). The main alterations found were congestion, vacuolation, and immune cell infiltration as presented in **Figure 3**. The SCOA treatment did not influence liver histology measures.

At 14 days of age, SH-challenged birds showed increased cecal villi height, villi thickness and villi surface area compared to the non-challenged group ($P < 0.05$; **Table 3**; **Figure 4**). Supplementing SCOA treatment did not affect the histological parameters in cecum having no interaction between SH challenge and SCOA supplementation ($P > 0.05$).

The results of mRNA expression of cytokines on liver at 14 days (**Figure 5**) showed higher IL-10 ($P > 0.05$) cytokines in SH-challenged birds compared to non-challenged, while IL-12 mRNA expression remained unaffected in all treatments.

The *in vitro* work suggests that SH UFPR1 strain is susceptible to amikacin, amoxicillin + clavulanate, ceftiofur, cephalexin, doxycycline and oxytetracycline and presents intermediary resistance to ampicillin + sulbactam, cephalothin, ciprofloxacin, enrofloxacin, and gentamycin (**Table 4**).

The whole genome of UFPR1 was sequenced to better understand the genotypic particularities of this strain and compared to the genomic sequence of a multidrug-resistant SH SL476 strain. As shown in **Figure 6**, the assembled genomic sequence from UFPR1 strain was 128 kb smaller than SH SL476 sequence, with important deletions of 11 chromosomal fragments in the Brazilian strain. Three of them were greater than 30, 40, and

TABLE 2 | Mean ± SD of feed intake (FI), body weight gain (BWG), and feed conversion (FC) during the periods of 1–21 days of age of broilers on Trials 1 and 2 in non-challenged and challenged birds.

	Period	Non-challenged	Challenged	*P*-value*
FI	1–21 days (Trial 1)	1,078.2 ± 19.03	1,088.3 ± 14.74	0.680
	1–21 days (Trial 2)	1,214.1 ± 33.20	1,197.4 ± 17.51	0.727
BWG	1–21 days (Trial 1)	884.98 ± 19.89	914.46 ± 25.32	0.367
	1–21 days (Trial 2)	836.58 ± 23.01	804.50 ± 24.08	0.341
FC	1–21 days (Trial 1)	1.221 ± 0.01	1.198 ± 0.02	0.468
	1–21 days (Trial 2)	1.452 ± 0.04	1.496 ± 0.03	0.499

**Tukey test.*

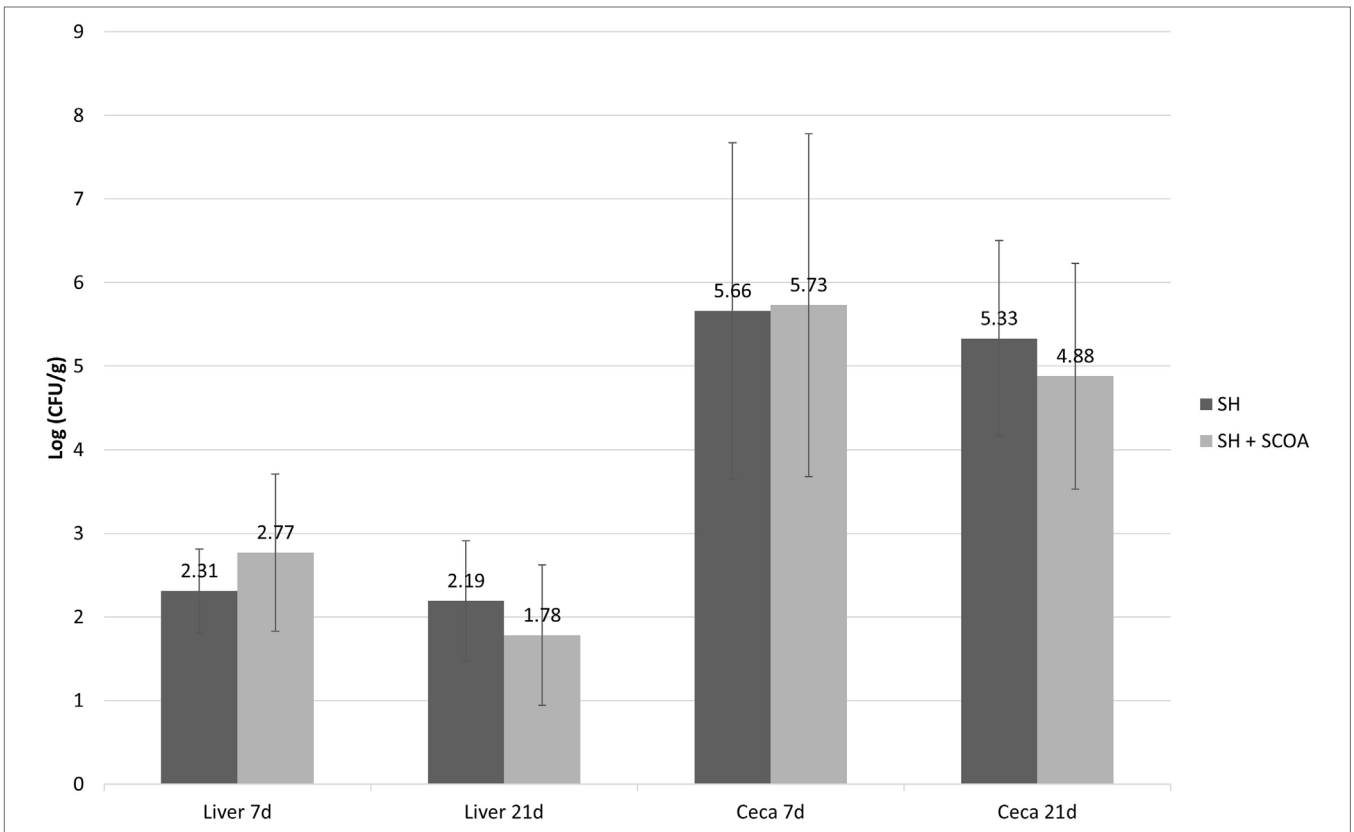

FIGURE 1 | Trial 1. *Salmonella* sp. quantification (Log CFU/g) in liver and cecum at 7 and 21 days of age in treatments challenged with *Salmonella* Heildelberg (SH) or *Salmonella* Heidelberg challenged + Short-Chain Organic Acids (SH + SCOA).

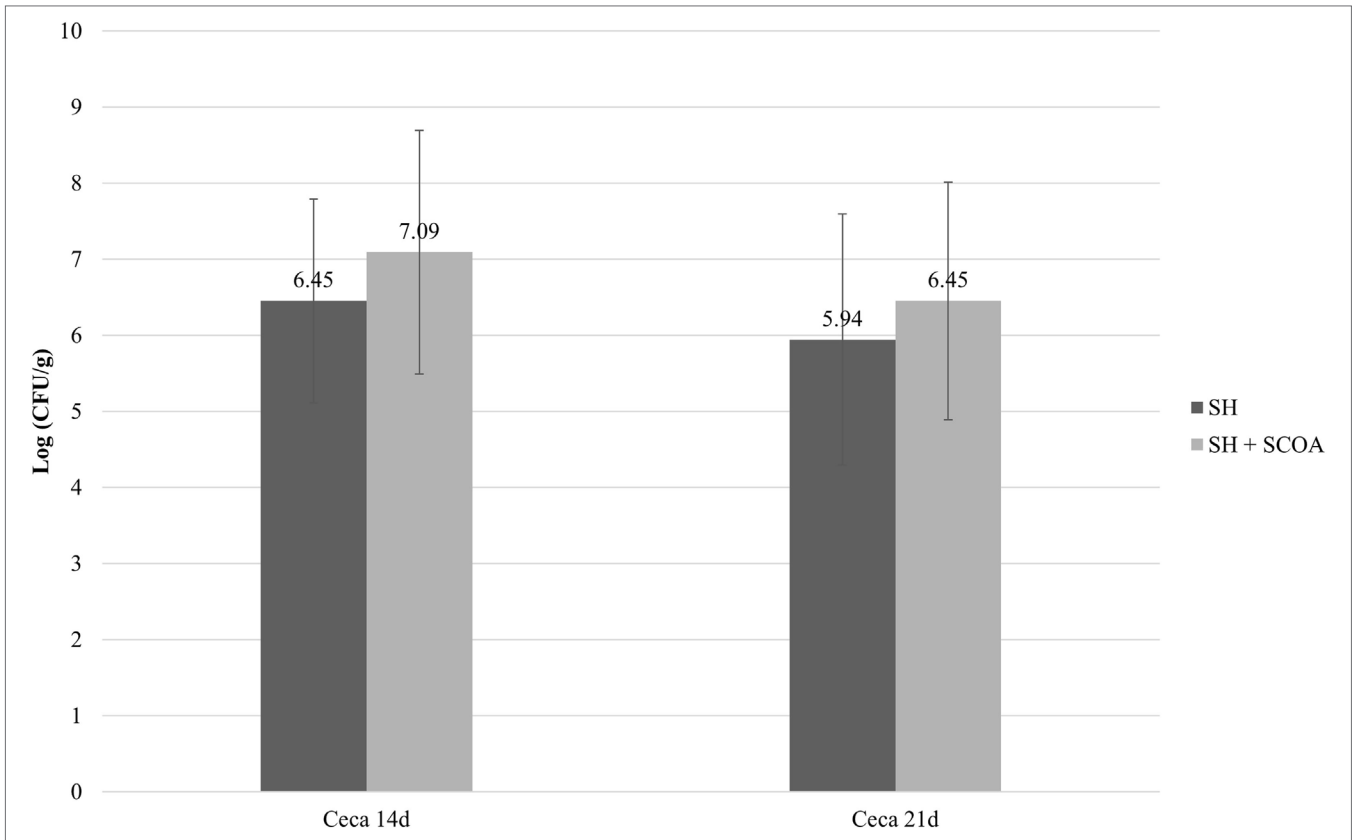

FIGURE 2 | Trial 2. *Salmonella* sp. quantification (Log CFU/g) in cecum at 14 and 21 days of age in treatments challenged with *Salmonella* Heidelberg (SH) or *Salmonella* Heidelberg challenged + Short-Chain Organic Acids (SH + SCOA).

TABLE 3 | Mean ± SE of histological alterations by I See Inside (ISI) methodology in liver and cecum at 14 days of age, evaluating the challenge and the use of feed additives interaction.

Treatment	ISI liver	Villus height (μm)	Villus thickness (μm)	Area (μm)
Non-challenge	9.93 ± 0.7[b]	164 ± 8.0[b]	91 ± 7.0[b]	14,924 ± 1,591[a]
S. Heildelberg Challenge (SHC)	20.31 ± 0.5[a]	176 ± 8.0[a]	108 ± 7.00[a]	19,008 ± 1,591[b]
NC + Short-chain organic acids (SCOA)	12.31 ± 0.7[b]	158 ± 8.0[b]	95 ± 7.00[b]	15,010 ± 1,591[a]
SHC + SCOA	19.10 ± 0.5[a]	175 ± 8.0[a]	106 ± 7.00[a]	18,550 ± 1,591[b]

[a,b]*Different letters in the same row indicate significant difference at P < 0.05.*

50 kb, encompassing several important genes (Datasheet S1 in Supplementary Material). Genomic regions without similar sequences in the compared genome can be observed by red dashes in **Figures 6A,B**. Nevertheless, the comparison between the genomes of these strains revealed high similarity with few translocation events and conserved synteny (**Figure 7**). Moreover, no plasmid-sequences were found in the assembled sequences from reads of the UFPR1 strain (BioProject NCBI number PRJNA378710), using Canu software v1.3 (33) to correct all input data.

