FOODBORNE ENTEROBACTERIACEAE OF ANIMAL ORIGIN: EPIDEMIC CHARACTERISTICS OF DRUG RESISTANCE, PATHOGENIC MECHANISMS, AND NOVEL CONTROL MEASURES

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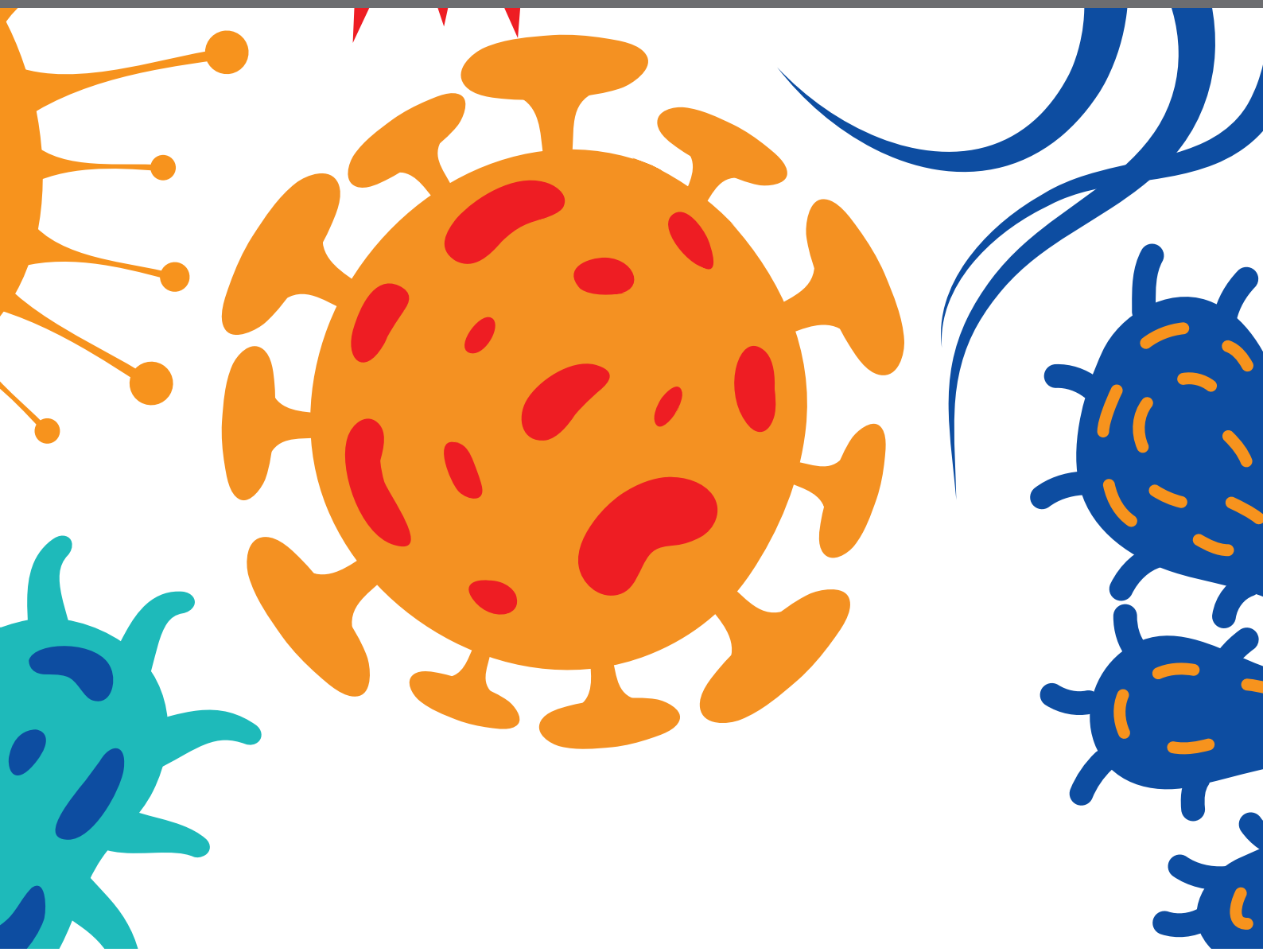

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FOODBORNE ENTEROBACTERIACEAE OF ANIMAL ORIGIN: EPIDEMIC CHARACTERISTICS OF DRUG RESISTANCE, PATHOGENIC MECHANISMS, AND NOVEL CONTROL MEASURES

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EDITORIAL
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Editorial: Foodborne Enterobacteriaceae of Animal Origin

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Keywords: foodborne Enterobacter bacteria, animal origin, epidemic characteristics, drug resistance, pathogenic mechanism, control measure

Editorial on the Research Topic

Foodborne Enterobacteriaceae of Animal Origin: Epidemic Characteristics of Drug Resistance, Pathogenic Mechanisms, and Novel Control Measures

INTRODUCTION

The main pathogens responsible for livestock and poultry products causing foodborne epidemics in people include *Salmonella*, *Campylobacter jejuni*, *Escherichia coli*, *Shigella*, *Proteus*, etc. These Enterobacteriaceae are important zoonotic foodborne bacteria capable of endangering human health. A high ongoing prevalence and occasional outbreaks of these bacteria cause global morbidity and mortality, leading to huge economic losses. The first objective of this Research Topic is to estimate the prevalence of resistance to common antimicrobial agents amongst Enterobacteriaceae isolated from livestock and poultry. The second objective is to advance food safety from farm to fork by understanding human health risks potentially posed by foodborne pathogens of animal origin, as well as to study pathogenic mechanisms and develop innovative strategies to minimize food safety risk. This topic contains 11 research and 2 review articles, covering a variety of topics, including epidemic characteristics of drug resistance, pathogenic mechanisms, and novel control measures.

EPIDEMIC CHARACTERISTICS OF DRUG RESISTANCE OF FOODBORNE ENTEROBACTERIACEAE

The global increase in antibiotic-resistant bacteria is a worldwide concern for human and animal health. In this topic, five research papers report epidemic characteristics of drug resistance in foodborne Enterobacteriaceae. Song et al. and Xu et al. investigated epidemiological characteristics of salmonellosis in breeder chickens in 10 Chinese provinces; among the *Salmonella* prevalent on poultry breeder farms in various provinces, *S. pullorum* and *S. enteritidis* continue to be major etiological agents. Most *Salmonella* strains isolated were multidrug-resistant (MDR), presenting a serious problem for animal and human. Therefore, it is necessary to monitor, control, and rationalize antimicrobial use on chicken farms to limit resistance against antimicrobial agents. These findings provide current information regarding the prevalence of antibiotic-resistant *Salmonella* on breeding poultry farms and inform the long-term goal of eradicating salmonellosis

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in China. Cui et al. focused on epidemiological characteristics of *Salmonella* in chicken flocks fed antibiotic-free diets. Herein, they investigated and compared *Salmonella* infections in three Chinese native breeders fed antibiotic-free diets and one conventional breeder, the Bairi chicken, in 2019. Antibiotic resistance rates and MDR rates in three chicken breeds fed antibiotic-free diets were significantly lower than that from a conventional Bairi chicken farm. In the article by Zhao et al., characteristics of Salmonella infections in the context of chicken mortality at hatching in Shandong were explored. *Salmonella* isolates with four sequence types (STs) were recovered from 6.7% of these embryos, with ST26 being the most prevalent; they inferred that *Salmonella* infections may be an important cause of chicken embryo mortality. In the article by Su et al., antibiotic resistance, molecular profiles, and intrinsic relationships of Shiga toxin-producing *E. coli* (STEC) O157:H7 isolates from cattle farms and abattoirs in Xinjiang were investigated. The bla_{CTX-M} gene encoding extended spectrum β-lactamases (ESBL) was first detected in STEC O157:H7 from cattle in Xinjiang. Furthermore, this gene was transferable under experimental conditions, highlighting potential dissemination of β-lactam resistance from cattle.

PATHOGENIC MECHANISMS OF FOODBORNE ENTEROBACTERIACEAE

In this topic, two research papers and one review focused on STEC. Shiga toxin (Stx) is the main virulence factor of STEC, with Stx2 being most frequently linked to severe illness. Ruminants, especially cattle, are the main reservoir of STEC. Pan et al. identified the correlation between *stx2* expression and expression of bovine host immune genes, i.e., *stx2* expression may be associated with expression of genes involved in B-cell proliferation and lymphotoxin beta production. Furthermore, they used artificial intelligence-based approaches to identify potential gene markers for *stx2* expression in cattle, shedding light on utility of host immune genes for predicting STEC colonization. In the article by Wang et al., microRNAomes of cattle intestinal tissues revealed possible miRNA-regulated mechanisms involved in *E. coli* O157 fecal shedding. The identified miRNAs and their functions increase understanding of molecular mechanisms regulating colonization of this foodborne pathogen *in-vivo*, which is vital to reduce cattle that are STEC O157 supershedders. Hwang et al. also provided a comprehensive review on STEC infection strategies, including mode of transmission of STEC and effects of Stx in humans and animals, STEC attachment and pathogenicity in the intestinal environment, and correlations between antimicrobial resistance (AR) and increased toxin gene expression in STEC.

Two papers focused on drug resistance mechanisms of foodborne Enterobacteriaceae. In the article by Luo et al., common ESBL encoding genes bla_{NDM} were profiled from chromosomal mobile genetic elements (MGE) of *Providencia rettgeri*, *P. mirabilis*, and *Klebsiella pneumoniae*. This study dealt with an extensive sequence comparison of 12 chromosomal genetic elements, including known integrative and conjugative elements (ICE), integrative and mobilizable elements (IME), bla_{NDM}-carrying transposons and newly sequenced bla_{NDM}-carrying ones. They determined that these 12 genetic elements encoded >51 resistance genes involved in resistance to 18 categories of antibiotics and heavy metals. In addition, 8 novel bla_{NDM}-carrying MGE were identified. This study provided deeper genetic insights into chromosomal integration of bla_{NDM}-carrying genetic elements in Enterobacteriaceae. Tran et al. identified MDR *E. coli* and *Salmonella* from broiler chicken production in Alberta, Canada, providing insights into potential horizontal gene transfer (HGT) events of AR genes between *E. coli* and *Salmonella* in broiler chickens. Moreover, certain MDR *E. coli* and *Salmonella* exhibited ESBL phenotypes encoded by transferable plasmids, suggesting HGT-associated MDR dissemination may readily occur in broiler chicken production.

Enteropathogenic *E. coli* (EPEC) continue to be the leading bacterial cause of infant diarrhea worldwide. Ledwaba et al. optimized a murine infection model that uses depletion of intestinal microbiota by antibiotics in weaned mice, enabling enhanced bacteria colonization; this will facilitate exploration of mechanisms involved in EPEC pathogenesis and development of vaccines or interventions.

The Mycotoxin Deoxynivalenol (DON) is a major health concern in poultry production, as it targets epithelial cells of the gastrointestinal tract and contributes to a loss of epithelial barrier function. Here, Ruhnau et al. reported that DON promoted *C. jejuni* multiplication in the intestine of broiler chickens, with consequences for bacterial translocation and gut integrity. Co-exposure of broilers to DON and *C. jejuni* can potentiate gut permeability, promoting *Campylobacter* colonization and its translocation across the intestinal epithelium.

NOVEL CONTROL MEASURES FOR FOODBORNE ENTEROBACTERIACEAE BACTERIAL DISEASES

To reduce both prophylactic and sub-therapeutic use of antibiotics, many studies have focused on alternatives with similar antibacterial and animal growth-promoting effects without depressing beneficial microbiota and minimizing development of antimicrobial resistance in animals. These alternatives include antimicrobial peptides (AMPs), probiotics, phytogenic compounds, etc. Protective effects of natural AMPs in food animals (i.e., swine, cattle and poultry), factors limiting the efficacy of these AMPs, and mitigating strategies were comprehensively reviewed by Fasina et al. It was concluded that AMP may partially replace conventional antibiotics in food animal production, thereby improving quality and microbiological safety of animal meat and egg products for human consumption.

CONCLUSIONS

The articles in this Research Topic enriched knowledge on epidemic characteristics of drug resistance and their genetic determinants, pathogenic mechanisms, and novel control measures of foodborne Enterobacteriaceae. This group contains a wide range of Gram-negative bacteria that commonly originate from food animals and are capable of entering the food supply chain, causing food poisoning and even life-threating illness. The complexity and degree of resistance possessed by these foodborne Enterobacteriaceae to clinically important antibiotics is vast and emerging. Therefore, there is an urgent need to address the AMR crisis from food animal origin by employing a "One Health" concept. There is a need for a broad range of research, including: monitoring management practices that promote AMR transfer at the animal, environment and human interface; understanding molecular mechanisms underlying persistence and transmission of foodborne pathogens in the animal food supply chain using multi-omics approaches; and mechanistic insights into their pathogenicity and AMR characteristics. As a final comment, the editors of this topic expresses their deep appreciation to all authors and reviewers for their great contributions to the collection.

AUTHOR CONTRIBUTIONS

FW, WZ, and DN edited the Research Topic of Foodborne Enterobacteriaceae of Animal Origin, wrote the manuscript, and approved it for publication. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Occurrence and Characterization of *Salmonella* Isolated From Chicken Breeder Flocks in Nine Chinese Provinces

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We investigated the prevalence of salmonellosis on 17 poultry breeding farms in nine Chinese provinces (Shandong, Jiangsu, Anhui, Zhejiang, Fujian, Guangdong, Yunnan, Sichuan, and Chongqing). Altogether, 3,508 samples from poultry breeding farms were collected in 2019, including 1,400 from cloaca swabs, 210 from feed, 1,688 from chicken embryos, and 210 from water. All the samples were subjected to bacterial isolation and culture, and bacterial species were identified by polymerase chain reaction. Serotyping, multilocus sequence typing (MLST), and drug-resistance phenotyping were performed on the isolates identified as *Salmonella*. Altogether, 126 *Salmonella* strains were detected in the 3,508 samples and the positivity rate for the samples was 3.59%. Among all the strains, 95 Salmonella isolates were selected for antimicrobial susceptibility test, resistance gene detection, serotyping, and genotyping. *S. gallinarum-pullorum* (57/95, 60.00%), *S. enteritidis* (22/95, 23.16%), and *S. agona* (16/95, 16.84%) serotypes were identified. The MLST classification showed that the 95 *Salmonella* strains fell into the following five sequence types (STs): ST92 (37/95, 38.95%), ST11 (22/95, 23.16%), ST2151 (19/95, 20.00%), ST13 (16/95, 16.84%), and ST470 (1/95, 1.05%). Apart from ST13, the other four STs shared close genetic relationships, and the genetic direction was ST11-ST470-ST92-ST2151. The resistance rates in the 95 isolates were 100% (95/95) for erythromycin, 68.42% (65/95) for tetracycline, and 53.68% (51/95) for streptomycin and ampicillin, respectively. The isolates were sensitive to polymyxin and sulfamethoxazole. Multi-drug resistance was seen in 70.53% (67/95) of the isolates. β-lactam-, aminoglycoside- and sulfonamide-encoding resistance genes were detected by PCR. The detection rate for bla_{TEM} and *sul3* was 100% (95/95), whereas *sul2* and *aaC4* had rates of 52.63 and 23.16%, respectively. These results indicate that some of the salmonellosis seen in Chinese breeding chicken farms may be caused by infection with *S. gallinarum-pullorum*, *S. enteritidis*, and *S. agona*. They also show that some *Salmonella* isolates have multi-drug resistance phenotypes and carry multi-drug resistance genes.

Keywords: *Salmonella*, multidrug resistance, MLST, phenotypes, chickens, breeder farms

INTRODUCTION

Salmonella is a food-borne disease-causing zoonotic pathogen (1), and its occurrence has frequently been reported in recent years (2, 3). More than 2,600 *Salmonella* serotypes have been identified and recorded (4). According to the World Health Organization (WHO), with nearly one-tenth of the world's population becoming infected each year and around 33 million deaths (http://www.who.int/mediacentre/factsheets/fs139/en/). Most cases occur in older adults and in immunocompromised people (5, 6), and it is estimated that in 2017 *Salmonella enterocolitis* was responsible for 95.1 million cases and killed 50,771 people (7–9). In addition to diarrhea, 535,000 cases of non-typhoidal *Salmonella* invasive disease have occurred, and an estimated 77,500 people have died (10). At present, antibiotic therapy remains the main prevention and treatment method for salmonellosis. However, the long-term and unreasonable use of antibiotics has caused *Salmonella* to develop widespread and strong resistance to these agents (11). Drug resistant bacteria can also spread to humans, leading to public health problems (12).

Various molecular typing techniques have been widely used in the field of microbiology to track the origins of pathogenic bacteria (13), the most widely of which used are multilocus sequence typing (MLST) and pulsed field gel electrophoresis (PFGE). MLST is fast and convenient, producing reliable high resolution data, and easy real-time internet sharing (14). PFGE is recognized by laboratories around the world for its high resolution and repeatability. But PFGE does not have a strict and uniform international naming standard, making it difficult to achieve data sharing with it. In recent years, a large number of epidemiological surveys on Salmonella have emerged in China. However, researchers were more inclined to conduct their epidemiological *Salmonella* surveys on commercial broiler sales chains where sick birds appeared than on healthier chains (15–17). Therefore, data from epidemiological surveys on *Salmonella* in domestic poultry farms, especially chicken breeder flocks, are incomplete.

Here, we collected 3,508 samples from 17 poultry breeding farms in nine Chinese provinces in 2019. We investigated the drug-resistance genes, drug-resistance phenotypes, and genetic relationships among the *Salmonella* isolates we collected. We comprehensively and systematically studied the epidemiological characteristics of *Salmonella*, with the aim of improving the molecular typing network database in China and providing relevant data with which to support the prevention and control of *Salmonella* in China.

MATERIALS AND METHODS

Sample Collection

From May to July 2019, 3,208 samples were collected from 14 poultry breeding farms in Jiangsu, Anhui, Zhejiang, Fujian, Guangdong, Yunnan, Chongqing, and Sichuan Province, China. They included 1,400 cloacal swab samples, 210 animal feed samples, 1,388 embryo samples and 210 water samples. An additional 300 chicken embryo samples were obtained from three chicken breeding farms in Shandong Province, China (**Figure 1A**).

Isolating and Serotyping *Salmonella*

Buffered peptone water (4.5 mL) (BPW, Haibo Biotechnology, Qingdao, China) was added to each sample (0.5 g) for pre-enrichment, according to a previously described method (18). After incubation at 37°C for 8–12 h, 0.5 mL of each pre-enriched culture was incubated in 4.5 ml of Tetrathionate Broth Buffer (TTB, Haibo Biotechnology) at 37°C for 24 h. After selective enrichment, one loopful of each broth culture was streaked onto xylose lysine tergitol 4 (XLT4, Haibo Biotechnology) agar and the plates were incubated at 37°C for 24–36 h. The presumptive *Salmonella* colonies were identified by polymerase chain reaction (PCR) assays using *invA* primers (19). The *invA* gene has a conserved sequence in *Salmonella* species and can therefore be used to detect and validate *Salmonella* strains (20). Here, *invA* primers (19) (F: 5′-ACAGTGCTCGTTTACGACCTGAAT-3′, R: 5′-AGACGACTGGTACTGATCGATAAT-3′) were used for PCR. The PCR cycling conditions were as follows: 1 denaturation cycle at 95°C for 5 min, 30 cycles of denaturation at 95°C for 30 s, followed by annealing at 56°C for 30 s and elongation at 72°C for 1 min, and a final 10 min elongation cycle at 72°C. Positive colonies were subsequently confirmed using a microbial mass spectrometer (IVD MALDI Biotyper, Bruker Bremen, Germany).

Salmonella Serotyping

After being resuscitated on XLT4 medium, all the *Salmonella* isolates were serotyped according to the Kauffmann-White scheme by slide agglutination with O and H antigens (Tianrun Bio-Pharmaceutical, Ningbo, China). Normal saline was used as a negative control, and plate agglutination reactions were considered *Salmonella* positive. The *Salmonella* strains were PCR-identified using DNA as the template and *Ipaj* (F: 5′-TACCTGTCTGCTGCCGTGA-3′, R: 5′-ACCCTGCAAACCTGAAATC-3′) (21) as primers for detecting *S. gallinarum-pullorum*.

Antimicrobial Susceptibility Testing

The Kirby-Bauer disk diffusion method, as described by the Clinical and Laboratory Standards Institute (CLSI), was used to test the susceptibility of the *Salmonella* isolates to 14 commonly used antibiotics, including polymyxin B (PB, 300 IU), sulfamethoxazole (SXT, 25 μg), florfenicol (30 μg), ofloxacin (5 μg), cefoxitin (30 μg), doxycycline (30 μg), amoxicillin (20 μg), tetracycline (TE, 30 μg), streptomycin (STR, 10 μg), gentamycin (10 μg), erythromycin (EM, 15 μg), ceftazidime (CAZ, 30 μg), ampicillin (AM, 10 μg), and gatifloxacin (5 μg). The results were interpreted according to the standard guidelines from the CLSI (22). *Salmonella* isolates that were resistant to more than three antimicrobial classes were defined as multidrug-resistant (MDR) isolates.