The genomic analysis of deleted regions revealed that 171 genes were present in SH SL476 strain but absent in UFPR1 strain (Datasheet S1 in Supplementary Material). The analyses of the 171 genes of SL476 showed that: (i) 46% were related to the codification of hypothetical proteins; (ii) 28% were mRNA sequences use to produce proteins with known functions involved in cell cycle regulation, DNA replication, virulence, drug resistance, and salt efflux; (iii) 16% correspond to encoded proteins related to the DNA recombination process (transposases and invertases genes), and (iv) 10% are encoded viral proteins (conjugal transfer, integrase, capsid, and tail proteins). Some of the genes in the absent regions were duplicated in SL476 genome or were located in another genomic region in UFPR1. Some genes were completely absent in UFPR1 strain (Datasheet S1 in Supplementary Material) such as (i) *aph3* and *aph6* genes that codify two isoforms of aminoglycoside O-phosphotransferase proteins; (ii) *tem-1* gene that codifies a protein associated with an antibiotic resistance mechanism in bacteria; (iii) *qacEΔ1* gene involved in resistance to a large spectrum of quaternary ammonium compounds (QAC); (iv) *sul1* gene involved in the sulfonamide resistance; (v) *tetB* gene linked to the efflux of tetracycline, and (vi) *lysR* gene that codifies the transcriptional activator of *lysA* gene, which encodes the diaminopimelate decarboxylase involved in a lysine synthesis pathway. *LysR* gene belongs to the LYSR-type

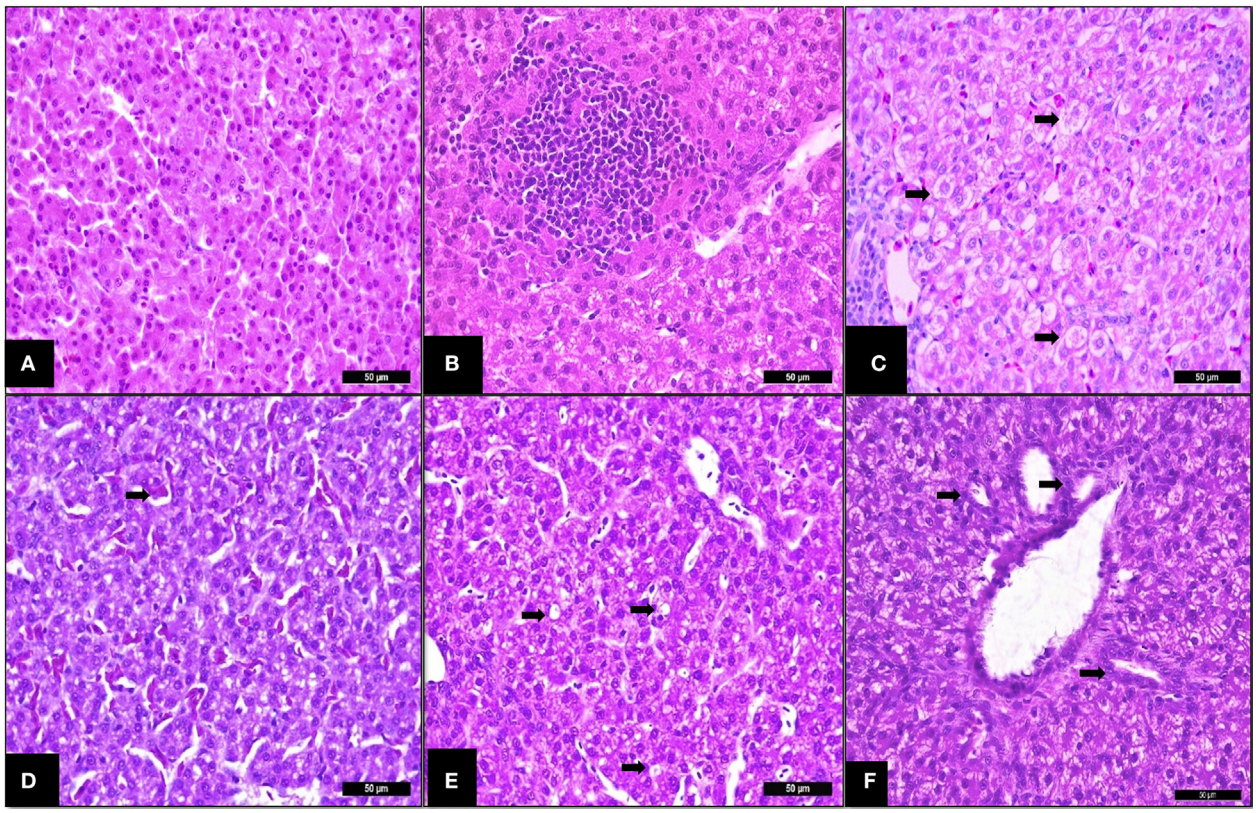

FIGURE 3 | Trial 2. Liver of broilers (14 days). **(A)** Non-challenged group—normal tissue (parenchyma), I See Inside (ISI) score 23. **(B)** SH-Challenged group—ISI score 25, cell infiltrate in parenchyma grade II. **(C)** SH-Challenged group—hydropic degeneration grade III. **(D)** SH-Challenged group—congestion grade II. **(E)** SH-Challenged group—Vacuolization grade II. **(F)** SH-Challenged group—bile-duct proliferation grade II. Hematoxylin and eosin, 400×.

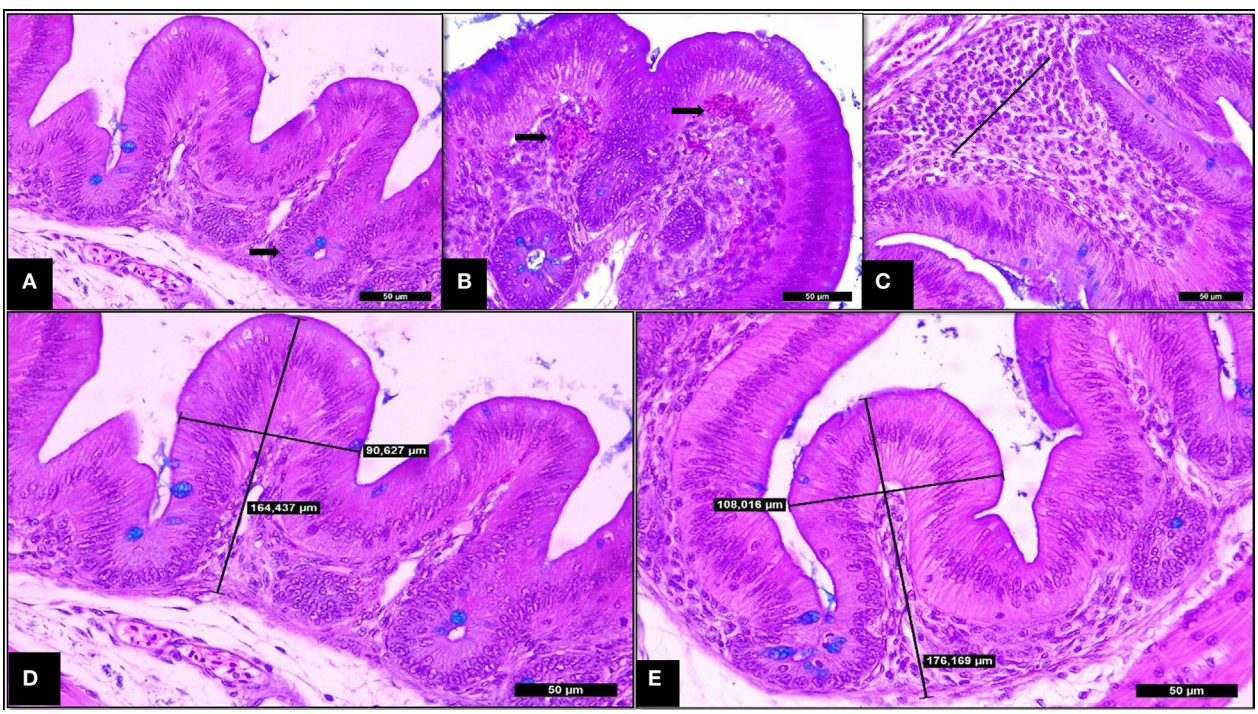

FIGURE 4 | Trial 2. Cecum of broilers (14 days). **(A)** Non-challenged group—normal villi and crypts of Lieberkhün. **(B)** SH-Challenged group—congestion grade III. **(C)** SH-challenged group—cell infiltrate in lamina propria grade II. **(D)** Non-challenged group—villus height and thickness axes of measurement. **(E)** SH-challenged group—villus height and thickness axes of measurement. Hematoxylin and eosin plus Alcian Blue, 400×.

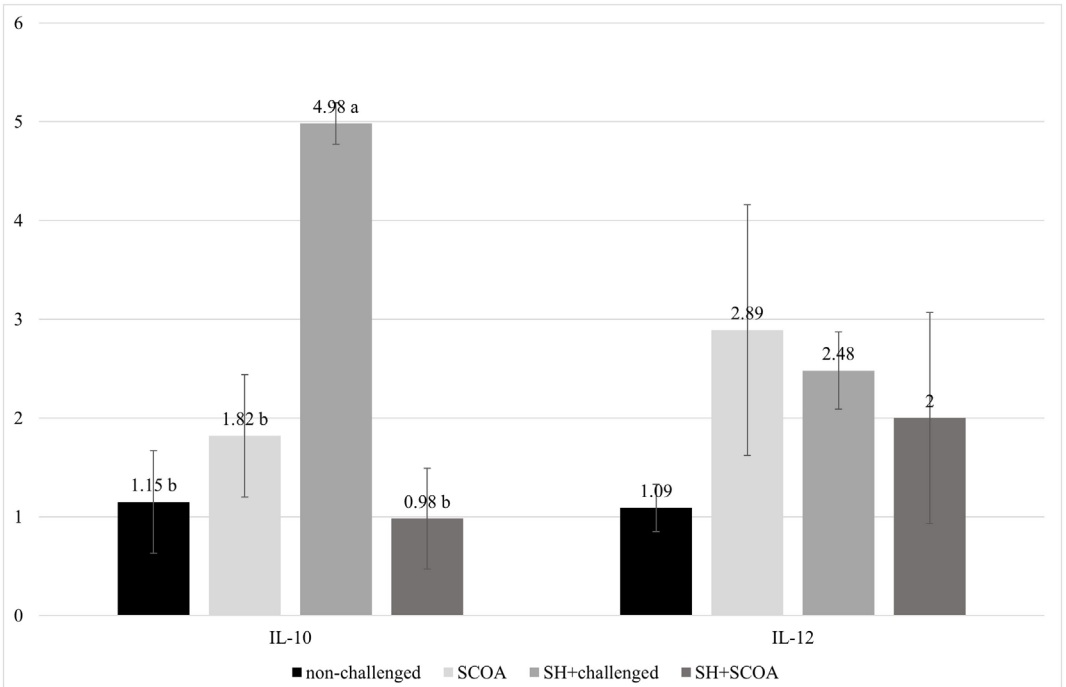

FIGURE 5 | Trial 2. mRNA expression of IL-10 and IL-12 at 14 days of age in non-challenged group, SH-challenged group, short-chain organic acids (SCOA) and SCOA + SH-challenged group. [a,b]Different letters indicate significant differences at $P < 0.05$ at Kruskal–Wallis test.

TABLE 4 | Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Brazilian *Salmonella enterica* serovar Heidelberg (UFPR1 strain).

Antibiotic	MIC (μg/mL)	MBC (μg/mL)	Breakpoint
Amikacin	1.90	61	Susceptible
Amoxicillin + clavulanate	≤0.06 + 0,15	875,000 + 218,750	Susceptible
Ampicillin + Sulbactam	24.41 + 3.05	6,250	Intermediate
Ceftiofur	1.52	25,000	Susceptible
Cephalexin	0.7625	–	Susceptible
Cephalothin	24.41	50,000	Intermediate
Ciprofloxacin	0.24	–	Intermediate
Doxycycline	1.40	718.75	Susceptible
Enrofloxacin	0.76	3,125	Intermediate
Gentamycin	1.22	9	Intermediate
Oxytetracycline	1.64	13,500	Susceptible

Inoculum SH UFPR1 strain: 2 × 10⁸ CFU/mL.

family transcriptional regulator, which regulates a varied set of genes involved in virulence, metabolism, quorum sensing, and motility (34). In the alignment of the genomes, the presence of five insertions was observed in the UFPR1 strain coding some genes like *bgt*, *bgr*, and *rpoS*. These genes are also present in the SL476 strain in other genomic regions and are correlated with important phenotypes found in UFPR1, such as virulence and organic acids resistance. Only five chromosomal fragments were found in UFPR1 compared to SL476 (Datasheet S2 in Supplementary Material); however, these fragments have been identified in several other strains demonstrating that they are not exclusive to UFPR1.

DISCUSSION

The results from the *in vivo* studies showed that SH UFPR1 strain do not seem to affect the zootechnical performance of broilers although these results should be taken with care given the fact that our experimental layout in terms of number of replicates was not designed to be highly sensible to changes in performance, but rather to be appropriate for all the other measurements described in this report.

No clinical signs were observed after SH UFPR1 oral challenge. Other studies have shown that non-typhoid *Salmonella* infection in chickens does not result in morbidity or severe clinical signs in spite of intestinal colonization, and liver and spleen bacterial infiltration (2, 3, 35). The SH UFPR1 strain infection produced mild histologic alterations in liver and cecum compared to non-challenged birds, mainly associated with inflammatory processes. No differences on IL-12 mRNA expression in liver on SH-challenged vs. non-challenged birds were observed as opposed to IL-10 mRNA liver expression which increased in the former group.