Detecting Drug-Resistance Genes

Bacterial DNA was extracted from the samples using a Bacterial Genome Kit (Bioteke, Beijing, China), according to the manufacturer's instructions. The β-lactam genes (bla_{TEM},

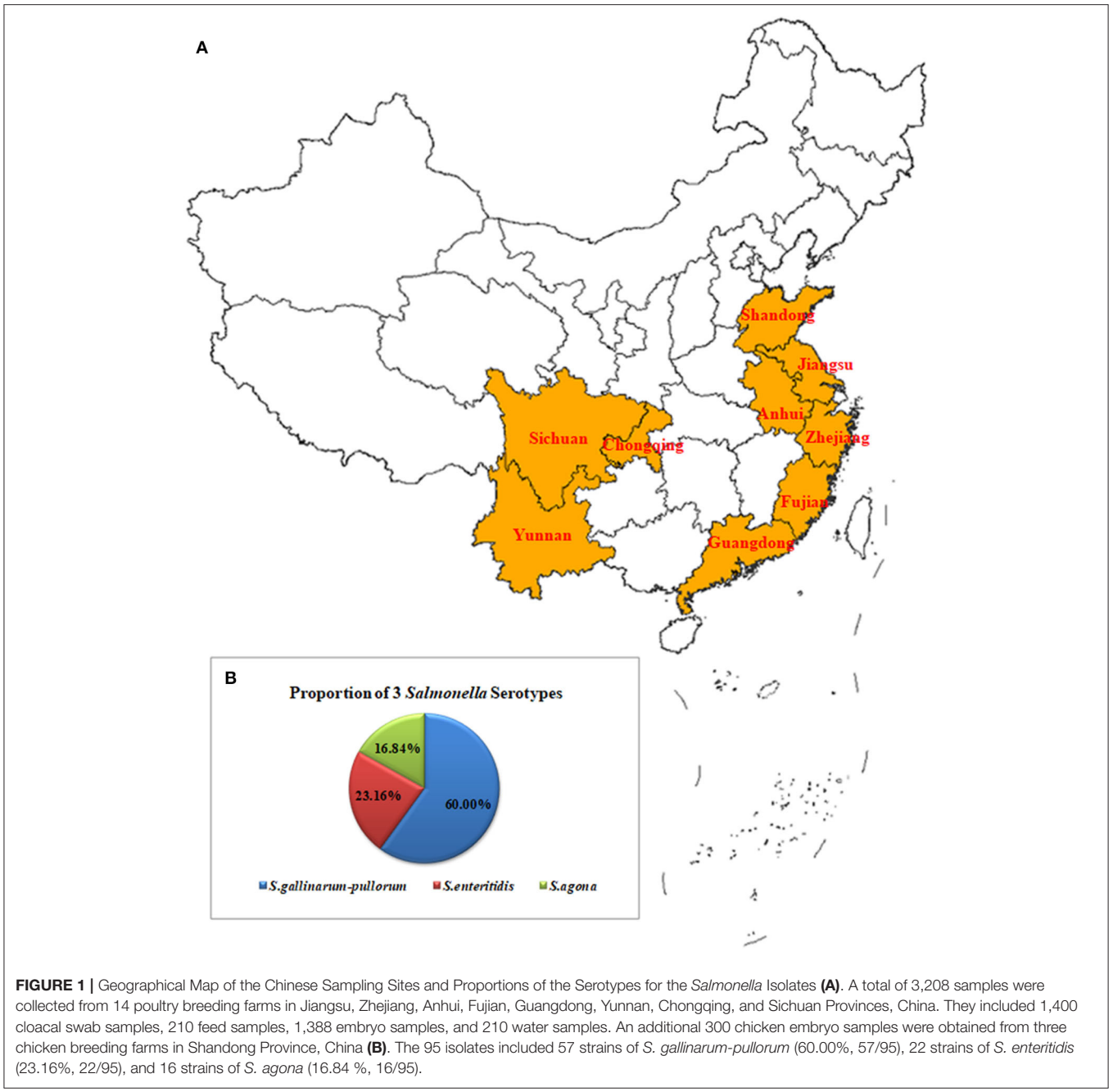

FIGURE 1 | Geographical Map of the Chinese Sampling Sites and Proportions of the Serotypes for the *Salmonella* Isolates **(A)**. A total of 3,208 samples were collected from 14 poultry breeding farms in Jiangsu, Zhejiang, Anhui, Fujian, Guangdong, Yunnan, Chongqing, and Sichuan Provinces, China. They included 1,400 cloacal swab samples, 210 feed samples, 1,388 embryo samples, and 210 water samples. An additional 300 chicken embryo samples were obtained from three chicken breeding farms in Shandong Province, China **(B)**. The 95 isolates included 57 strains of *S. gallinarum-pullorum* (60.00%, 57/95), 22 strains of *S. enteritidis* (23.16%, 22/95), and 16 strains of *S. agona* (16.84 %, 16/95).

bla_{SHV}, bla_{PSE}, bla_{CTX-M}, and bla_{OXA}) and other genes associated with resistance to aminoglycosides (*aaC1, aaC3, and aaC4*), quinolones (*qnrB, qnrC, qnrD, qnrS,* and *aac*(6′)*-Ib-cr*), tetracyclines (*tetA*, *tetB*), and sulfonamides (*sul1*, *sul2*, and *sul3*) were PCR-detected. The relevant primers are listed in **Table 1**.

Detecting Class I Integrons

Class I integron-targeting primers were designed based on previously described sequences (28). After DNA gel extraction kit (Vazyme Biotech, Nanjing, China) purification, the class I integron-positive DNA samples were sequenced (Sangon Biotech, Shanghai, China). The DNA sequences obtained were compared with those in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) using the Basic Local Alignment Search Tool to determine the gene cassettes within the variable region of the class I integrons.

MLST

Seven housekeeping genes (*aroC, dnaN, hemD, hisD, purE, sucA,* and *thrA*) were used to characterize the *Salmonella* isolates using MLST (http://mlst.warwick.ac.uk/mlst/). The protocols from the MLST homepage were used, including the PCR conditions

TABLE 1 | Primers used to detect antimicrobial-resistance genes.

Resistance gene category	Resistance gene	Primer sequence	References
β-lactams	bla_{TEM}	F: 5′- ATAAAATTCTTGAAGACGAAA – 3′	(23)
		R: 5′- GACAGTTACCAATGCTTAATC – 3′	
	bla_{SHV}	F: 5′- TTATCTCCCTGTTAGCCACC – 3′	(23)
		R: 5′- GATTTGCTGATTTCGCTCGG – 3′	
	bla_{PSE}	F: 5′- TAGGTGTTTCCGTTCTTG-3′	(24)
		R: 5′- TCATTTCGCTCTTCCATT-3′	
	bla_{CTX-M}	F: 5′- CGCTTTGCGATGTGCAG-3′	(23)
		R: 5′- ACCGCGATATCGTTGGT-3′	
	bla_{OXA}	F: 5′- TCAACTTTCAAGATCGCA-3′	(23)
		R: 5′- GTGTGTTTAGAATGGTGA-3′	
Quinolone	*qnrB*	F: 5′- GATCGTGAAAGCCAGAAAGG-3′	(23)
		R: 5′- ACGATGCCTGGTAGTTGTCC-3′	
	qnrC	F: 5′- GGTTGTACATTTATTGAATC-3′	(23)
		R: 5′- TCCACTTTACGAGGTTCT –3′	
	qnrD	F: 5′- AGATCAATTTACGGGGAATA-3′	(23)
		R: 5′- AACAAGCTGAAGCGCCTG – 3′	
	qnrS	F: 5′- ACGACATTCGTCAACTGCAA-3′	(23)
		R: 5′- TAAATTGGCACCCTGTAGGC-3′	
	aac(6′)-Ib-cr	F: 5′- TTGCGATGCTCTATGAGTGGCTA – 3′	(23)
		R: 5′- CTCGAATGCCTGGCGTGTTT – 3′	
Aminoglycosides	*aaC1*	F: ACCTACTCCCAACATCAGCC-3′	(25)
		R: ATATAGATCTCACTACGCGC-3′	
	aaC3	F: CACAAGAACGTGGTCCGCTA-3′	(25)
		R: AACAGGTAAGCATCCGCATC-3′	
	aaC4	F: CTTCAGGATGGCAAGTTGGT-3′	(25)
		R: TCATCTCGTTCTCCGCTCAT-3′	
Tetracycline	*tetA*	F: 5′- GCGCCTTTCCTTTGGGTTCT-3′	(25)
		R: 5′- CCACCCGTTCCACGTTGTTA-3′	
	tetB	F: 5′- CATTAATAGGCGCATCGCTG-3′	(25)
		R: 5′- TGAAGGTCATCGATAGCAGG-3′	
Sulfonamides	*sul1*	F: 5′- CTTCGATGAGAGCCGGCGGC-3′	(26)
		R: 5′- GCAAGGCGGAAACCCGCGCC-3′	
	sul2	F: 5′- GCGCTCAAGGCAGATGGCATT-3′	(26)
		R: 5′- GCGTTTGATACCGGCACCCGT-3′	
	sul3	F: 5′- AGATGTGATTGATTTGGGAGC-3′	(27)
		R: 5′- TAGTTGTTTCTGGATTAGAGCCT-3′	

and primer sequence information (http://mlst.warwick.ac.uk/mlst/dbs/Senterica). PCR products were purified and sequenced (Sangon Biotech). The nucleic acid sequences corresponding to the allele values of the seven pairs of *Salmonella* housekeeping genes were downloaded from Pubmlst (https://pubmlst.org/), and BioEdit v7.0.9 software was used to construct a local gene bank for them. Genes from the gene library were aligned to obtain the allele values of the isolated strains and their corresponding ST types. The sequence types were assigned according to the MLST online scheme (http://mlst.warwick.ac.uk/mlst/dbs/Senterica), and the results of the eBURST map were constructed by Phyloviz (https://online.phyloviz.net/index).

RESULTS

Isolation and *Salmonella* Serotyping

As described in the methods section, samples of the diseased materials were subjected to two-step enrichment using BPW and TTB, and a single colony was obtained by a three-line method on XLT4 medium. Colorless, translucent small colonies were observed on the plates, and some had smooth black cores and very narrow transparent bands around them. DNA was extracted from the isolated bacterial colonies, and PCR was performed with *invA* primers for *Salmonella* detection. After the PCR detection and the microbiological mass spectrometer verification, 126 *Salmonella strains were isolated, with the detection rate of* 3.59% (126/3508; **Table 2**). The isolating rates among different sample source were shown as: 6.27% (104/1688) in chicken embryo samples, 2.86% (6/210) in water samples, 1.14% (16/1400) in cloaca swabs, 0% (0/210) in feed samples. *Salmonella* was not detected in samples from Jiangsu and Sichuan provience. Among all the isolates, 54 sratins were recovered from samples of Shandong province, with the highest isolation rate of 18% (54/300), while a total of 72 *Salmonella* isolates were isolated from the other 6 provinces. In this study, a total of 95 strains, consisted of 23 randomly selected Shandong isolates and the aforementioned 72 isolates, were collected for subsequent tests.

TABLE 2 | Isolation rate and distribution of chicken *Salmonella* isolates.

Sampling area	Source				Isolation rate
	Feed	Water	Cloaca swabs	Embryo[a]	
Jiangsu	0 (0/30)[b]	0 (0/30)	0 (0/200)	0 (0/200)	0% (0/460)
Zhejiang	0 (0/30)	6.67% (2/30)	7.00% (14/200)	4.00% (8/200)	5.22% (24/460)
Anhui	0 (0/30)	10% (3/30)	0.50% (1/200)	2.00% (4/200)	1.74% (8/460)
Fujian	0 (0/30)	0 (0/30)	0 (0/200)	13.00% (26/200)	5.65% (26/460)
Guangdong	0 (0/30)	0 (0/30)	0.50% (1/200)	4.00% (8/200)	1.96% (9/460)
Yunnan	0 (0/30)	3.33% (1/30)	0 (0/200)	1.00% (2/200)	0.65% (3/460)
Chongqing	0 (0/15)	0 (0/15)	0 (0/100)	2.00% (2/100)	0.87% (2/230)
Sichuan	0 (0/15)	0 (0/15)	0 (0/100)	0 (0/88)	0% (0/218)
Shandong	-	-	-	18.00% (54/300)	18.00% (54/300)[c]
Total	0 (0/210)	2.86% (6/210)	1.14% (16/1400)	6.27% (104/1688)	3.59% (126/3508)

[a] *All chicken embryos were dead embryos collected randomly from the hatchery that fail to hatch.*
[b] *Numbers in parentheses are positive/total.*
[c] *We randomly selected 23 strains from 54 strains for in-depth study.*

Salmonella Serotyping Results

The Ningbo Tianrun Serum Diagnostic Kit test results showed that the O antigens of the 95 *Salmonella* strains were O9, O12 (79/95) and O4, O12 (16/95). The results from the semi-solid puncture tests showed that 38/95 isolates had flagella and 57 had no flagella. The serum diagnostic kit results showed that the H antigens from the 38 isolates were Hg, m (22/38) and Hs (16/38). The kit's results when combined with the PCR results showed

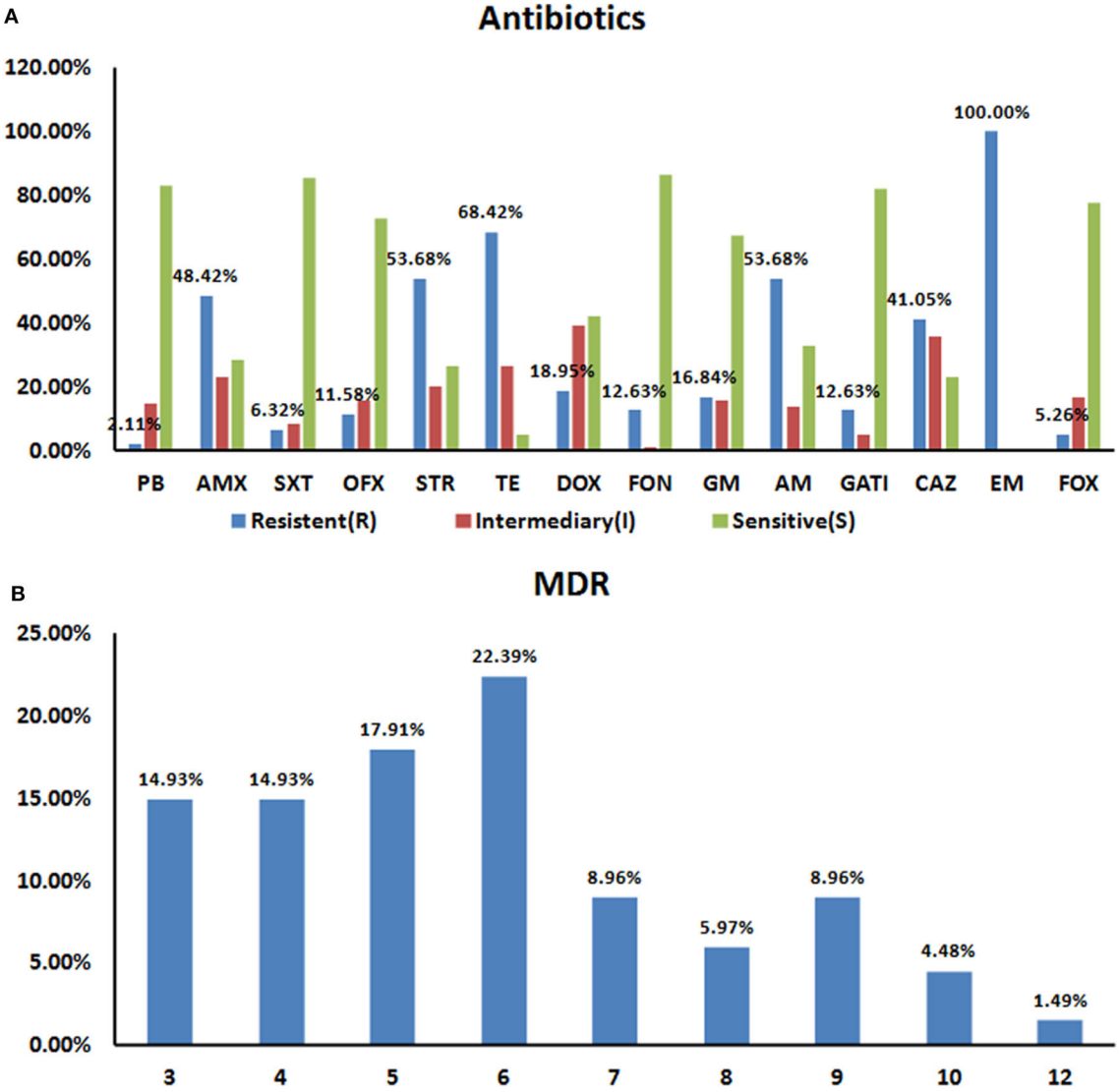

FIGURE 2 | Resistance Characteristics of the 95 *Salmonella* Strains to 14 Antibiotics **(A)**. Rates of antibiotic resistance amongst *Salmonella* isolates. The drug resistance rates for all 95 isolates were 100% for EM (95/95), 68.42% (65/95) for TE, and 53.68% (51/95) for STR and AM, respectively. All 95 strains showed polymyxin and sulfamethoxazole sensitivity **(B)**. Prevalence of multidrug resistance amongst 95 *Salmonella* isolates. 70.53% (67/95) of the tested *Salmonella* isolates were resistant to at least three antibiotics. About 36.8% (35/95) strains resistant to 6–12 drugs.

that the 95 isolates included 57 *S. gallinarum-pullorum* strains (60.00%, 57/95), 22 *S. enteritidis* strains (23.16%, 22/95), and 16 *S. agona* strains (16.84 %, 16/95) (**Figure 1B**).

Antibiotic Resistance and MDR Profiles

The KB method was used to count the diameters of the inhibition zones and to determine growth inhibition to 14 common antibacterial drugs in the 95 *Salmonella* strains according to the 2018 edition of the CLSI (22) standards. The resistance rates in the 95 isolates were 100% (95/95) for erythromycin, 68.42% (65/95) for tetracycline, and 53.68% (51/95) for STR and AM, respectively. The AM resistance rate for the 95 isolates was 53.68% (51/95), and they were all sensitive to PB and SXT (**Figure 2A**).

We next statistically analyzed the drug resistance spectrum and multiple drug resistance in the isolated bacteria (**Figure 2B**). The MDR statistics showed that 70.53% (67/95) of the strains were resistant to three or more drug types, indicating that drug resistance in the isolated bacteria is prevalent with 38 different resistance patterns identified (**Table 3**). The drug-resistance spectrum was broad, and the most common pattern in seven of the isolates involved AMX-STR-TE-AM-CAZ-EM. The serotypes and resistance patterns were not significantly correlated. According to the resistance profile heat map (**Figure 3**), drug-resistant *Salmonella* isolated from the three

TABLE 3 | Antimicrobial resistance patterns of the 95 *Salmonella* isolates.

No. of drugs	Antimicrobial resistance patterns	Isolate no.
12	AMX-SXT-OFX-STR-TE-DOX-FON-GM-AM-GATI-CAZ-EM	1
10	AMX-OFX-STR-TE-FON-GM-AM-GATI-CAZ-EM	2
	AMX-STR-TE-FON-GM-AM-GATI-CAZ-EM-FOX	1
9	AMX-STR-TE-FON-GM-AM-GATI-CAZ-EM	5
	AMX-OFX-STR-FON-GM-AM-GATI-CAZ-EM	1
8	PB-AMX-STR-TE-DOX-AM-CAZ-EM	2
	AMX-SXT-STR-TE-FON-AM-CAZ-EM	1
	AMX-OFX-STR-TE-AM-CAZ-EM-FOX	1
7	SXT-STR-TE-DOX-GM-AM-EM	1
	AMX-OFX-STR-TE-AM-CAZ-EM	4
	AMX-SXT-OEX-STR-TE-DOX-EM	1
6	AMX-STR-AM-CAZ-EM-FOX	2
	AMX-STR-TE-AM-CAZ-EM	7
	AMX-TE-DOX-AM-CAZ-EM	1
	AMX-TE-GM-AM-CAZ-EM	1
	STR-TE-DOX-AM-GATI-EM	1
	TE-GM-AM-CAZ-EM-FOX	1
	AMX-OFX-TE-DOX-AM-EM	1
	STR-TE-FON-AM-GATI-EM	1
5	STR-TE–GM-CAZ-EM	2
	SXT-STR-TE-DOX-EM	1
	STR-TE-DOX-CAZ-EM	1
	AMX-STR-TE-AM-EM	5
	AMX-TE-AM-CAZ-EM	1
	AMX-STR-TE-DOX-EM	2
4	STR-TE-AM-EM	1
	AMX-STR-TE-EM	1
	TE-DOX-AM-EM	1
	TE-AM-CAZ-EM	1
	AMX-TE-DOX-EM	1
	AMX-TE-AM-EM	1
	AMX-STR-AM-EM	3
	STR-AM-CAZ-EM	1
3	TE-DOX-EM	5
	TE-CAZ-EM	1
	TE-AM-EM	2
	STR-TE-EM	1
	AMX-AM-EM	1

chicken farms in Shandong Province had the highest drug-resistance rates.

Prevalence of Antibiotic Resistance Genes

Using the DNA from each isolate as the template, PCR was used to detect the carriage status of the isolates to 18 drug resistance genes. Four different drug resistance genes were detected in DNA from the isolates, including those encoding β-lactams, aminoglycosides, and sulfonamides. The highest detection rates were seen for bla_{TEM} and *sul3* (100%, 95/95), followed by *sul2* and *aaC4* (52.63 and 23.16%, respectively) (**Figure 4**).

Detection of Class I Integrons

PCR testing detected no class I integrants in the DNA from the isolates; therefore none of the strains from this study carried class I integrants.