Shanmugasundaram et al. (36) showed that CD4+CD25+ (Treg) cells increase in number in the cecum of chickens infected with *Salmonella* Enteritidis. These cells collected from cecal tonsils of *S.* Enteritidis-infected birds and re-stimulated *in vitro* with *Salmonella* antigen had higher IL-10 mRNA content compared to those in the control group ($P < 0.05$). The CD4+CD25+ cells were associated with suppressing the immune response and maintaining the *Salmonella* infection in the host (4). The Treg cell was not marked in this study, but the absence of clinical signs,

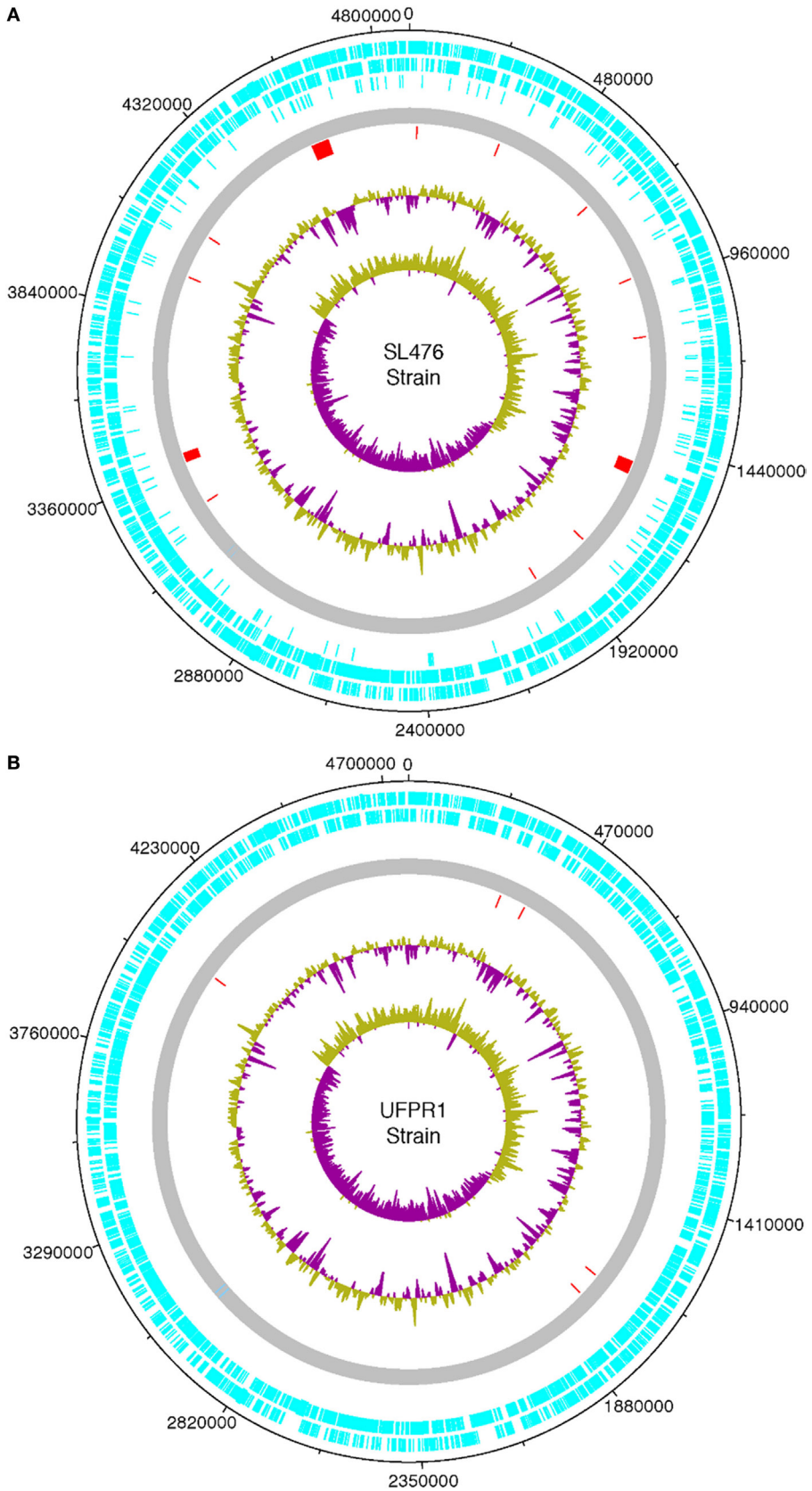

FIGURE 6 | Continued

FIGURE 6 | Continued
Chromosome features of a Brazilian UFPR1 strain **(B)** compared to SL476 strain **(A)** isolate. The circular map was drawn using DNA Plotter. Different features are shown in different colored bars. The coding sequences are shown in light blue (forward and reverse). The complete genome is shown in gray, the red dashes represents unique chromosome regions that have no homologous sequence in the genome of the other strain, green and purple in the major circle represent the GC content, while in the central circle show the GC skew [(G – C)/G + C]. Regions with GC content below the average are shown in purple and those with content above the average are shown in green.

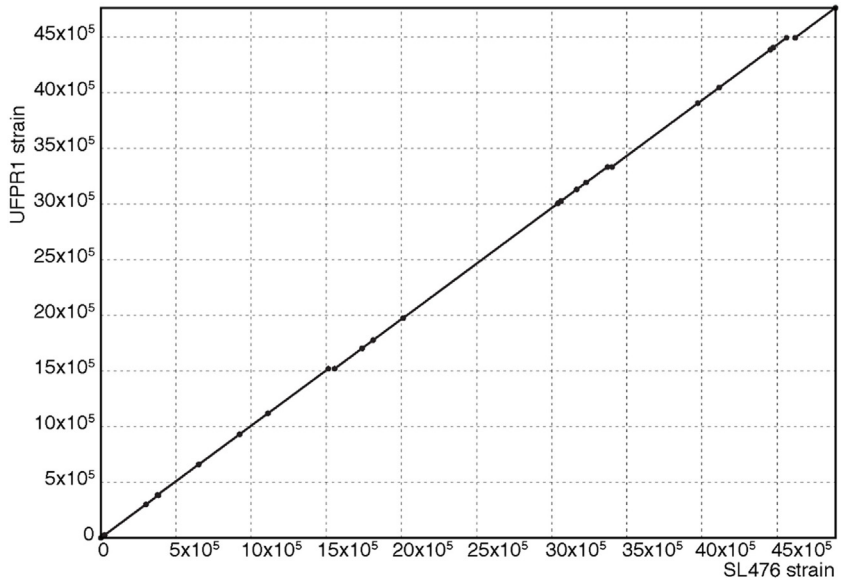

FIGURE 7 | Alignment between genomic sequences from SL476 and UFPR1 strains. This dot-plot was generated with Mummer Software. The exact matches between genomic sequences are represented on the diagonal, showing the high conservation between the genomes with few missing fragments, as shown in **Figure 6**.

the apparent lack of effect on zootechnical parameters, along with the increase of IL-10 mRNA found in the present work suggest that this mechanism could be similar in the UFPR1 strain.

Associating phenotypic observations with genomic sequence information of an organism can contribute to understand some of its key biologic aspects, such as mechanisms for genetic information storage, genome organization, the effect of deletion, insertion, inversion, and translocation on the genome function. To that aim, the maximum number of possible genome sequences is necessary.

Whole-chromosome alignments made in the present genomic study showed that, besides phenotypic differences, the UFPR1 strain has a genome very similar to that of the multidrug resistance SH SL476 strain overall (**Figure 6**). However, several chromosomal fragments that harbor various important genes were lost in UFPR1 (Datasheet S1 in Supplementary Material). The *aph3* and *aph6* genes that were deleted in UFPR1, encode two isoforms of aminoglycoside O-phosphotransferase that participate in the primary mechanism of resistance to aminoglycosides, such as kanamycin, gentamycin, streptomycin, and neomycin. Both genes are frequently found together with transposable elements (37). The *tem-1* gene, which codifies the β-lactamase protein in bacteria, is associated with β-lactam antibiotic resistance and was also deleted in UFPR1. The protein produced by the translation of this mRNA fragment is able to hydrolyze penicillin and first-generation cephalosporin (38). Fragments of chromosome in which genes related to the production of proteins involved in DNA replication, such as DNA polymerase, DNA helicase, DNA resolvase, and DNA topoisomerase, were also found to be deleted in UFPR1 with copies of these genes present in other genomic DNA regions, indicating that their deletion did not affect UFPR1's replication. In agreement with this observation, UFPR1 presented normal growth when cultured *in vivo* in the present report.

Likewise, deletion of *qacEΔ1, sul1*, and *tetB* genes were found in SH UFPR1 strain. The former gene has been associated to resistance against a large spectrum of cationic compounds such as intercalating dyes, diamidines, and biguanides (39), *sul1* to tolerance to sulfonamide (40) and *tetB* to tetracycline efflux (41). Although other genes linked to tetracycline tolerance were found in UFPR1 strain, such as *tetA* class B and *tetA* class C genes, the deletion of *tetB* gene could explain the intermediate tetracycline resistance observed in this report. The deletion of *LysR* gene in UFPR1 may be linked to its low pathogenicity since microorganisms lacking it have been found to be less virulent (34, 42).

Gene deletion is used as an evolutionary process in bacteria in which small genomes have evolved from large genomes, with natural selection acting as a significant driver of gene loss and reductive genome evolution (43). However, bacteria genome could be increased by the acquisition of genetic fragments transferred horizontally (44).

Interestingly, no sequence from plasmids among those assembled was observed. Genome stability is threatened by transposons, which are able to create repetitive sequence islands that can initiate ectopic recombination (45). The present results showed that UFPR1 has several deletions of genes of various transposases suggesting that DNA transposition could be decreased in this strain. The UFPR1 genome presented five different fragments that are absent in SL476 but present in other *Salmonella* genomes evidencing the fact that they are not exclusively of UFPR1. In those five fragments, important genes related to some of the phenotypic characteristics reported herein in UFPR1 were found, such as the *Bgt* and *Bgr* genes that are related to serotype transformation; and the *rpoS* gene, linked to sensitivity to lower temperatures. The proteins produced by the expression of *Bgt* and *Bgr* genes are related to the glucosylation of the O-antigen repeated units of lipopolysaccharide (LPS) and are correlated to serotype conversion in *Shigella flexneri* and also in *Salmonella* (46). The presence of this gene is also correlated to the increase of virulence and resistance to oxidative stress (47).

Activation of *rpoS* gene is involved in cold sensitivity of *Salmonella enterica* serovar Typhimurium (48). The *rpoS* gene codifies for an alternative sigma factor that regulates many cellular responses to environmental conditions, such as heat, alkaline, and acid stress. Mutations of this gene have been detected in pathogenic bacteria (49). Bacteria are subjected to acid stress situations such as to the extreme low pH of the stomach and to the organic acids that are produced in large quantities in the gut including acetic, propionic, and butyric acids. In both situations, bacteria activates mechanisms for acid tolerance response (ATR), for which *rpoS* is a key regulator, thus minimizing the lethal effects of the acid stress. However, *Salmonella rpoS* mutant fails to provide the same level of protection when compared to a wild-type strain. Therefore, *rpoS* mutant is ineffective to sustain the ATR resulting in rapid cell death when exposed to pH 3.0 (50, 51). The product of the *rpoS* gene regulates the virulence gene expression in *Salmonella* Typhimurium in response to conditions encountered in the host tissue. Mutations in the *rpoS* gene yield that bacteria unable to develop a complete ATR significantly reducing its virulence potential (51). The presence of *rpoS* gene in the UFPR1 strain could be involved to the resistance to SCOA found. It has been observed that the alternative sigma factor clearly plays an important role in protecting *Salmonella enterica* serovar Typhimurium against weak acids (52).

In a recent study, Dhanani et al. (15) demonstrated in an *in vivo* experiment that the resistant genes found in SL476 may explain its pathogenicity, colonization ability and persistence in chickens. The absence of several genes involved in tolerance to antibiotics and in the efflux of salt reported in this paper, could explain why UFPR1 was in general susceptible to antibiotics, but resistant to SCOA likely due to the presence of *rpoS* gene.

The use of SCOA can be effective for the control of *Salmonella* in broilers being an important tool for the poultry industry (6, 21). However, testing for the presence of the ATR gene in *Salmonella* strains could help avoiding the misuse of these substances.

In this report, we used comparative genomics to study some of the genotypic peculiarities of UFPR1 linking those findings to phenotypic observations such as its tolerance to SCOA and sensitivity to various antibiotics. The comparison of several genome sequences could reveal important aspects of the evolution of the different *Salmonella* strains, and in a more accurate analysis, help identifying single nucleotide polymorphisms involved in potentially unknown pathways that could be relevant to the study of the metabolism of *Salmonella* and its control. Our findings can also help developing effective strategies to control this agent in broilers, thus preventing food-borne disease in humans.

Salmonellosis causes great economic damage to the poultry industry worldwide from strains that are either pathogenic and non-pathogenic to humans. Developing methodologies that reliably and promptly differentiate pathogenic from non-pathogenic strains could ameliorate that economic loss.

CONCLUSION

The infection of *Salmonella enterica* serovar Heildelberg UFPR1 in broilers did not affect zootechnical performance and promoted a mild inflammatory reaction on cecum and liver.