MLST

MLST classification showed that the 95 *Salmonella* strains fell into five ST types: ST92 (37/95, 38.95%), ST11 (22/95, 23.16%), ST2151 (19/95, 20.00%), ST13 (16/95, 16.84%), and ST470 (1/95, 1.05%) (**Figure 5A**). The results of the eBURST map showed that isolate ST11 was a cloned progenitor, isolates ST92 and ST2151 were subcloned groups from isolate ST11. Apart from ST13, the other four STs have close genetic relationships. The genetic direction was determined to be ST11-ST470-ST92-ST2151 (**Figure 5B**), with ST11 being the core sequence type.

DISCUSSION

An important transmission route for *Salmonella* is vertical transmission. Therefore, chicken breeder farms are required to actively prevent, control, and work toward eliminating *Salmonella* infections on them. Once breeder chickens become infected with *Salmonella*, a "magnification effect" can occur as the generations expand. China has listed Salmonellosis as a priority disease control species in the National Medium and Long-Term Animal Disease Prevention and Control Plan (2012–2020) (http://www.gov.cn/zwgk/2012-05/25/content_2145581.htm), which requires all chicken farms across the country to meet the standards required to eliminate chicken salmonellosis by 2020. Hence, investigating *salmonella* epidemics on breeding farms is particularly important.

To investigate the prevalence and characteristics of *Salmonella* on Chinese poultry breeding farms requires information about drug-resistance genes, and the phenotypic characteristics of the isolates collected from them and their relationships. In this study, from May to July 2019, 3,508 samples from breeding farms were collected, including 1,400 cloaca swabs, 210 feed samples, 1,688 chicken embryo samples, and 210 water samples. The samples came from 14 breeder farms in eight provinces (Jiangsu, Anhui, Zhejiang, Fujian, Guangdong, Yunnan, Chongqing, and Sichuan) and three breeder flocks in Shandong Province. We detected 126 *Salmonella* strains in the 3,508 samples, a positivity rate of 3.59%. The positive isolation rate was very low, which may reflect the fact that the efforts made to prevent and control animal diseases in China are transitioning from effective control to gradual purification and elimination. In this investigation, especially in several breeder flocks in Jiangsu and Sichuan, *Salmonella* elimination has been done well. In contrast, the three local breed chicken breeder flocks in Shandong were more seriously contaminated with *salmonella*. It indicated that more attention should be paid on *salmonella* purification in local chicken breeds.

From the 1990s onwards, pullorum disease has seriously threatened the poultry industry (29). *S. pullorum* infection is still threatening the poultry farming industry and related industries in developing countries including China (30), resulting

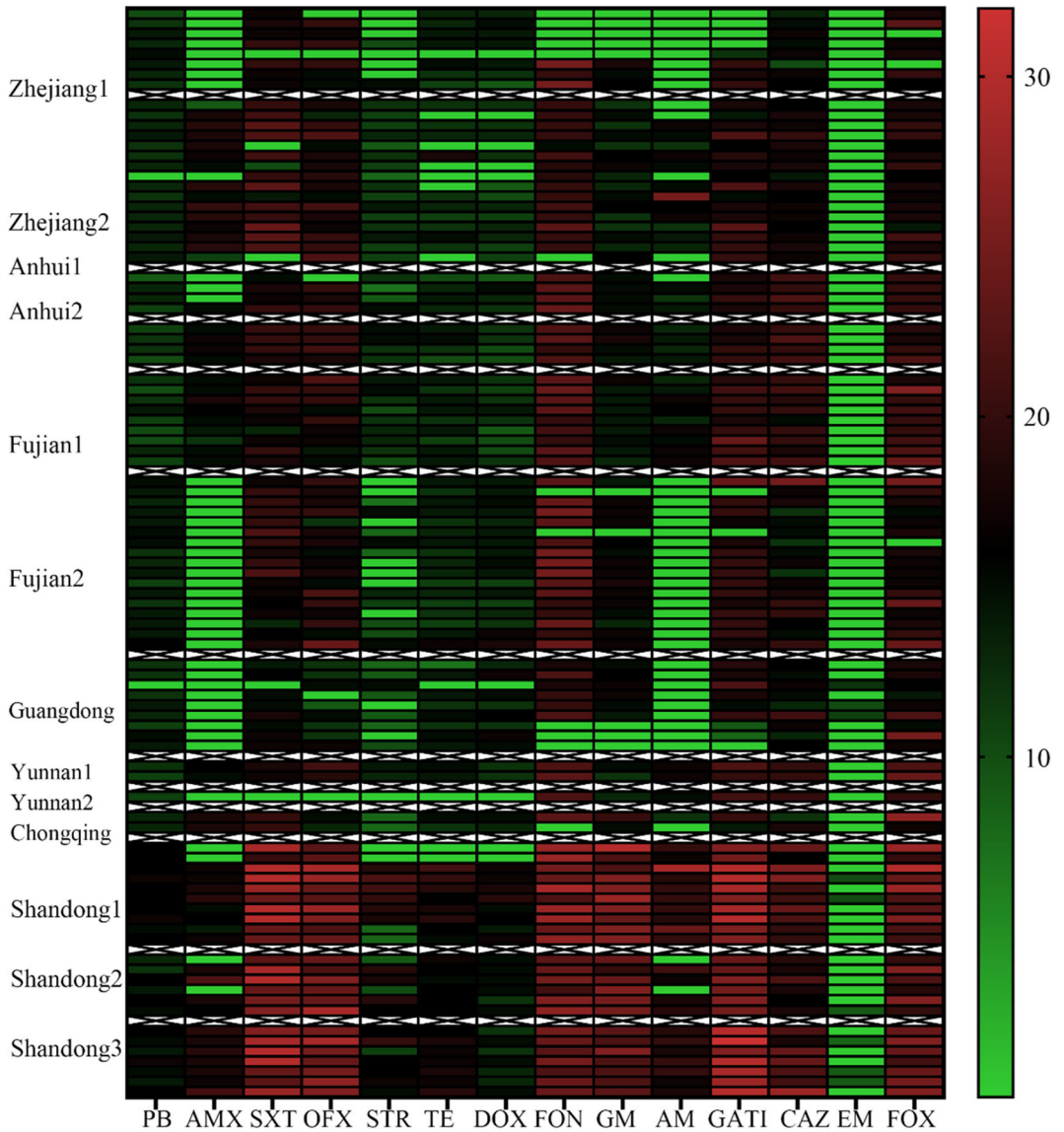

FIGURE 3 | Antibiotic Resistance Heat Map for the 95 *Salmonella* Strains. Heat map showing the antibiotic-resistance distribution for the 95 *Salmonella* strains isolated from samples from broiler chicken farms in nine provinces to 14 antibiotic types. The color scale of the individual cells represents the resistance rate, as a percentage. PB, polymyxin B; AMX, amoxicillin; SXT, sulfamethoxazole; OFX, ofloxacin; STR, streptomycin; TE, Tetracycline; DOX, doxycycline; FON, florfenicol; GM, Gentamycin; AM, ampicillin; GATI, Gatifloxacin; CAZ, Ceftazidime; EM, erythromycin; and FOX, cefoxitin.

in huge economic losses in these countries. Among the three serotypes identified in this study, *S. gallinarum-pullorum* (57/95, 60.00%) still dominates, with *S. enteritidis* (22/95, 23.16%) and *S. agona* (16/95, 16.84%) ranking second and third. In contrast, *S. enteritidis*, *S. Indiana*, and *S. typhimurium* are more common in the Chinese chicken retail industry.

After serotype identification, we used molecular typing to further analyze the genetic relationships among the isolates. Various molecular typing techniques are widely used in the microbiology field to track the origin of pathogenic bacteria (13), from which the most commonly used techniques are MLST and PFGE. The high resolution and repeatability of PFGE has been recognized by laboratories around the world, but PFGE lacks a strict and uniform international naming standard, so it is difficult to achieve data sharing with this technique. With its convenience, high resolution, reliable data, and easy real-time internet sharing, MLST is widely used (14). In this study, the MLST typing results showed that the 95 *Salmonella* strains we isolated fell into five ST types (ST92, ST11, ST2151, ST13, and ST47) (1/95, 1.05%). The following correlations between STs and *Salmonella* serovars were founded: ST13 with *S. agona*, ST11 with *S. Enteritidis*, and ST92, *ST2151*, and ST470 with *S. gallinarum-pullorum*. Among them, ST92 (37/95, 38.95%) was the most common, a finding consistent with the results of a

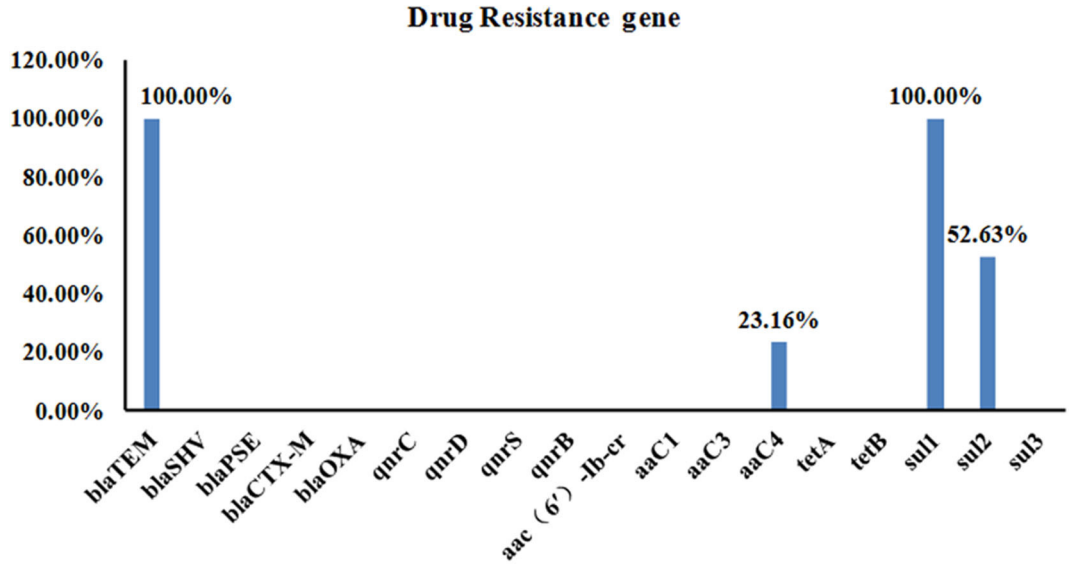

FIGURE 4 | Carriage of 18 Drug Resistance Genes in the 95 *Salmonella* Strains. A total of four different drug resistance genes were detected in the DNA from the isolates, including those encoding β-lactams, aminoglycosides, and sulfonamides. The highest detection rate was for bla_{TEM} and *sul3* (both were 100%, 95/95), followed by *sul2* and *aaC4* with detection rates of 52.63 and 23.16%, respectively.