The use of different SCOA in drinking water or feed was ineffective against *Salmonella enterica* serovar Heildelberg UFPR1 strain in the present layout.

The genomic findings showed several differences between SH UFPR1 strain and the pathogenic SL476 strain.

The absence of several genes involved in antibiotics resistance and in salt efflux; along with the presence of *rpoS* gene, could explain the overall high susceptibility of UFPR1 to the antibiotics tested, and its resistance to SCOA. This information can help the poultry industry on designing SH control programs targeted against this specific strain.

Understanding the phenotypic and genotypic differences among *Salmonella* strains could help improving our knowledge on their metabolism, which could ultimately lead to their effective control.

ETHICS STATEMENT

The experiments were approved by the Ethical Committee of Agricultural Sector of Federal University of Parana under approval number: 037/2016 and 014/2016.

AUTHOR CONTRIBUTIONS

ES—isolation of *Salmonella* Heildelber strain UFPR1, design *in vivo* and *in vitro* trial, analysis and interpretation of data, drafting and revising it critically, and final approval of the version.

RH—design *in vivo* and *in vitro* trial, analysis of data, drafting the work and revising it critically, and final approval of the version. JW—carry on the *in vivo* and *in vitro* trial, analysis of data, drafting the work and revising it critically, and final approval of the version. RG-E—design *in vivo* and *in vitro* trial, analysis, interpretation of data for the work, drafting the work, and final approval of the version. MC, CF, PM, AC—analysis, interpretation of genomic data and revising it critically, and final approval of the final version.

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The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fvets.2017.00184/full#supplementary-material.

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Impact of Built-up-Litter and Commercial Antimicrobials on *Salmonella* and *Campylobacter* Contamination of Broiler Carcasses Processed at a Pilot Mobile Poultry-Processing Unit

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The small-scale mobile poultry-processing unit (MPPU) produced raw poultry products are of particular food safety concern due to exemption of USDA poultry products inspection act. Limited studies reported the microbial quality and safety of MPPU-processed poultry carcasses. This study evaluated the *Salmonella* and *Campylobacter* prevalence in broiler ceca and on MPPU-processed carcasses and efficacy of commercial antimicrobials against *Campylobacter jejuni* on broilers. In study I, straight-run Hubbard × Cobb broilers (147) were reared for 38 days on clean-shavings (CS, 75) or built-up-litter (BUL, 72) and processed at an MPPU. Aerobic plate counts (APCs), coliforms, *Escherichia coli*, and yeast/molds (Y/M) of carcasses were analyzed on petrifilms. Ceca and carcass samples underwent microbial analyses for *Salmonella* and *Campylobacter* spp. using the modified USDA method and confirmed by API-20e test (*Salmonella*), latex agglutination immunoassay (*Campylobacter*), and Gram staining (*Campylobacter*). Quantitative polymerase chain reaction (CadF gene) identified the prevalence of *C. jejuni* and *Campylobacter coli* in ceca and on carcasses. In study II, fresh chilled broiler carcasses were spot inoculated with *C. jejuni* (4.5 $\log_{10}$ CFU/mL) and then undipped, or dipped into peroxyacetic acid (PAA) (1,000 ppm), lactic acid (5%), lactic and citric acid blend (2.5%), sodium hypochlorite (69 ppm), or a H_2O_2–PAA mix (SaniDate® 5.0, 0.25%) for 30 s. Surviving *C. jejuni* was recovered onto Brucella agar. APCs, coliforms, and *E. coli* populations were similar ($P > 0.05$) on CS and BUL carcasses. Carcasses of broilers raised on BUL contained a greater ($P < 0.05$) Y/M population (2.2 $\log_{10}$ CFU/mL) than those reared on CS (1.8 $\log_{10}$ CFU/mL). *Salmonella* was not detected in any ceca samples, whereas 2.8% of the carcasses from BUL were present with *Salmonella*. Prevalence of *Campylobacter* spp., *C. jejuni* was lower ($P < 0.05$), and *C. coli* was similar ($P > 0.05$) in CS-treated ceca than BUL samples. Prevalence of *Campylobacter* spp., *C. jejuni*, and *C. coli* was not different ($P > 0.05$) on CS- and BUL-treated carcasses. All antimicrobials reduced *C. jejuni* by 1.2–2.0 log CFU/mL on carcasses compared with controls. Hence, raising broilers on CS and applying post-chilling antimicrobial treatment can reduce *Salmonella* and *Campylobacter* on MPPU-processed broiler carcasses.

Keywords: broiler carcasses, mobile poultry-processing unit, litter, antimicrobials, *Salmonella*, *Campylobacter*

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INTRODUCTION

Since July 2011, new performance standards have been established by the United States Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS) in response to national baseline studies that required routine testing for *Salmonella* and *Campylobacter* in all processing plants. These new performance standards state that the percentage of *Salmonella*-positive samples must be <7.5% and *Campylobacter*-positive samples should be <10.4% (1). With the implementation of more rigorous standards for pathogen reduction by the USDA-FSIS, it is necessary for poultry processors to employ new or additional pre- and post-harvest interventions for effective control of *Salmonella* and *Campylobacter* throughout chicken processing.

Demand for locally grown products has increased due to consumer interest in sustainable agriculture and an expectation of improved flavor and nutrition. Interest in pastured poultry production and on-farm mobile slaughter of poultry has increased dramatically in the last 20 years. In the previous 5–10 years, some Mid-Atlanta states (Kentucky, Pennsylvania, Ohio, and Massachusetts) have offered mobile poultry-processing units (MPPUs) to small-scale farmers to facilitate production and processing of ≤1,000 broilers per year for local and intrastate, direct sale to consumers under the inspection exemption by the USDA-FSIS *Poultry Products Inspection Act*. According to the West Virginia Department of Agriculture (WVDA), no small-scale poultry-processing facilities (including MPPUs) exist at West Virginia (WV). The lack of small-scale poultry-slaughtering facilities limits small-scale poultry producers in WV to local/intrastate selling of ≤1,000 birds per year. Small-scale farmers who wish to slaughter and sell poultry products locally must have them slaughtered and processed in an out-of-state USDA-FSIS-inspected facility (2). To continue to grow small-scale local poultry industries at WV, the WVDA is planning to assist small-scale poultry processors to install MPPUs at state-wide areas (Personal communication with Mr. Jerry Ours, Poultry Program Coordinator of WVDA). Therefore, it is important to conduct research projects from pre-harvest to post-harvest process to identify food safety risks associated with locally produced broilers, to provide supporting documentation for implementation of an MPPU, to secure local production and distribution of safe poultry meat in WV, and eventually to decrease/eliminate health disparities through optimized local food systems in WV and the mid-Atlantic region.

From the pre-harvest prospective, locally small-scale poultry growers often reuse litter to rear consecutive broiler flocks. Litter is often reused for 1–2 years before full cleanout and replacing with new litter (3). Therefore, food safety concerns are raising about the reusing of litter especially for the challenge of control *Campylobacter* during poultry raising. There is limited research on the comparison of broilers reared on clean-shavings (SC) vs. built-up-litter (BUL) regarding the microbial quality and safety of broilers, including the colonization and contamination of *Salmonella* and *Campylobacter* spp. on broiler carcasses. The literature shows that the welfare, health, performance, and carcass quality of poultry are affected directly by litter quality (4). The samples utilized for the present study were collected from a small-scale broiler trial that compared the performance of industry-standard broilers reared on CS or BUL.

From the post-harvest processing prospect, the slaughter and carcass processing in MPPUs are carried out on a more manual basis instead of using industry-scale, large, automated commercial processing lines. Their products differ based on the variety of available equipment, producer resources, and facilities. This diversity, along with the absence of regulatory guidance, has failed to yield the data needed to validate the safety of raw chicken/broiler carcasses and chicken parts produced by MPPUs. The limited application of antimicrobial intervention plus a final ice water-chilling process without application of post-chilling decontamination treatments makes locally grown MPPU-processed poultry products more vulnerable to infection by *Salmonella* and *Campylobacter*. Lactic acid (LA), peroxyacetic acid (PAA), sodium hypochlorite (SH), and a blend of lactic and citric acid (LCA) have been approved by USDA-FSIS to control food-borne pathogens during industry-scale poultry processing (5). The data available currently on industry-scale poultry processing have reported the efficacy of various commercial antimicrobials to control *Salmonella* and *Campylobacter* in the processing of poultry meat (6, 7). However, few studies have validated the efficacy of commercial antimicrobial interventions on MPPU-produced broiler meat.

Therefore, the present study had two main objectives. First, we wished to ascertain the populations of aerobic plate counts (APCs), total coliforms (TCCs), generic *Escherichia coli*, yeast, and molds on raw broiler carcasses and evaluate the prevalence of *Salmonella* and *Campylobacter* spp. in the ceca and on the carcasses of broilers processed at a university pilot-scale MPPU. Second, we wished to evaluate the efficacy of commercial antimicrobial agents against *Campylobacter jejuni* on MPPU-processed broiler carcasses.

MATERIALS AND METHODS

Raising Broilers

Broilers sampled for the present study were obtained from a study conducted at the West Virginia University (WVU) Poultry Farm, as reported previously by Glover et al. (8). Broilers were cared for according to guidelines set by the Animal Care and Use Committee of WVU. Briefly, 736 1-day-old straight-run Hubbard × Cobb chickens were obtained from a local hatchery and raised for 38 days with 174 chickens using in this study. Broilers had access to food and water *ad libitum*. Broilers were fed with a high-by product protein diet containing a 30% inclusion of wheat (high in non-starch polysaccharides). The diet formulated did not contain any antibiotics or coccidiostats. Litter was bagged and stored at the end of each replicate (three consecutive identical replicates were conducted) to allow each room to be disinfected appropriately between each replicate. Once rooms had been disinfected, litter and CS were redistributed. Two rooms within the WVU Poultry Farm Research Facility were utilized to completely remove litter and broilers to eliminate cross-contamination. One room was termed "CS" (**Figure 1**), and the second room was called "BUL" (**Figure 1**). There were 16 pens in CS or BUL

FIGURE 1 | Broilers were reared in "clean-shavings" and "built-up-litter" room.

room with 23 birds per pen, and a stocking density was 0.06 m^2/bird. At the end of each replicate, three to four broilers from eight pens from each room (CS or BUL) were collected and processed at the WVU pilot processing facility that mimicked an MPPU, which was replicated three times with a total of 174 broilers. For each replication, 25 broilers were from CS room, and 24 broilers were from BUL room. Litters from CS and BUL treatment were analyzed for *Salmonella* in a commercial microbial testing lab and no *Salmonella* (<1 CFU/25 g litter) was detected in litters.

Processing Broilers in an MPPU Facility

The processing of aforementioned broilers was in an MPPU facility at WVU poultry farm with no application of antimicrobial agents. No *Salmonella* spp. was sampled from the MPPU facility according to the real-time polymerase chain reaction (PCR) test of the InvA gene (internal unpublished data). Broilers were killed with a hand knife and allowed for bleeding for 2 min. After scalding and defeathering, the evisceration was conducted manually on a stainless-steel table with glove hands. Broiler carcasses were then rinsed in warm (50°C) tap water before chilling in a static container with ice water for 24 h. Ceca samples of each processed broilers were collected for later microbial analyses.

Preparation of Broiler Carcasses and Ceca Samples

After chilling for 24 h, carcasses were added to a sterile chicken-sampling bag (Nasco, Fort Atkinson, WI, USA) rinsed with 400 mL of buffered peptone water (BPW; Hardy Diagnostics, Santa Maria, CA, USA) and followed by vigorous shaking for 60 s (9). Ceca samples were prepared by vertical cutting, addition into a sterile filtered Whirl-Pak@ bag (Nasco) with 60 mL of BPW, followed by homogenization in a masticator (IUL Instruments, Barcelona, Spain) for 2 min. The 60 mL of ceca solution was equally split into two tubes for further testing *Salmonella* and *Campylobacter* spp.

Numeration of APCs, *E. coli*/TCCs, and Yeast/Molds (Y/M)

The rinsate of each carcass sample was serially diluted 10-fold into 0.1% BPW and plated onto APCs, *E. coli*/TCCs, and Y/M petrifilm (3M Microbiology, Saint Paul, MN, USA) for enumeration of the total population of aerobic bacteria, generic *E. coli*, TCCs, and Y/M, respectively, according to manufacturer instructions. Petrifilms were incubated at 25°C for 72 h (APCs), 35°C for 48 h (*E. coli*/TCCs), and 25°C for 120 h (Y/M) followed by manual counting of colonies.

Isolation of *Salmonella* spp.

The isolation of *Salmonella* spp. was used modified FDA-BAM methods (10) as described in the previous study of Li et al. (11). The aforementioned broiler BPW rinsate and 30 mL of ceca BPW solution were pre-enriched for 24 h at 35°C. Then, 0.1 mL was transferred into a 10 mL of Rappaport–Vassiliadis broth for secondary enrichment (24 h, 35°C). This was followed by streak plating onto XLT-4 agar and HardyCHROM™ agar (Hardy Diagnostics) and incubation for 24 h at 35°C. The one to two presumptive typical *Salmonella* colonies from XLT-4 agar and HardyCHROM agar were confirmed using a *Salmonella* Latex Agglutination Test kit (Oxoid, Basingstoke, UK) and API 20E Test kit (bioMėrieux, Durham, NC, USA). *Salmonella* Typhimurium ATCC 14028 was used as a positive control from a biochemistry and immunology test.

Isolation of *Campylobacter* spp.

The isolation of *Campylobacter* spp. was according to the previous study of Scheinberg et al. (9). 30 mL of broiler-carcass rinsate or ceca sample solution was mixed with 30 mL of 2× Bolton's broth (Hardy Diagnostics). These mixtures were incubated for 48 h at 42°C under microaerophilic conditions (5.0% O_2, 10% CO_2, and 85% N_2) in a 2.5-L microaerophilic jar (Oxoid). Following incubation, a loopful of Bolton's broth was streaked on modified Campy-Cefex Agar (Hardy Diagnostics) and incubated for 72 h at 42°C under the microaerophilic conditions described above. Presumptive colonies on the modified Campy-Cefex Agar gar were confirmed using the Campy-latex Agglutination Test (Oxoid), oxidase test (Hardy Diagnostics), and Gram staining to observe for "corkscrew" morphology.

Identification of *C. jejuni* and *Campylobacter coli*

The identified *Campylobacter* colonies were regrown into 10 mL of Bolton's broth for 48 h at 42°C under the microaerophilic conditions described above. Then, the growing solutions were used to test for the presence of *C. jejuni* and *C. coli* in ceca or carcass samples using a TaqMan® kit (Fisher Scientific, Fair Lawn, NY, USA) following the manufacturer instruction. Total DNA was extracted according to the method described in Li et al. (11) followed by the real-time PCR detection of CadF gene (12). Reactions were conducted in a total volume of 20 μL, which included 10 μL of 2× qPCR MasterMix, 1 μL of *C. jejuni* or *C. coli* primer/probe mix, 1 μL of internal extraction control primer/probe mix, 3 μL of RNAse/DNAse free water, and 5 μL of

extracted DNA. Amplification of the CadF gene was done on a 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Conditions for the amplification were 37°C for 15 min, 95°C for 2 min, and 30 cycles of 95°C for 10 s and 60°C for 1 min.

Preparation of *C. jejuni* Inoculum

Strains RM5032, RM1188, and RM1464 of *C. jejuni* (kindly supplied by Dr. Nereus Gunther from USDA-ARS, Wyndmoor, PA, USA) were used in this study. Each individual *C. jejuni* strain was maintained on Brucella agar (Hardy Diagnostics) at 4°C under microaerophilic conditions (5.0% O_2, 10% CO_2, and 85% N_2) in a 2.5-L microaerophilic jar (Oxoid). The colonies grown on Brucella agar were verified by a Campy-latex Agglutination Test kit. To prepare the inoculum, single colonies of each *C. jejuni* strain were inoculated individually into 10 mL of Bolton's broth and incubated for 48 h at 42°C under the microaerophilic environment described above. Before experimentation, the three cultures of *C. jejuni* were combined, harvested by centrifugation (5,000 × *g*, 15 min, room temperature), duplicate washed with 0.1% BPW to remove residual media, centrifuged, and resuspended in 0.1% BPW. The bacterial population of the final inoculum suspension was 7 log colony-forming units (CFU)/mL.

Inoculation of *C. jejuni* on Broiler Carcasses

The MPPU-processed carcasses from WVU poultry farms were transferred to a food microbiology laboratory at WVU and used in experiments within 24–48 h. Broiler carcasses were assigned randomly to a treatment group and inoculated with the three-strain mixture of *C. jejuni*. This was achieved by addition of five drops of 200 μL of the bacterial mixture on medial and lateral sides (13) and placement on foil paper in a biohazard hood for 20 min to allow bacterial attachment. The final inoculation level of the organism on carcasses was 4.54 ± 1.24 log CFU/mL of carcass rinsate.

Antimicrobial Treatment of Broiler Carcasses

The *C. jejuni*-inoculated broiler carcasses were left untreated (control) or immersed in antimicrobial solutions: PAA (0.1%; pH, 3.0; 15.7°C; Birko, Henderson, CO, USA), LA (5%; pH, 2.0; 15.3°C; Birko), LCA (2.5%; Chicxide®), SH (freely available chlorine, 67–69 ppm; pH, 9.1; 14.4°C; Birko), and a PAA/hydrogen peroxide mix (SaniDate® 5.0, 0.25%; pH, 7.25; 15.2°C; Arbico Organics, Tucson, AZ, USA). Treatment involved immersing three carcasses into a 10-L prepared antimicrobial solution with manual agitation (≈500 rpm) for 30 s with draining for 2 min. The tested concentration of PAA, LA, and LCA was in the range allowed in USDA-FSIS Directive 7120.7 (5). PAA concentration was determined using a Titration Drop Test kit (LaMotte Co., Chestertown, MD, USA) (14). The concentration of LA, LCA, and SaniDate 5.0 was calculated according to factsheet supplied by the manufacturer. The initial and residual free-chlorine concentration was measured using the *N*,*N* diethyl-1,4 phenylenediamine sulfate method (15). For SH solution, after 30-s treatment of broiler carcasses, the initial free-chlorine concentration was 67–69 ppm, and the residual free-chlorine concentration was 11.8 ppm. Therefore, the mean initial and final residual free-chlorine concentration was ≈40 ppm (i.e., <50 ppm and in accordance with USDA-FSIS Directive 7120.7) (5). The pH and temperature of antimicrobial solutions were measured using a digital pH meter (Fisher Scientific).

Microbiological Analyses

Numeration of *C. jejuni* on broiler carcasses was done according to the methods described by Nagel et al. (14) and Gunther et al. (16). Carcasses were placed in a sterile chicken-sampling bag (Nasco) and rinsed with 200 mL of BPW supplemented with 0.1% sodium thiosulfate (Fisher Scientific) followed by vigorous shaking for 60 s (14). After 10-fold serial dilution in Bolton's broth, the dilution liquid was spread plated onto Brucella agar (16) and incubated for 48 h at 42°C in the microaerophilic jar (5.0% O_2, 10% CO_2, and 85% N_2) before manual counting of colonies. The growth of *Campylobacter* colonies on Brucella agar was also confirmed using the Campy-latex Agglutination test.

Data Analyses

In study I, three replications were conducted for the experiment. For each replication, treatments of CS (25 broilers) and BUL (24 broilers) were organized in a split-plot design consisting of a 2 × 2 factorial arrangement in a randomized block design for broilers reared at the WVU Poultry Farm. In study II, the antimicrobial intervention test was repeated twice with three carcasses per treatment per repeat (a total of six samples of carcasses per treatment). A chi-square test (significance level at 0.05) from JMP® was done to compare differences in the percentage of *Salmonella*, *Campylobacter* spp., *C. jejuni*, and *C. coli* on broiler carcasses between treatment of CS and BUL. Data on microbial quality (converted to log CFU/mL) of broiler carcasses (APCs, *E. coli*, TCCs, and Y/M) were analyzed using Student's *t*-test by SAS v9.2 (SAS Institute, Cary, NC, USA). One-way ANOVA of SAS v9.2 was used to analyze the survival population and reduction of *C. jejuni* on broiler carcasses after antimicrobial treatment. To compare the level of reduction of the *C. jejuni* response to various antimicrobial agents, reduction data were determined using the following equation:

$$\text{Reduction ratio} = \log_{10}(N_0 / N)$$

where N_0 is the mean control plate counts and N is the plate count of each individual antimicrobial-treated sample. Mean values were compared with a significance level of $\alpha = 0.05$ as determined by Tukey's honest significant difference test.

RESULTS

Microbial Quality of Broiler Carcasses

As indicators of microbial hygiene, the population of APCs, TCCs, *E. coli*, and Y/M of broiler carcasses from CS and BUL groups is quantified in **Table 1**. There was no significant difference ($P > 0.05$) in APCs, TCCs, and *E. coli* between carcasses in the CS room and BUL room (**Table 1**). The mean value (in log CFU/mL) of APCs was 3.4–3.5, TCCs was 2.2–2.5, and

TABLE 1 | Mean ± SD of microbial populations (log CFU/mL of sample rinsate) measured as aerobic plate counts (APCs), total coliforms (TCCs), *Escherichia coli*, and yeast/molds (Y/M) on broiler carcasses in "clean-shavings (CS)" and "built-up-litter (BUL)" rooms.

Treatment	APCs	TCCs	*E. coli*	Y/M
CS (n = 75)	3.4 ± 0.2[a]	2.5 ± 0.3[a]	2.1 ± 0.6[a]	1.8 ± 0.3[a]
BUL (n = 72)	3.5 ± 0.2[a]	2.2 ± 0.4[a]	2.1 ± 0.5[a]	2.2 ± 0.4[b]

Mean values with different lowercase letters within a column are significantly different ($P < 0.05$).

E. coli was 2.1 of all carcasses (**Table 1**). The total population of Y/M on CS broiler carcasses was lower by 0.4 log CFU/mL ($P < 0.05$) than BUL carcasses (**Table 1**).

Prevalence of *Salmonella* spp. in Broiler Ceca and on Carcasses

The presence of *Salmonella* spp. on broiler carcasses was tested and confirmed by the *Salmonella* Latex Agglutination Test and API 20E strips with a biochemical profile code 6704752 (17). There was no contradiction in results between these two tests. Overall, a *Salmonella* spp. was not detected on any ceca samples tested regardless of CS and BUL treatments, suggesting that *Salmonella* spp. was not colonized in all broilers tested. *Salmonella* spp. was not detected on CS-treated carcasses, and it was present on 2.8% (2 of 72 samples) of carcasses in the BUL room (**Table 2**).

Prevalence of *Campylobacter* spp. in Broiler Ceca and on Carcasses

Overall, the prevalence of *Campylobacter* spp. in broiler ceca (64.6–84.6%) and on carcasses (50–56.2%) was shown in **Table 3**. In general, the prevalence of *Campylobacter* spp. in the ceca in the CS room were lower ($P < 0.05$) than those in the BUL room (**Table 3**) but similar ($P > 0.05$) on the carcasses of broilers compared to the samples in BUL room (**Table 3**). Among the broilers in the CS room, *Campylobacter* spp. was colonized in 64.6% (49 of 75) of ceca samples, and was present on 50% (37 of 75) of carcasses (**Table 3**). Among BUL-treated samples, 84.6% (61 of 72) of ceca samples were colonized with, and 56.3% (41 of 72) of carcasses were carrying *Campylobacter* spp. (**Table 3**).

Quantitative PCR revealed that the prevalence of *C. jejuni* was lower ($P < 0.05$) in the ceca (14.7 vs. 30.6%) but similar on carcasses (19.4 vs. 28.6%) of CS broilers compared to the BUL samples (**Table 3**). *C. coli* was present at a similar level ($P > 0.05$) in the ceca (36.0 vs. 30.6%) and ($P > 0.05$) on the carcasses (19.4 vs. 25.7%) of CS and BUL-treated samples (**Table 3**).

Antimicrobial Efficacy in Inactivation of *C. jejuni*

The survival and reduction values of *C. jejuni* on post-chilled broiler carcasses treated with 0.1% PAA, 5.0% LA, 2.5% LCA, 69 ppm SH, or 0.25% SaniDate 5.0 are shown in **Table 4**. The initial level of *C. jejuni* recovered on inoculated broiler carcasses was 4.54 log CFU/mL. All tested antimicrobial treatments reduced the *C. jejuni* on broiler carcasses significantly ($P < 0.05$) compared with the untreated control. Specifically, 0.1% PAA reduced *C. jejuni* by 2.04 log CFU/mL compared with the control, which was better ($P < 0.05$) than all the other antimicrobials (**Table 4**). In the present study, dipping carcasses in 5.0% LA reduced the *C. jejuni* population by 1.43 log CFU/mL compared with the untreated control ($P < 0.05$) (**Table 4**). Broiler carcasses dipped into SH (69 ppm), 2.5% LCA, and 0.25% SaniDate 5.0 reduced the *C. jejuni* population by 1.65, 1.43, and 1.26 log CFU/mL, respectively, and there were no significant difference ($P > 0.05$) between these treatments (**Table 4**).

TABLE 2 | Prevalence of *Salmonella* spp. in the ceca and on the carcasses of broilers in "clean-shavings (CS)" and "built-up-litter (BUL)" rooms and processed in a mobile poultry-processing unit.