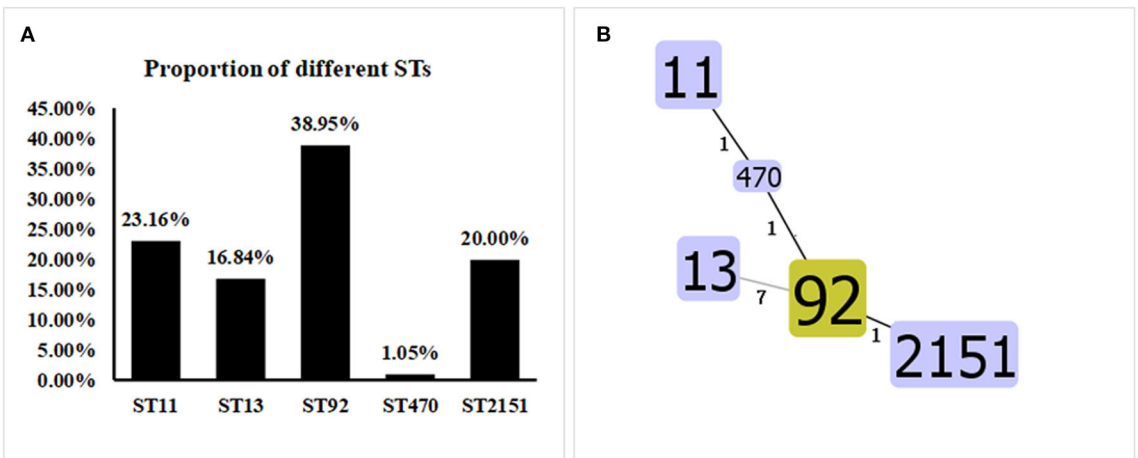

FIGURE 5 | Proportions and Allelic Map of the Five STs in the 95 *Salmonella* Isolates. **(A)** MLST classification showed that the 95 *Salmonella* strains fell into the following five STs: ST92 (37/95, 38.95%), ST11 (22/95, 23.16%), ST2151 (19/95, 20.00%), ST13 (16/95, 16.84%), and ST470 (1/95, 1.05%). **(B)** Apart from ST13, the other four STs shared close genetic relationships. The genetic direction was found to be ST11-ST470-ST92-ST2151, and ST11 is the core sequence type.

study on *S. enteritidis* in China from 2011 to 2016 (30), thereby confirming that the current *Salmonella* type still dominates. In addition to ST13, the *Salmonella* isolates displayed four other close ST kinships, with the genetic direction being ST11-ST470-ST92-ST2151.

In this study, β-lactams, quinolones, aminoglycosides, tetracyclines, and sulfonamides were tested on the 95 isolates. Among them, β-lactam-, aminoglycoside-, and sulfonamide-resistance genes were detected by PCR. Four resistance genes were detected, among which the detection rate for the β-lactam bla_{TEM}resistance gene and the *sul3* sulfonamide resistance gene was 100% (95/95) for both, whereas those of *sul2* and *aaC4* were 52.63 and 23.16%, respectively. Quinolone resistance genes and tetracycline resistance genes were not detected. This result is consistent with the results of a study (31) on sulfonamide-resistance genes in *Pasteurella multocida*, where the detection rate for *sul2* (and *sul1*) exceeded 97%. Furthermore, Zhu et al. (32) identified sulfonamide-resistance genes (*sul1*, *sul2*, and *sul3*) in 89 (97.8%) Salmonella isolates. These findings indicate that sulfonamide-resistance genes are relatively stable in the environment. Extended-spectrum β-lactamase (ESBL)-producing *Salmonella* is a significant

clinical and food safety concern worldwide (33). *Salmonella* isolates that harbor ESBL-encoding genes are able to hydrolyze penicillin as well as most of the first, second, and third generation cephalosporins, and even carbapenems (34, 35). In the present study, all the *Salmonella* isolates carried bla_{TEM}, a β-lactam resistance gene, highlighting their potential pathogenesis and the risk posed by this pathogen to Chinese poultry breeding farms.

The Chinese Veterinary Pharmacopeia report (30) pointed out that the drugs tested in the present study, including EM, TE, SXT, and AM, have been widely used in poultry production in China. The resistance rate of these 95 isolates to AM, STR, TE, and EM ranged from 53.68 to 100%. The *Salmonella* isolates from each province in this article were resistant to at least three common antibacterial drugs, and the proportion of MDR strains was 70.53% (67/95), which is consistent with the results from previous surveys (15, 36). This result is consistent with the findings of previous reports from Iran and the United States (37, 38). It is worth noting that should strains with the same MDR genotypes as those identified in the *Salmonella* isolates be transferred to humans via chickens and their derivatives and with different degrees of resistance to 14 antibacterial drugs as was shown for our isolates, this would constitute a major public health threat.

A recent study indicated that integrins and multiple drug resistance genes on mobile plasmids may be responsible for the stable spread of MDR genes in *Salmonella* (39). Previous studies on class I integrants in *Salmonella* in Hubei, Shandong, and Shanxi (18, 39, 40) provinces have found that class I integrants displaying the various antibiotic resistance gene cassettes that are commonly found in *Salmonella* isolates are associated with provinces, markets, and storage conditions differ. Multiple antibiotic resistance in isolates carrying class I integrons is significantly higher than that seen in isolates lacking these integrons, and they usually show a corresponding antibiotic resistance spectrum to the antibiotic resistance gene cassette contained in their class I integrants. In the present study, the detection rate for class I integrants was 0 (0/95), and the influence of gene mutations and other factors relating to drug resistance in *Salmonella* was not investigated. This may be related to the stricter control measures of breeding poultry farms compared to commercial chicken farms, bacterial infectious diseases are better controlled, and the application of antibiotics is greatly reduced. At the same time, it should be noted that poultry farming often uses antibiotics, which may explain the resistance patterns we observed with the tested antibiotics. Therefore, reducing antibiotic use is particularly important for limiting the emergence of super multidrug resistant organisms so that good health in humans and animals can be maintained.

CONCLUSIONS

The *Salmonella* detection rate for the 17 breeder farms in the Chinese provinces we surveyed was 3.59%. Five STs, ST92, ST11, ST2151, ST13, and ST470 were identified. Our results indicate that among the *Salmonella* prevalent in the poultry breeder farms in the various provinces, *S. gallinarum*, *S. enteritidis*, and *S. agona* still occupy the main positions. All of the *Salmonella* isolates carried bla_{TEM} and *sul3*, and 64.21% were MDR strains, suggesting that antibiotic use in the poultry breeder farms still requires attention. The correlation between the serotypes and the molecular subtypes of the *Salmonella* isolates suggests that the results of a comprehensive analysis of different methods may provide more useful epidemiological information related to this infection. Our findings will update current knowledge on the prevalence of antibiotic resistance on poultry breeding poultry farms in China and provide information and technical support toward the long-term goal of eradicating salmonellosis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

SS, FW, and XG conceived and designed the experiments and analyzed the data. YanS, FZ, LZ, and FW performed the experiments and contributed the reagents. YL performed data validation. FW and YanyS wrote the manuscript. All authors have read and approved the final manuscript.

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Epidemiological Investigation and Antimicrobial Resistance Profiles of *Salmonella* Isolated From Breeder Chicken Hatcheries in Henan, China

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The emergence of antimicrobial-resistant (AR) *Salmonella* has a major concern worldwide. This study was designed to determine the AR profiles and serovars distribution of *Salmonella enterica* isolated from different breeds of breeder chickens in the province of Henan, China. For this, 2,139 dead embryo samples were collected from 28 breeder chicken hatcheries, representing two domestic and four foreign breeds. The samples were subjected to the isolation and identification of *Salmonella* by PCR. The confirmed strains were serotyped according to the Kauffmann-White scheme and their AR profiles against 20 antimicrobial agents were determined by Kirby-Bauer (K-B) disc diffusion method. The results of this study showed the prevalence of *Salmonella* in 504 strains (23.56%) with a high abundance in southern regions of Yellow River (28.66%, $n = 495$, $N = 1{,}727$) compared to the northern regions (2.18%, $n = 9$, $N = 412$) ($p < 0.0001$). The domestic breeds were more contaminated than imported breeds ($p < 0.0001$). However, the contamination rate of samples recovered from M-hatcheries was the highest ($p < 0.0001$). Serotyping method identified 12 serovars, with the dominance of *S.* Pullorum (75.79%), followed by *S.* Enteritidis (7.14%). The AR assay showed high resistant to ciprofloxacin (77.00%), sulfisoxazole (73.00%), and ampicillin (55.60%), as well as 98.81% ($n = 498$) of the isolated strains, were resistant to at least one antimicrobial and 69.64% ($n = 351$) were resistant to three or more antimicrobials. Among them, one strain of *S*. Thompson was resistant to 15 antimicrobial agents belonging to eight different classes. In conclusion, *Salmonella* strains isolated in this study were multidrug-resistant (MDR), presenting a serious problem for human and animal health. Therefore, it is necessary to monitor, control, and rationalize the use of antimicrobials agents in chicken farms in order to limit the increasing resistance against the recent antimicrobial agents.

Keywords: *Salmonella*, chicken, embryo, hatchery scale, antimicrobial resistance, prevalence, ma breed, san huang breed

INTRODUCTION

Globally, salmonellosis is considered as one of the most important zoonotic diseases. According to the estimation of the World Health Organization (WHO), non-typhoidal salmonellosis was responsible for about 1.6 billion cases of acute gastroenteritis or diarrhea, causing 3 million deaths each year (Mahmud et al., 2011). In China, a study based on the literature review estimate that the incidence of non-typhoidal salmonellosis was 626.5 cases per 100,000 persons (Mao et al., 2011; Pan et al., 2018a; Yue et al., 2020). However, many studies have reported *Salmonella* as the responsible agent of many foodborne outbreaks in China and elsewhere (Wang et al., 2007; Cleary et al., 2010; Hedican et al., 2010; Le Hello et al., 2012; Guo et al., 2015; Moffatt et al., 2016; Jourdan-Da Silva et al., 2018). Currently, animals, in particular, poultry and eggs are considered to be the primary cause for salmonellosis and numerous other foodborne outbreaks (Gieraltowski et al., 2016; Keerthirathne et al., 2017; Biswas et al., 2019, 2020; Yu et al., 2020). Generally, *Salmonella* grow in animal farms may contaminate eggs and/or meat during the slaughtering process before being transferred to humans through food chain. Indeed, previous studies have been reported the isolation of *Salmonella* from foods of animal origin as well as human samples (Ed-Dra et al., 2018; Paudyal et al., 2018, 2020; Jiang et al., 2019; Elbediwi et al., 2020). Moreover, other studies were reported the consistency relationship between *Salmonella* strains causing human diseases and those isolated at farms and/or food products (Painter et al., 2013; Pan et al., 2018b, 2019; Paudyal et al., 2019; Wang et al., 2019).

Salmonella are Gram-negative rod-shaped bacteria, facultatively anaerobic, and belong to the family Enterobacteriaceae. They are generally mobile, capsule-less, not spore-forming, and colonize the digestive tract of many vertebrates (Baird-Parker, 1990; Bernal-Bayard and Ramos-Morales, 2018). So far about 2,600 serovars have been discovered, many of them were implicated in human and animal diseases (Feasey et al., 2012; Gong et al., 2014; Paudyal et al., 2019). In poultry farms, *Salmonella* may contaminate the flocks causing severe diseases with a high level of mortality. *Salmonella* Gallinarum biovar Pullorum and Gallinarum are host-specific for avian species, causing Pullorum disease and fowl typhoid, resulting in huge economic losses to the poultry industries every year (Gast and Porter, 2020). However, to treat and control the bacterial infectious diseases in poultry farms, farmers use many antimicrobials for therapeutic and prophylaxis practices. Unfortunately, the antimicrobials abuse were the main driver for the emergence of multidrug-resistant (MDR) bacteria in China, which has become a serious problem to public health (Yue, 2016; Paudyal and Yue, 2019). Generally, bacteria acquired resistance in animal farms before being transferred to humans through the food chain (Mehdi et al., 2018; Elbediwi et al., 2019; Paudyal et al., 2019; Zhang et al., 2019).

China is a major consumer of chicken and its products overall the world. Henan province, located in the central part of China with convenient transportation, is also a major breeding province in China. In 2012, the export of meat, eggs, and milk from Henan province accounted for 19.14% of the country's total exports. However, foods of animal origin, in particular, contaminated poultry products (eggs and poultry meat) have been considered the main vehicles of *Salmonella* infection and were clearly associated with worldwide epidemics (Hedican et al., 2010; Guo et al., 2015; Moffatt et al., 2016). Currently, there are few studies regarding *Salmonella* of chicken origin in Henan province. Additionally, breeder farms are less studied compared to commercial farms, slaughterhouses, and markets. Therefore, in order to fill up the epidemiological gaps concerning the distribution of *Salmonella* in breeder chicken hatcheries, 28 sampling sites belonging to nine cities of Henan province were subjected to the isolation, identification, and serotyping of *Salmonella* isolates. The antimicrobial resistant (AR) patters of the isolated strains were also determined. In this study, we compared the prevalence of *Salmonella* among different hatcheries and breeds as well as investigated the prevalence of different serovars and their AR patterns for guiding the prevention and control of *Salmonella* in animals and foodborne transmission toward humans.

MATERIALS AND METHODS

Sample Collection

From August 2014 to April 2015, a total of 2,139 dead chicken embryos were collected as the samples from randomly selected 28 hatcheries for breeding chickens in nine cities of Henan province: Zhengzhou, Xuchang, Pingdingshan, Hebi, Anyang, Zhoukou, Shangqiu, Xinyang, and Luohe, including two domestic breeds (Ma and San huang) and four foreign breeds (Hyline, Cobb, Ross 308, and Arbor Acres). According to the breeding scale, these chicken hatcheries were divided into three small-scale hatcheries (S-hatcheries, housing $\leq$10,000 chicken embryos), 22 medium-scale hatcheries (M-hatcheries, housing $>$10,000, $\leq$50,000 chicken embryos) and two large-scale hatcheries (L-hatcheries, housing $>$50,000 chicken embryos). The breeding scale of Zhoukou-3 hatchery is not available.

Isolation

The embryo surface was disinfected with ethanol for 2 min and then placed on the sterile tray of the clean bench. Sterile forceps and scissors were used to find the yolk sac of the chicken embryo and extract the samples. The yolk sac solution was placed onto *Salmonella-Shigella* agar using sterile cotton swabs and streaked using disposable sterile inoculating loops then the plates were incubated for 24 h at 37° C. The translucent colorless or black center colonies were considered presumptive of *Salmonella* and were selected and inoculated into Luria-Bertani broth for serotyping and genomic DNA preparation (Han et al., 2011; Xin et al., 2020).

Genomic DNA Preparation

The raw genomic DNA sample was extracted by boiling method. Briefly, 400 μL of the culture of presumptive *Salmonella* were placed in 1.5 mL tubes and centrifuged for 2 min at 12,000 rpm, then the supernatant was discarded and 200 μL of ddH_2O were added to resuspend the culture of *Salmonella*. The suspension was centrifuged at low speed for 1 min, boiled for 10 min and

transferred immediately into ice for 10 min. Then, another centrifugation was performed at 12,000 rpm for 2 min and the obtained supernatant (template DNA) was analyzed for checking the purity and stored at −20° C until use.

PCR Identification

For the identification of *Salmonella*, amplification of enterotoxin *stn* gene was performed by PCR in a final volume of 20 μL (Xiong et al., 2018), including 10 μL of 2 × Master Mix, 1.5 μL of F/R primers (primer concentration is 5 μM, primer sequences (5′-3′) were *stn* F: TATTTTGCACCACAGCCAGC and *stn* R: CGACCGCGTTATCATCACTG), 2 μL of template DNA, and 5 μL of ddH_2O. The PCR amplification was performed under the following reaction conditions: initial denaturation at 94° C for 5 min; 35 sequential cycles of denaturation at 94° C for 30 s, annealing at 58° C for 30 s, and extension at 72° C for 20 s; and a final extension at 72° C for 5 min. The PCR products were subjected to gel electrophoresis, and those presented a targeted band of about 260 bp were considered positives.

Serotyping

Salmonella isolates confirmed by PCR were serotyped by slide agglutination test with O and H antigens (Tianrun Bio-Pharmaceutical, Ningbo, China), according to the manufacturer's instructions. The results were analyzed and interpreted according to the Kauffmann-White scheme. Molecular identification and discrimination of *S.* Pullorum and *S.* Gallinarum was performed as recommended previously (Zhu et al., 2015).

Antimicrobial Susceptibility Test

Antimicrobial susceptibility profiles of the isolated *Salmonella* strains were determined according to the Kirby-Bauer (K-B) disc diffusion method and the recommendations of the American Clinical Laboratory Standards Institute (CLSI, 2015). For control strains, *Escherichia* coli ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used. Twenty different antimicrobials belonging to 12 classes were used for this assay. The isolates showing a decrease in susceptibility (intermediate) were ranged with the resistant group for the sake of clarity and to facilitate analysis. The classes of antimicrobials used in our assay were as follow: aminoglycosides (kanamycin: KAN 30 μg, gentamicin: GEN 10 μg, amikacin: AMK 30 μg), penicillin (ampicillin: AMP 10 μg), beta-lactam combination (amoxicillin-clavulanic acid: AMC 20/10 μg), cephems (ceftriaxone: CRO 30 μg, ceftazidime: CAZ 30 μg, cefazolin: CFZ 30 μg), carbapenems (meropenem: MEM 10 μg, imipenem: IPM 10 μg), monobactams (aztreonam: ATM 30 μg), tetracyclines (tetracycline: TET 30 μg, oxytetracycline: OTC 30 μg), polypeptide (colistin: CST 10 μg), phenicol (chloramphenicol: CHL 30 μg), quinolones (enrofloxacin: ENR 5 μg, ciprofloxacin: CIP 5 μg), sulphonamides (sulfamethoxazole-trimethoprim: SXT 23.75/1.25 μg, sulfisoxazole: SIZ 250 μg), nitrofurans (nitrofurantoin: NIT 300 μg).

Statistical Analysis

SPSS 26.0 software was used to perform statistical analysis on the prevalence of *Salmonella* in different regions, hatcheries, breeds, and serovars and biovars by using Chi-square test. The two-way ordinary ANOVA analysis was used to compare the difference in the cumulative prevalence of *Salmonella* recovered from L-hatcheries and M-hatcheries with respect to the individual antimicrobial.

RESULTS

Prevalence, Breed, and Serovar Distribution

During the 9 months of sampling, from August 2014 to April 2015, a total of 2,139 dead chicken embryo samples were collected from 28 breeder chicken hatcheries and analyzed for the presence of *Salmonella*. The sampling framework was designed to cover a large geographical area of Henan province; in fact, 28 sampling sites belonging to nine cities were selected for this study (**Figure 1**).

The results of this study showed that the average prevalence of *Salmonella* was 23.56% (n = 504, N = 2,139) (95% CI: 21.8–25.4). Among the total samples collected in this study (N = 2,139), 142 were from S-hatcheries, 1,759 were from M-hatcheries and 180 were from L-hatcheries, in addition to 58 samples collected from an unknown-scale hatchery. The results showed that the prevalence of *Salmonella* in M-hatcheries and L-hatcheries were 27.52% [95% CI: 25.4–29.7 (n = 484, N = 1,759)] and 11.11% [95% CI: 6.9–16.6 (n = 20, N = 180)], respectively. Whereas, *Salmonella* was not detected in S-hatcheries (n = 0, N = 142) and the unknown-scale hatchery (n = 0, N = 58) (**Table 1**). The difference in the prevalence of *Salmonella* between different hatcheries was studied by using Chi-square test which revealed that the higher prevalence in M-hatcheries was statistically significant ($p < 0.0001$). Moreover, our results showed that the prevalence of *Salmonella* in the south of the Yellow River (28.66%, n = 495, N = 1,727) was much higher than the north region (2.18%, n = 9, N = 412) ($p < 0.0001$).

Six different breeds were studied, including two domestic breeds (Ma and San huang) and four imported breeds (Hyline, Cobb, Ross 308, and Arbor Acres). The distribution of these breeds at different hatcheries is given in **Table 2**. The results showed that the isolation rate of *Salmonella* was more than 40% in seven hatcheries; all of them were San huang breeder chicken hatcheries and Ma breeder chicken hatcheries. *Salmonella* was not detected in hatcheries of imported breeds, including two Cobb hatcheries, five Hyline hatcheries, one Arbor Acres hatchery, and one Ross 308 hatchery. The statistical analysis showed that the prevalence of *Salmonella* in domestic breeds was much higher than that of foreign breeds ($p < 0.0001$). The breed San huang was the most contaminated one (48.60%, n = 173, N = 356), followed by the breed Ma (34.60%, n = 282, N = 815), while the breed Hyline present the lowest contamination rate (0.33%, n = 1, N = 302). However, among the four imported breeds, Ross 308 and Arbor Acres were more susceptible to *Salmonella* contamination than Hyline and Cobb ($p < 0.0001$).