Treatment	Ceca	Carcasses
CS	0% (0/75)[a]	0% (0/75)[a]
BUL	0% (0/72)[a]	2.8% (2/72)[a]

Mean values with the same lowercase letters within a column are not significantly different ($P > 0.05$).

TABLE 3 | Prevalence of *Campylobacter* spp., *Campylobacter jejuni*, and *Campylobacter coli* in the ceca and on the carcasses of broilers in "clean-shavings (CS)" and "built-up-litter (BUL)" room and processed in a mobile poultry-processing unit.

Treatment	Ceca			Carcass		
	C. spp.	*C. jejuni*	*C. coli*	*C.* spp.	*C. jejuni*	*C. coli*
CS	64.6% (49/75)[a]	14.7% (11/75)[a]	36% (27/75)[a]	50% (37/75)[a]	19.4% (14/75)[a]	19.4% (14/75)[a]
BUL	84.6% (61/72)[b]	30.6% (22/72)[b]	30.6% (22/72)[a]	56.3% (41/72)[a]	28.6% (21/72)[a]	25.7% (18/72)[a]

Mean values with different lowercase letters within a column are significantly different ($P < 0.05$).

TABLE 4 | Survival and reduction (mean ± SD) of *C. jejuni* (counts on Brucella agar) recovered from inoculated broiler carcasses left untreated or treated with peroxyacetic acid (PAA, 0.1%, pH 3.0, 15.7°C), lactic acid (LA, 5%, pH 2.0, 15.3°C), lactic and citric acid (LCA) blend (2.5%, pH 2.7, 15.2°C), sodium hypochlorite (SH, 67–69 ppm, pH 9.1, 14.4°C), a PAA and hydrogen peroxide mixer (SaniDate® 5.0, 0.25%, pH 7.2, 15.2°C) for 30 s.

Treatment	Survival (log CFU/mL)	Reduction (log CFU/mL)
Control	4.54 ± 1.24[a]	–
PAA	2.49 ± 0.77[b]	2.04 ± 0.77[a]
LA	3.11 ± 0.70[b]	1.43 ± 0.70[ab]
LCA	3.11 ± 0.17[b]	1.43 ± 0.71[ab]
SH	2.89 ± 0.15[b]	1.65 ± 0.15[ab]
SaniDate® 5.0	3.28 ± 0.51[b]	1.26 ± 0.51[b]

"–" indicates reduction data are not available.
Mean values with different lowercase letters within a column are significantly different ($P < 0.05$).

DISCUSSION

Aerobic plate counts are used to assess the total microbial population on broiler carcasses. The coliform population

(especially the generic *E. coli* population) indicates the potential fecal contamination on processed meat and poultry products according to USDA-FSIS (9). The population of Y/M of processed broiler carcasses has not been reported widely. The value for APCs was similar to, but that for TCCs and *E. coli* was higher than the value noted by Scheinberg et al. (9). They reported that the value (in log CFU/mL) for APCs, TCCs, and *E. coli* was approximately 4.0, 1.5, and 0.9, respectively, in whole chickens at farmers' markets in Pennsylvania (9). Northcutt et al. (18) found a similar value (in log CFU/mL) for APCs (3.2) and *E. coli* (1.7) on post-chilled conventional chicken rinsates to our results. Although the yeast and molds population recovered from broiler carcasses of CS treatment is significantly lower than the BUL treatment, a 0.4-log difference is generally not considered biologically significant (19). Overall, the levels of APCs, TCCs, *E. coli*, and Y/M found on MPPU-processed broiler carcasses in the present study suggest that small-scale growers of broilers who use MPPUs should implement antimicrobial interventions during processing or apply post-chilling interventions to reduce the background microflora on broiler surfaces.

A high level of *Salmonella* spp. on chickens processed at locally commercialized poultry facility has been reported in other studies (9, 20). For example, Trimble et al. (20) reported that 43% of chicken carcasses processed in an USDA-inspected facility were *Salmonella*-positive. Also Scheinberg et al. (9) found 20–28% of *Salmonella*-positive broiler carcass samples from farmers' markets and local supermarkets in Pennsylvania. The *Salmonella* present on those small, locally processed broiler carcasses may be attributed to variances in farm management and lack of regulatory guidance. In the present study, very low percentage of *Salmonella* spp. was identified in broiler ceca and on carcasses regardless of CS and BUL treatment, which is in agreement with the studies of Killinger et al. (21) and Trimble et al. (20). They reported that *Salmonella* was not detected on carcasses processed in the university pilot-scale MPPU in the states of Washington (21) and Arkansas (20). These results might be explained by the following four reasons. First, applying good cleaning and sanitization practices could reduce *Salmonella* spp. effectively on broilers (22). The WVU poultry-raising room and pilot MPPU facility was cleaned repeatedly with hot water along with physically sweeping and applying commercial detergent and chlorinated water afterward. Second, compared to the commercial poultry-processing facility, the university pilot-scale MPPU was less frequently used, therefore less cross-contamination would occur. Third, due to budgetary restraints only a limited sample size (23.9%, 174 of 736) of ceca and broiler samples were tested for *Salmonella* spp. Therefore, the results may not accurately reflect the *Salmonella* profile of the entire raised broilers. Finally, in this study, *Salmonella* and *Campylobacter* both occupy the same gastrointestinal tract of broilers; therefore, it is possible that *Campylobacter* was present in significant amounts and *Salmonella* was not detected.

Campylobacter spp., especially *C. jejuni* and *C. coli*, are the two major *Campylobacter* species and commonly cause human gastroenteritis if undercooked poultry meat is eaten (12). The percentage of *Campylobacter* spp. on MPPU-processed broilers has not been studied widely. Overall, the prevalence of *Campylobacter* spp. in broiler ceca and on carcasses was much higher than the percentage of *Salmonella*. Findings are in accordance with the study of Trimble et al. (20), which suggest that for small-scale broiler producers, the management practices used to control *Salmonella* effectively might have only a slight effect on *Campylobacter* due to the difference in the physiology and ecology of these two pathogens in production and processing environments (20, 23). The high percentage of *Campylobacter* spp. observed in the present study may have been due to (1) use of a single-stage static scalder; (2) the practice of manual evisceration; (3) a single, static chilling tub without any antimicrobial agents (which may have resulted cross-contamination of broiler carcasses during the pilot-scale MPPU process).

Ceca is the main source of *Campylobacter* colonization in broilers. The impact of CS and BUL on the colonization of *Campylobacter* spp., *C. coli*, and *C. jejuni* in ceca of broilers were investigated in this study. CS reduced the percentage of *Campylobacter* spp. and *C. jejuni* but did not affect the percentage of *C. coli* compared to the BUL treatment. This mixed result might be explained by the two reasons. On one side, BUL was bagged, stored, and maintained the same litter throughout each trial, whereas CS was replaced if "caking" occurred. This process may have allowed for increased colonization of *Campylobacter* spp. and *C. jejuni* in BUL-treated cecum. On the other side, the colonization of *C. coli* might be caused by the complex factors of the broiler cecum, rather than attributed to the practices of litter including using old "dirty" litter repeatedly (24). No significant difference of *Campylobacter* spp., *C. jejuni*, and *C. coli* detected on broiler carcasses regardless of they were reared on BUL or CS treatment indicate that simply application of litter treatment including replacing old dirty litter with CS in the raising room did not directly influence the levels of *Campylobacter* on broiler carcasses. A further antimicrobial intervention is necessary during the post-harvest broiler processing to control *Campylobacter* level in the final broiler carcasses.

Results of this study showed that *Campylobacter* was dominant on MPPU-processed broiler carcasses; therefore, validation of the efficacy of various commercial antimicrobial agents against this pathogen after chilling was important. Nagel et al. (14) and Chen et al. (25) also reported that PAA (0.04–0.1%) is the most effective antimicrobial agent used during post-chilling dipping to decontaminate *Campylobacter* on poultry products compared with chlorine, cetylpyridinium chloride, and lysozyme. Nagel et al. (14) reported that 0.1% PAA achieved a reduction of 2.03 log CFU/mL of *C. jejuni* on broiler carcasses processed in an industry-scale pilot post-chilling dipping tank. Chen et al. (25) found that 0.1% PAA reduced *Campylobacter* by ≈1.5 log CFU/g in ground chicken meat. PAA is a combination of peracetic acid and hydrogen peroxide, and it denatures proteins and disrupts bacterial cell walls (26). PAA at <2,000 ppm (0.2%) has been approved by USDA-FSIS for application on poultry carcasses since 2001 (6, 7), and it is the most prevalent antimicrobial agent used in the poultry industry (7). Small-scale poultry producers in Pennsylvania and the WV area who currently own or will purchase MPPUs wish to know the antimicrobial efficacy of PAA due to the concerns

of "organic" processing. The present study provides important, validated data for them.

Lactic acid at <5.0% is approved by the USDA-FSIS as an antimicrobial agent applied on broiler carcasses before or after chilling (5). In the present study, dipping carcasses in 5.0% LA reduced the *C. jejuni* population by 1.43 log CFU/mL compared with the untreated control ($P < 0.05$) (**Table 4**). Coşansu and Ayhan (27) dipped the legs and breasts of chickens into 1 and 3% LA and achieved reductions of 0.36–1.36 and 1.27–1.98 log MPN/cm^2, respectively. Burfoot et al. (28) sprayed 4 and 8% LA onto chicken carcasses and reduced the *Campylobacter* on skin surfaces by 0.4–0.8 and 1.9 log CFU/g, respectively. Potential undesirable sensory and quality concerns have been raised upon application of LA on broiler carcasses (29).

In the present study, broiler carcasses dipped into SH (69 ppm), 2.5% LCA and 0.25% SaniDate 5.0 reduced the *C. jejuni* population by 1.65, 1.43, and 1.26 log CFU/mL, respectively, and there were no significant difference ($P > 0.05$) between these treatments (**Table 4**). Recently, SH (commonly referred to as "free chlorine") has lost its dominant position as an antimicrobial agent used in poultry-meat processing due to: the requirement of a high concentration; rapid reaction with organic matter; a poultry-meat trade issue between the USA and Russia (7, 14, 25). There is a growing interest in the development and evaluation of other chemical antimicrobials as chlorine alternatives. LCA (Chicxide; a buffered blend of LA and citric acid) at ≤2.5% is approved for use on poultry-meat surfaces (5), and its antimicrobial efficacy against *Salmonella* spp. has been evaluated on broiler carcasses (30). SaniDate 5.0 contains 23% hydrogen peroxide and 5.3% PAA and has been shown to control food-borne pathogens on food-contact surfaces effectively. It is also recommended by the WV Small Farm Center for use on poultry meat for small-scale poultry growers in WV (personal communication with Dr. Tom McConnell, Program Leader of the WV Small Farm Center). Results of the present study suggest a similar reduction effect on *Campylobacter* by LCA and SaniDate 5.0 compared with SH. Hence, LCA and SaniDate 5.0 could be used by local, small-scale MPPU poultry processors during post-chilling.

In conclusion, results of this study suggest that the development of good clean and sanitizing practices may control *Salmonella* on broiler carcasses effectively. Broilers reared on CS could be beneficial for the pre-harvest control of *Campylobacter* compared with broilers reared on BUL. Results of the present study confirmed that application of post-chilling antimicrobial-dipping treatments (especially PAA) could be a potential intervention approach to control *Campylobacter* on locally processed broilers using an MPPU. These results could contribute to the development of the new USDA-FSIS 5-year strategic plan for control of *Salmonella* in poultry-meat products (31). Our data could also assist WV state and local regulatory agencies to assess the potential risk and develop control strategies for *Salmonella* and *Campylobacter* in the application of MPPU processes for local poultry growers.

ETHICS STATEMENT

Broilers sampled for the present study were obtained from a study conducted at the WVU Poultry Farm, as reported previously by Glover et al. (8). Broilers were cared for according to guidelines set by the Animal Care and Use Committee of WVU. Briefly, 736 1-day-old straight-run Hubbard × Cobb chickens were obtained from a local hatchery and raised for 38 days with 174 chickens using in this study. Broilers had access to food and water *ad libitum*. Broilers were fed with a high-by product protein diet containing a 30% inclusion of wheat (high in non-starch polysaccharides). The diet formulated did not contain any antibiotics or coccidiostats. Litter was bagged and stored at the end of each replicate (three consecutive identical replicates were conducted) to allow each room to be disinfected appropriately between each replicate. Once rooms had been disinfected, litter and CS were redistributed. Two rooms within the WVU Poultry Farm Research Facility were utilized to completely remove litter and broilers to eliminate cross-contamination. One room was termed "CS" (**Figure 1**) and the second room was called "BUL" (**Figure 1**). There were 16 pens in CS or BUL room with 23 birds per pen, and a stocking density was 0.06 m^2/bird. At the end of each replicate, three to four broilers from eight pens from each room (CS or BUL) were collected and processed at the WVU pilot processing facility that mimicked an MPPU, which was replicated three times with a total of 174 broilers. For each replication, 25 broilers were from CS room, and 24 broilers were from BUL room. Litters from CS and BUL treatment were analyzed for *Salmonella* in a commercial microbial testing lab, and no *Salmonella* (<1 CFU/25 g litter) was detected in litters.

AUTHOR CONTRIBUTIONS

KL conducted poultry processing, microbial pathogen testing, reference organization, and drafted manuscript. LL conducted antimicrobial validation study and data collection and analysis. BG raise all broiler carcasses and revised the manuscript. JM managed the poultry farm and coordinated the project. CS generated the idea of this project and drafted, revised, and finalized the manuscript.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Listeria Occurrence in Poultry Flocks: Detection and Potential Implications

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Foodborne pathogens such as *Salmonella, Campylobacter, Escherichia coli,* and *Listeria* are a major concern within the food industry due to their pathogenic potential to cause infection. Of these, *Listeria monocytogenes,* possesses a high mortality rate (approximately 20%) and is considered one of the most dangerous foodborne pathogens. Although the usual reservoirs for *Listeria* transmission have been extensively studied, little is known about the relationship between *Listeria* and live poultry production. Sporadic and isolated cases of listeriosis have been attributed to poultry production and *Listeria* spp. have been isolated from all stages of poultry production and processing. Farm studies suggest that live birds may be an important vector and contributor to contamination of the processing environment and transmission of *Listeria* to consumers. Therefore, the purpose of this review is to highlight the occurrence, incidence, and potential systemic interactions of *Listeria* spp. with poultry.

Keywords: *Listeria*, poultry, live production, isolation, detection

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INTRODUCTION

Microorganisms such as *Salmonella*, *Campylobacter*, and *Listeria* represent a considerable concern within the food industry due to their pathogenic properties and their potential to establish infections in humans. It is imperative that intervention strategies be established to reduce risk of foodborne illness to consumers, specifically *Listeria monocytogenes*. However, little is known about the prevalence of *Listeria* spp. throughout the poultry production and processing continuum. *Listeria* species are Gram-positive, non-spore forming, rod-shaped bacteria that are naturally found in the environment, including soil, sewage, feces from animals and birds, and surface water (1, 2, 3, 4). *Listeria* are persistent facultative anaerobes that ideally proliferate in temperatures of 30 to 37°C, but can withstand temperatures between 0 and 43°C (4, 5). In addition to being able to survive a wide range of temperatures, *Listeria* spp. can grow in a variety of salt concentrations, high osmotic pressure, and low pH environments, but succumb to pasteurization (6). There are at least six known species: *Listeria grayi*, *Listeria seeligeri*, *Listeria welshimeri*, *Listeria ivanovii*, *Listeria innocua*, and *L. monocytogenes*. Of the six species, *L. ivanovii* is pathogenic to animals and *L. monocytogenes* is the only species pathogenic to humans. While *Listeria* commonly colonize multiple mammalian hosts, it remains unclear the exact relationship of *Listeria* with avian species. While a frequent contaminant of ready to eat meats, the relationship with the live bird production aspect is much less clear. In this review, the occurrence,

incidence, and potential systemic interaction of *Listeria* spp. with chickens and other avian species will be discussed.

Listeria AND FOODBORNE DISEASE

Listeria monocytogenes can be subdivided into different phylogenic evolutionary lineages (I, II, III, and IV) based on ecology, genomic content, and recombination rates (7). Phylogenetic serotypes are based on cell wall antigen expression and thus can be used to identify variations among all 13 serotypes (7). Lineage I consists of serotypes 1/2b, 3b, 3c, and 4b strains, while lineage II includes serotypes 1/2a, 1/2c, and 3a (8, 9). Phylogenetic evidence has indicated that rare serotypes may have evolved recently, or multiple times, from one of the major serotypes (10). Lineage III belongs to two groups, formerly lineages IIIA/C and IIIB/C known as lineages III and IV, with serotypes 4a and 4c strains (11). However, limited knowledge exists for lineage IV due to rarity and low strain variability, which contributes to seven unclear serotypes. Similarly, little is known about the lineage status of serotype 7 due to lack of availability of such strains (10, 12, 13).

Serotypes that are most commonly associated with human cases are 11 (1/2b) in chicken meat, C1-056 (1/2a) in human, sporadic case, N1-225 (4b) causing human epidemic, and N1-227 (4b) (14). Variations in strains of *L. monocytogenes* are characterized according to their physiological properties and phenotypic characteristics, such as growth behavior, acid tolerance, and resistance to various stresses (14). There has also been identification of strain variance due to heat resistance (14), which could be directly related to those involved in food contamination that carries over to the consumer due to resistance to heat treatments.

Previous reports have shown an increase in the prevalence of *L. monocytogenes* in ready-to-eat (RTE), vacuum packaged, sliced meat products where 95% of all *L. monocytogenes* belonged to Lineage II, serotype 1/2a, with the remaining 5% varying between serotypes 1/2b, 3b, and 4b (15). Kramarenko et al. (16) reported that 93% of all *L. monocytogenes* isolates obtained from meat products belonged to serotype 1/2a and 1/2c (16). Therefore, this suggests that variations between stress and exposure influence which lineage, serotype, and strain is ultimately responsible for contamination.

Listeria monocytogenes is the causative agent of listeriosis, where those considered to be most susceptible include the elderly, immunocompromised, and pregnant women (1, 13). Although one of the less common foodborne illnesses, it frequently requires hospitalization (17–20) and has a mortality rate of 20 to 30% (6). The Centers for Disease Control and Prevention Morbidity and Mortality Weekly Report states that of the 123 cases that occurred in 2013, 91% of reported cases resulted in hospitalization (21). There are a variety of symptoms that may arise upon infection, including septicemia, meningitis, and gastroenteritis (22). In pregnant women, it may cause spontaneous abortion, premature labor, and neonatal disease (23). Serotypes 1/2a (lineage II) and 1/2b and 4b (lineage I) are responsible for a majority of the *L. monocytogenes* hospitalized cases (10, 12, 13). Lineage I is responsible for cases among outbreaks of human clinical listeriosis (10, 13), while Lineage II strains exhibit a significantly higher prevalence among food isolates, the environment and animal clinical cases (9). Lineage III and IV strains account for approximately 1% of human listeriosis cases in humans but are more prominent in animals (24). Most sporadic human listeriosis cases appear to be caused by serotype-4b and -1/2a strains, while most human listeriosis outbreaks have been linked to serotype-4b strains (11). Outbreaks have rarely occurred because of non-4b serotypes but do happen (8)). For example, a serotype-1/2a outbreak of gastrointestinal listeriosis was linked to sliced turkey in the United States (8).

In the United States, there is a zero-tolerance policy for *L. monocytogenes* in food that requires the recall of any adulterated food product (18). *L. monocytogenes* has been investigated in various food products such as seafood, dairy products, meats, and RTE products. In the past, there were listeriosis outbreaks in the United States linked to contaminated cantaloupes, soft cheeses, RTE turkey deli meat, ice cream, unpasteurized milk, candied apples, packages and frozen vegetables, and most recently, soft raw milk cheeses (25).

There has been limited focus on *Listeria* spp. related to poultry and poultry products. To date, there have been no poultry (chickens, specifically)-related listeriosis outbreaks; however, though uncommon, poultry flocks can be contaminated with *L. monocytogenes* and result in sporadic listeriosis cases (3, 5, 26). In the few studied cases where the disease was attributed to poultry sources, symptoms have included septicemia or localized encephalitis (5). Poultry flocks can serve as a reservoir and can contaminate the litter and surrounding environments (3, 5, 27–29). Although rare cases of human listeriosis from raw poultry meat has stemmed from contamination and unhygienic practices of processing environments (4, 5), little is known about the prevalence within poultry and poultry products. Therefore, the purpose of the remainder of this review is to take a farm-to-fork approach and highlight the possible issues and places of potential *Listeria* spp. contact during the production of poultry and the importance for continued research of *Listeria* spp. in poultry to reduce the potential of future outbreaks.

LIVE POULTRY PRODUCTION PRACTICES

Consumers have repeatedly expressed concerns about animal welfare related to intensive chicken farming. They want to be sure that all animals being raised for food are treated with respect and are properly cared for during their lives. Farmers and companies share the public's concern and recognize that they have an ethical obligation to make sure that the animals on their farms are well cared for (30). Guidelines for poultry management include hatchery operations, appropriate housing and space, proper nutrition and feeding, health care, and monitoring, and these guidelines are provided within handbooks to poultry breeders (31), industry association protocols (30), or legislative texts (32).

Housing type and management are dictated by the type of poultry being produced (broiler versus layers), economics and the preferences in a particular region and climate. Globally, poultry housing must provide comfortable and protective shelter for the birds and effective measures must be established to protect flock health and minimize any negative impact on bird welfare (30). Several housing systems are used including conventional cages,

furnished/enriched cages, barn systems, or free-range systems which exist for layers (33). The most common housing systems around the world remain cages with a space allowance ranging from 300 to 750 cm^2 according to the legislation of the country (34). Broilers are generally held in groups in environmentally controlled housing, open and naturally ventilated poultry houses or on free range (34). In grow-out houses, the minimum space should be one-half square foot per bird as stated by the Council for Agricultural Science and Technology (CAST) (30). Whatever the rearing system, poultry housing and equipment must be designed to protect the birds from environmental conditions. Appropriate ventilation and heater systems are needed to regulate seasonal temperatures to keep air moving throughout the house and to provide optimal air quality at any time (30, 31). The bedding material must be of good quality and the litter must be kept clean. Ammonia emissions must be monitored and appropriate measures be taken, if necessary to reduce to the minimum allowable level by appropriate measures, if necessary (30, 31, 35).

Nutrition and water requirements for poultry depend on a range of factors including the commercial goals of the poultry enterprise, type of bird, breed and age, or stage of development (30, 31, 36). A good-quality dietary feed will help the birds stay healthy and grow well. Birds should have unlimited access to clean, fresh, and good quality drinking water. All feeding and drinking systems must be checked for proper operation daily and must be adjusted in height as the birds grow. Precise and complete guidelines about the nutrition and water requirements, as well as the feeding and drinking systems, are given by poultry breeders (31). A good poultry health management is an important component of flock management and meat or egg production (37). Good hygienic conditions within the poultry house must be achieved through the implementation of correct biosecurity, cleaning, and vaccination programs. A working relationship with an avian veterinarian is an integral part of health management (30, 31).

Listeria spp. WITHIN THE HATCHERY ENVIRONMENT

Live poultry production includes the hatchery and the grow-out farm environments. These two steps of poultry production may contribute to the contamination of live birds with *L. monocytogenes* and potentially lead to the contamination in the food processing plant and the poultry meat. The hatchery is responsible for the incubation of fertile eggs obtained from parent breeders and the hatching of chicks. The hatchery is the first production stage where eggshell surfaces, embryos, and chicks can be contaminated by pathogenic bacteria. Very few studies have investigated the occurrence of *Listeria* spp. and *L. monocytogenes* in the hatchery environment, Over 200 samples collected in three commercial broiler hatcheries in northern Georgia, USA, only 1% of chick paper pads and 6% of eggshell fragments were positive for *L. monocytogenes* (38). In Thailand, 32 hatcheries were inspected for *L. monocytogenes* over a 5-year period. Incubator trays of equipment used in hatcheries were swabbed (548 samples) and meconium from 10-day-old chicks were collected (523 samples). *L. monocytogenes* was not detected in 1,071 samples over this 5-year period (39).

Overall, limited data are available on the occurrence of *Listeria* spp. and *L. monocytogenes* in the hatchery environment. The vertical transmission of *L. monocytogenes* from the parent flock to the day-old chicken leaving the hatchery has not fully been investigated, contrary to other foodborne pathogens such as *Salmonella* (40, 41) and *Campylobacter* (42, 43). Since *L. monocytogenes* contamination of poultry products has focused on the processing stage (44, 45) and RTE products (1, 6), the hatchery environment has not been viewed as important to poultry meat contamination processes. However, there is no evidence to exclude the hatchery environment in the early contamination of chicks and consequently the final product.

Listeria spp. WITHIN THE GROW-OUT FARM ENVIRONMENT

Once the 1-day-old chicks leave the hatchery environment, they are shipped to the grow-out farms to reach pre-determined size/weight based on the processing/final product requirements. Studies reporting the prevalence of *Listeria* spp. and *L. monocytogenes* in the grow-out farm environment vary according to the country, the number of farms and flocks examined, the breed of bird, and the type of samples collected (**Table 1**). To evaluate the contamination rate of *Listeria* spp. and *L. monocytogenes* on grow-out farms, studies have either investigated its prevalence in environmental samples surrounding or within the production area (soil, grass, dust, litter, feed, drinking water, layer egg shells, nest boxes) or in bird ceca and feces. In studies focusing on environmental samples, *Listeria* spp. prevalence ranged from as low as 1.4 to 53% (5, 6, 46–49). According to the sample type, 9.8 to 52.5, 70, 10, 30, and 6 to 42.8% of the samples were positive for *Listeria* spp. in broiler litter, farm feed, farm drinking water, soil, and grass samples, respectively (5, 46–48). Milillo et al. (46) demonstrated that environmental samples collected from the pasture before broiler introduction were rarely positive for *Listeria* spp. (5%), whereas samples collected after broiler exposure were significantly more likely to contain *Listeria* spp. (53%) (46). These findings implicate poultry as a source of the *Listeria* spp. being found in these environmental samples. Not only *Listeria* spp. have been isolated from environmental samples from grow-out farms but also they have also been isolated directly from the poultry. An investigation in Danish broilers and the broiler house environment of 71 flocks. *Listeria* spp. was identified in 9.8% of litter samples and 17% of fecal samples yielding an overall prevalence of 14% (10/71) in broiler flocks (47). A lower prevalence of *Listeria* spp. (4.7%) was found in 150 fresh fecal droppings collected at four chicken farms in the suburbs of Tokyo (50). *Listeria* spp. were more likely to be isolated from young broilers, suggesting that as the birds' intestinal microbiota develop, their levels of *Listeria* spp. decline (46). *L. innocua* is the predominant species found on grow-out farms, representing ≤78% of all isolated *Listeria* spp. (5, 46, 47, 50, 51). *L. innocua* is important because it is closely related to *L. monocytogenes* and both are genetically similar (6, 52). Other *Listeria* species, such as *L. ivanovii*, *L. welshimeri*, and

TABLE 1 | Prevalence of *L. monocytogenes* during live production in grow-out farm environments.

Country	Number of farms or flocks involved		Sample collected	*L. monocytogenes* prevalence (%)			*L. monocytogenes* lineages/serovars	Reference
				Per sample	Per flock			
Denmark	71 broiler flocks		Wet litter Feces	1.5% (1/67) 2.1% (1/48)	3% (2/71)		ND	(47)
Denmark	236 parents, 5 flocks 2078 broilers, 90 flocks		Cecal content	4.7% (11/236) 0% (0/2,078)	ND ND		ND	(53)
Egypt	20 farms, 200 samples		Litter Poultry feed Drinking water	2.5% (2/80) 0% (0/20) 0% (0/20)	ND		ND	(54)
France	84 cage-layer flocks		Feces Dust	28.6% (total)	30.9% (26/84)		ND	(55)
	142 broiler chicken flocks		Boot swabs	46.2%	31.7% (45/142)			
France	200 laying hen flocks	88 caged-flocks	Feces Dust	10.5% (45/429) 5% (7/139)	29.5% (26/88)	15.5% (31/200)	1/2a, 1/2b and 4e, 4b	(56)
		112 floor-reared flocks	Dust	2.9% (6/206)	4.5% (5/112)		1/2a	
	145 broiler flocks	85 conventional flocks	Boot swabs	12.7% (54/425)	28.2% (24/85)	31.7% (46/145)	1/2a, 1/2b and 4e, 4b	
		60 free-range flocks		36.7% (22/60)	36.7% (22/60)		1/2a, 1/2b and 4e, 4b	
France	75 breeding turkey flocks 86 fattening turkey flocks		Feces	4.8% (18/375) 2.6% (11/428)	12% (9/75) 9.3% (8/86)		1/2a 1/2a, 4b	(57)
Japan	4 chicken farms		Feces	0% (0/150)	ND		ND	(50)
Spain	60 free-range chicken flocks		Feces	ND	26.7% (16/60)		4b or 4e	(58)
Thailand	43 breeder farms 1,331 broiler farms		Litter Poultry feed Drinking water	0% (0/2,504) 0% (0/2,215) 0% (0/2,398)	ND		ND	(39)
USA	340 broilers 280 layers		Grass Soil Ceca	4.8% (1/21) 0% (0/20) 1% (4/399)	Lineage II (1/2a, 1/2c, 3a) Lineage III (4a, 4b, 4c)		ND	(6, 46)
United Kingdom	Local farm		Litter Hens feces Duck feces	11.1% (1/9) 20% (1/5) 16.7% (1/6)	ND		ND	(51)

ND, not determined.

L. seeligeri, have also been identified in environmental farm samples or chicken feces but their detection remain infrequent (5, 50).

Listeria monocytogenes has been isolated on grow-out farms from litter (51, 59), dust (56), grass (46), feed (60), feces (56, 61, 62), and cecal (46, 53) samples, with an overall contamination rate ranging from 0 to 46.2% in those samples. The prevalence of *L. monocytogenes* in poultry ceca and feces is generally low but can be highly variable, ranging from 0 to 32% (39, 47, 50, 51, 53, 56). Moreover, only a small percentage of birds may be long-term carriers of the organism (63). Intestinal carriage of *L. monocytogenes* may be transient, most likely resulting from ingestion of *Listeria*-contaminated feed, soil, and/or drinking water. Indeed, it has rarely been proven that poultry feed and drinking water can be contaminated with *Listeria* spp. generally (54) or *L. monocytogenes* specifically (60, 64). Broiler flock contamination rates for *L. monocytogenes* can range from 3 to 32% (47, 55, 56, 58), with similar contamination rates for layer hens and turkey flocks (55–57). The number of positive samples within positive flocks is generally low, with most of the positive flocks (32 to 55.6%) represented by only one positive sample (55–57). These results suggest that poultry may not represent a common reservoir for *L. monocytogenes* in the grow-out farm environment, although variability in its prevalence among broiler flocks makes poultry a potential source of *L. monocytogenes* that should be further investigated.

Poultry can shed *Listeria* spp. and *L. monocytogenes* in fecal material and contaminate elements of the grow-out farm environment, including the poultry house where studies have shown that ≤52.5 and ≤25% of litter samples were positive for *Listeria* spp. and *L. monocytogenes*, respectively (51, 54, 59). Chemaly et al. (56) showed that *L. monocytogenes* detection within samples collected from caged laying hen flocks was dependent on sample type. When only *L. monocytogenes*-positive flocks were considered, the difference between dust and fecal samples were strongly significant, with a greater detection in feces than in dust samples (56). This may be attributed to dust samples being contaminated by contact with fecal materials shed on the floor. A potential transfer of *Listeria* spp. between broilers and their environment has been shown by Milillo et al. (46). The circulation of *Listeria* spp., especially *L. monocytogenes*, between animals and farm environment has also been observed in ruminant farming systems (65, 66).

Poultry farm characteristics and management practices also have an influence on the prevalence of *L. monocytogenes* in bird

flocks. These risk factors are mostly related to the hygienic status of the house and sanitary measures applied to the flocks. Aury et al. (55) identified six risk factors significantly associated with *L. monocytogenes* contamination at the end of the broiler flocks production cycle (55). The risk of *L. monocytogenes* contamination was increased when (i) farmers did not respect the principle of two areas (clean and dirty) at the poultry house entrance, (ii) disinfection was not carried out between flocks by spraying, (iii) there was an absence of pest control of the poultry house before the arrival of the next flock, (iv) litter was not protected during storage, (v) farm staff cared for other broiler houses, and (vi) the watering system did not consist of nipples with cups. Within the same study, three risk factors significantly increased *L. monocytogenes* contamination in caged hen flocks at the end of the laying period: (i) presence of pets in the production site, (ii) type of feed (use of meal rather than crumbs/crumbles), and (iii) insufficient or incomplete removal of fecal droppings (e.g., conveyor belt with deep pit storage or deep pit only methods not used) (55).

The prevalence of *L. monocytogenes* contamination may also be dependent of the type of production system. No significant differences in the prevalence of *Listeria* spp. were found on and within eggs and in the environment of a sister flock of conventional cage and free-range laying hens. In this study, *L. monocytogenes* represented 28.5% (2/7) of the *Listeria* spp. isolated (48). This result is supported by Schwaiger et al. (49) who compared cloacal swabs from 20 conventional and organic egg farms in Germany and found no significant difference in *Listeria* spp. between production methods (49). Conversely, Chemaly et al. (56) showed a significant difference between caged- and floor-reared hens with a greater detection of *L. monocytogenes* in dust samples from floor-reared hens, in *L. monocytogenes*-positive flocks (56). Alternatively raised broilers (e.g., all natural, pastured) represent management systems with unknown food safety implications, considering these poultry are raised in less controlled environments than conventionally raised birds (67). Poultry farms frequently have other animals (beef cattle, sheep, goats, or swine) and pets present on the production site (67). These animals can be reservoirs for and play a role in the multiplication and excretion of *L. monocytogenes* into the environment. The presence of other animals during grow-out was found to increase the risk of *L. monocytogenes* contamination in laying hen flocks (55). Because *L. monocytogenes* is commonly associated with other farm animals and is a natural saprophyte, the occurrence of *L. monocytogenes* in alternatively raised poultry is of particular interest.

Few studies have identified *L. monocytogenes* from poultry farms at the serogroup level. From these, serovar 1/2a represented a dominant proportion of the isolates regardless of the bird species (46, 56, 57). To a lesser extent, serovars 1/2b and 4e/4b were also identified in laying hen, broiler and turkey flocks (56–58). Although serovar 1/2a did not differ between caged- and floor-reared hens, or between standard and free-range systems for broilers, serovars 1/2b and 4e/4b were significantly more prevalent in broilers (56). Although rarer in poultry than other important foodborne pathogens (*Salmonella, Campylobacter*), the above discussions show that *Listeria* spp., and specifically *L. monocytogenes*, can be present both within the birds (ceca and feces), and the grow-out farm environment. These data highlight the potential for the live birds to be a vector for this pathogen to enter the processing environment, which is the side most commonly viewed as the greatest risk for *L. monocytogenes* contamination.

POTENTIAL FOR LISTERIC INFECTIONS IN POULTRY

Although poultry can be an asymptomatic carrier of *L. monocytogenes*, they can also develop, in rare cases, listeric infections (27, 62). Only a few sporadic clinical outbreaks have been described. An outbreak of listeriosis was reported in a backyard poultry flock in Washington State was attributed to serotype 4b the source of the infection (26). *L. monocytogenes* serovar 4b was also involved in an outbreak of listeriosis in a pheasant breeder farm of Jingzhou, Hubei Province, China (68). There is greater evidence for potential systemic *Listeria* infection in other avian species, such as turkeys. Huff et al. (69) demonstrated in young turkey poults inoculated with a high or low dose of *L. monocytogenes* Scott A in the air sac that the high dosed birds reached 100% mortalities in 2 weeks, and the *Listeria* challenge strain could be isolated from the liver, pericardium, brain, both knee joints, suggesting that *L. monocytogenes* Scott A could be invasive through the respiratory system of susceptible turkey poults. In a follow-up challenge study comparing oral or oculonasal routes, Huff et al. (70) demonstrated that the oculonasal route led to greater mortalities and lower body weights than orally challenged birds. Stress may be a factor as well. When Huff et al. (71) exposed 13-week-old male turkeys to an immunosuppressive treatment and stress associated with transport, they observed an increase *Listeria* colonization in older birds. There are no comparable studies conducted with commercial poultry but a recent study by Jarvis et al. (72) demonstrated that *L. monocytogenes* strains could infect HD11 chicken macrophage-like cells and that infection leads to an initial halt in growth of the HD11 cells for at least 11 h before the HD11 cells begin to lyse. The authors used this as evidence to suggest there could be sufficient time for *Listeria* infected macrophages to circulate in the blood and potentially infect other tissues in the chicken. Therefore, it would be interesting to compare cellular mechanisms of these *Listeria* infected HD11 cells with other avian species such as turkeys as well non-avian macrophages such as those from mice.

CONCLUSION

Listeria is considered one of the major bacterial foodborne pathogens but it is often not considered epidemiologically important in poultry production, although there is nothing about the poultry production and processing environments that preclude the survival and persistence of *Listeria* spp. While sporadic and very isolated cases of listeriosis have been attributed to poultry, *Listeria* spp., and specifically *L. monocytogenes* have been isolated from all stages of the poultry production and processing continuum. Grow-out farm studies show that live birds are an important potential vector for *Listeria* contamination of the processing environment. Different studies have described factors related to the survival of *Listeria* within processing facilities, but there is a paucity of evidence linking live production and processing environments. The following food

safety-related question must be asked: Does *Listeria* contamination of poultry meat come from poultry or its environment? To address this question, there is a need to better understand the genetics of *Listeria* spp. and *L. monocytogenes* isolated from poultry environments as compared to other sources and listeriosis outbreaks attributed to poultry. If the epidemiological and genetic factors related to *Listeria* prevalence and pathogenicity can be elucidated, there is an opportunity to better assess the potential public health effects of *Listeria* from the poultry industry and develop management practices or treatments to mitigate these effects.

AUTHOR CONTRIBUTIONS

The authors declare that there is no conflict of interest and contribution was equally distributed among authors.

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