Among 504 *Salmonella* isolates, 12 different serovars and two biovars of serovar Gallinarum were identified (**Table 1**). Serovars distribution showed the dominance of *S.* Pullorum (17.86%, n

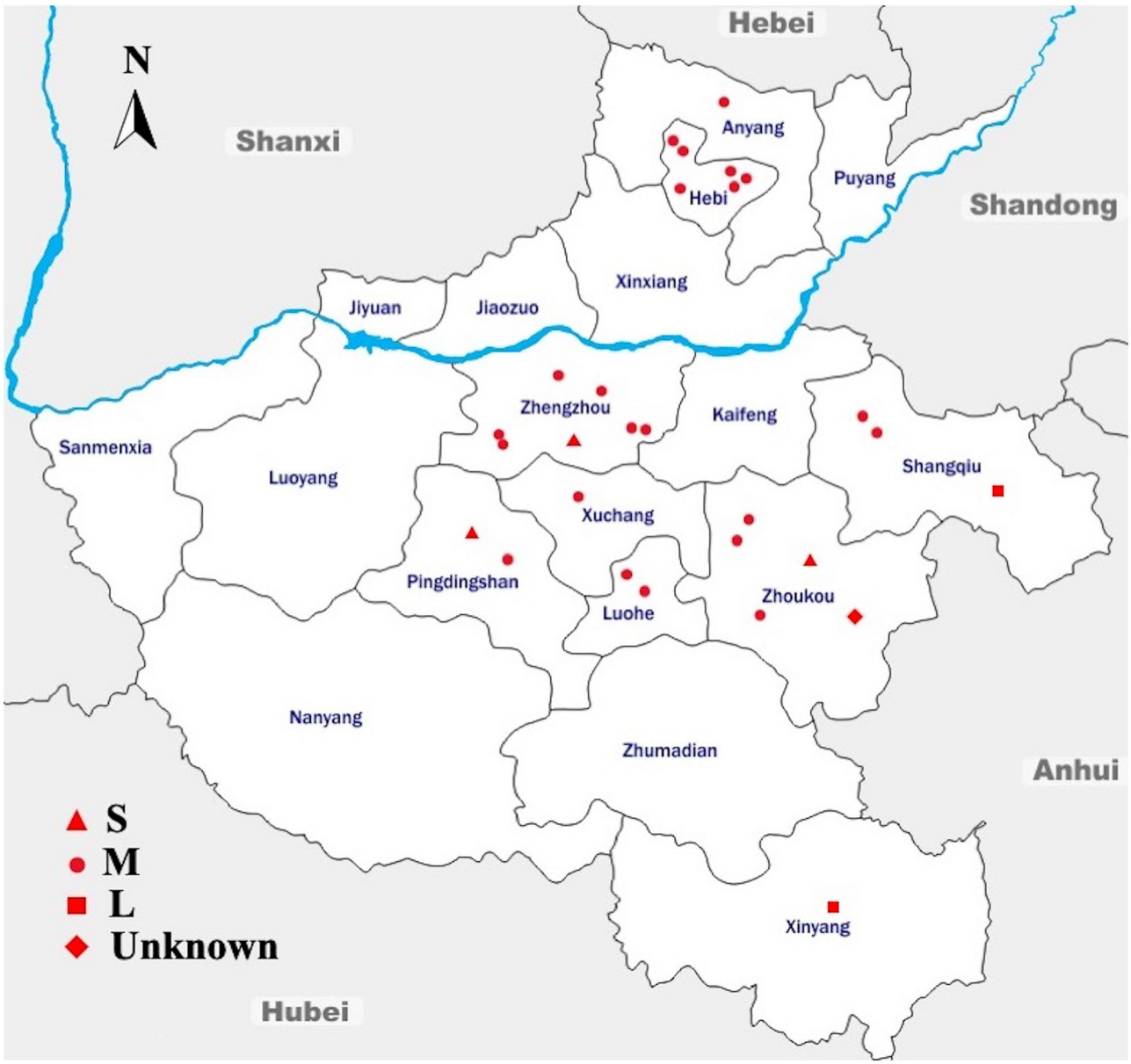

FIGURE 1 | Geographic Distribution of the Sampling Sites Across Different Regions in Henan, China. Sampling sites are denoted as small-scale hatcheries (S), medium-scale hatcheries (M), large-scale hatcheries (L), and unknown-scale hatcheries (Unknown). The blue line denotes the Yellow River, flowing through the province to the east into the Bohai Sea. The arrow shows the geographical north. This map is not drawn to scale.

= 382), followed by *S.* Enteritidis (1.68%, $n = 36$), while the lowest prevalent serovars were Entebbe, Tamilnadu, Fillmore and Gatuni (0.05%, $n = 1$). *S.* Gallinarum was detected only in L-hatcheries, while many serovars like Entebbe, Edinburg, Thompson, Tennessee, Tamilnadu, Fillmore, Gatuni, Enteritidis, Kimpese, and Cerro were isolated only from M-hatcheries. *S.* Pullorum was isolated at the highest frequency both in M-hatcheries (20.75%, $n = 365$) and L-hatcheries (9.44%, $n = 17$), while *S.* Blegdam was more prevalent (0.97%, $n = 17$) in M-hatcheries. The prevalence of *Salmonella* serovars according to the hatchery scale variation was presented in **Figure 2** and revealed that the difference in prevalence rate between M-hatcheries and L-hatcheries was due to *Salmonella* Gallinarum biovars Pullorum ($p < 0.001$) and Gallinarum ($p < 0.0001$).

Antimicrobial Resistance and Multidrug Resistance Pattern

AR patterns of the isolated *Salmonella* strains were determined by Kirby-Bauer (K-B) disc diffusion assay for 20 antimicrobial agents, representing 12 different classes, and the results were presented in **Table 3**. These results showed that *Salmonella* isolates had high resistance against quinolones (ciprofloxacin,

TABLE 1 | The distribution of twelve serovars at different hatcheries.

Hatcheries	Total samples	Serovars	Positive samples	%Prev. (HL)[a]	%Prev. (Ov.)[b]
Scale unknown	58 (1)	–	0 (0)	**0.00**	**0.00**
S-hatcheries[c]	142 (3)	–	0 (0)	**0.00**	**0.00**
M-hatcheries[d]		Entebbe	1	0.06	0.05
		Edinburg	17	0.97	0.79
		Thompson	14	0.80	0.65
		Tennessee	9	0.51	0.42
		Tamilnadu	1	0.06	0.05
		Fillmore	1	0.06	0.05
		Gatuni	1	0.06	0.05
		Pullorum	365	20.75	17.86
		Enteritidis	36	2.05	1.68
		Blegdam	17	0.97	0.84
		Kimpese	4	0.23	0.19
		Cerro	18	1.02	0.84
Subtotal M-hatcheries	1759 (22)		484 (17)	**27.52****	
L-hatcheries[e]		Pullorum	17	9.44	
		Gallinarum	2	1.11	0.09
		Blegdam	1	0.56	
Subtotal L-hatcheries	180 (2)		20 (1)	**11.11**	
Total hatcheries	**2139 (28)**		**504 (18)**		**23.56**

[a]Prevalence at hatchery level; [b]Overall prevalence; [c]Small-scale hatcheries; [d]Medium-scale hatcheries; [e]Large-scale hatcheries.
*****$p < 0.0001$, statistical difference in Salmonella prevalence between M- and L-hatcheries.*

TABLE 2 | The distribution of six breeds at different hatcheries.

Hatcheries	Breeds		Positive samples	%Prev. (BL)[1]	%Prev. (Ov.)
Scale unknown	Ross 308		0	0.00	0.00
S-hatcheries	Hyline		0	0.00	0.00
M-hatcheries	Hyline****a,b	**Foreign**	1	0.33	0.05
	Cobb		1	0.47	0.05
	Arbor acres		6	6.12	0.28
	Ross 308		41	11.48	1.92
	San huang	**Domestic**	173	48.60	8.09
	Ma		262	34.60	13.18
L-hatcheries	Hyline		0		
	Ma		20		

*[1]Prevalence at breed level; ****$p < 0.0001$, [a]statistical difference in Salmonella prevalence between foreign and domestic breeds; [b]statistical difference in Salmonella prevalence between Arbor Acres, Ross 308 and Hyline, Cobb.*

77.00%) and sulphonamides (sulfisoxazole, 73.00%), but low resistance against phenicols (chloramphenicol, 1.00%), while they were susceptible to carbapenems (meropenem and imipenem) and polypeptides (colistin). Moreover, our results showed that the overall average resistance of *Salmonella* isolated from M-hatcheries was higher than that of L-hatcheries ($p = 0.0013$), while statistical analysis performed by using two-way ordinary ANOVA, showed that the difference in resistance to the individual antimicrobial was statistically highly significant ($p < 0.0001$) (**Figure 3**).

The AR patterns related to different serovars or biovars are given in **Figure 4**. These results showed that the variation in the resistance among serovars was statistically significant ($p < 0.05$). It should be noted that only one strain of serovar Entebbe, Tamilnadu, Fillmore, and Gatuni was isolated in this study. Moreover, the resistance of the same serotype was statistically significant when it was compared between L-hatcheries and M-hatcheries ($p < 0.01$) (**Figure 5**).

The AR analysis showed that 98.81% ($n = 498$) of the isolates were resistant to at least one antimicrobial and 69.64% ($n = 351$)

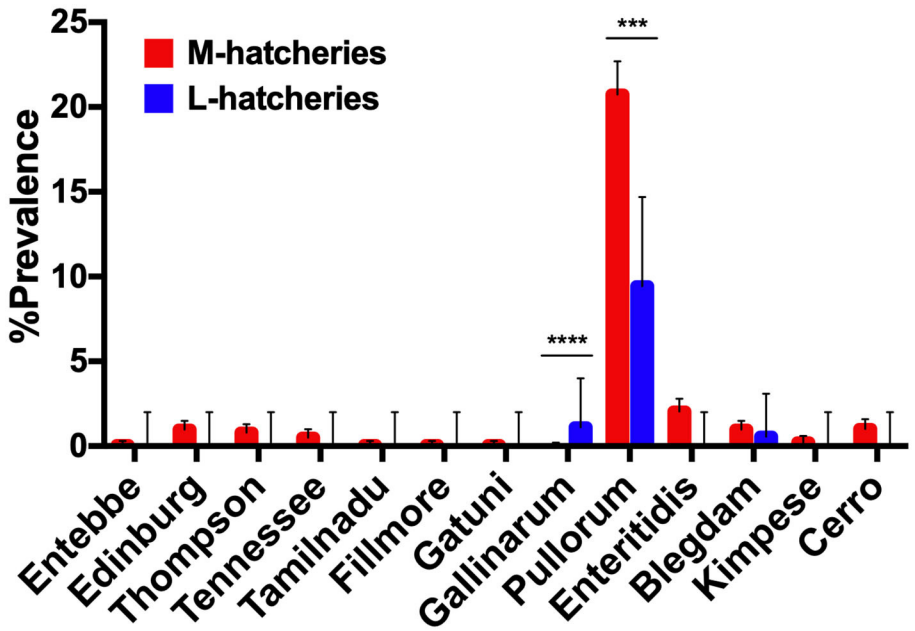

FIGURE 2 | The hatchery scale variation in the distribution of prevalence of 12 serovars. Serogroup: O:4(B), Entebbe; O:7(C1), Edinburg/Thompson/Tennessee/Tamilnadu; O:8(C2-C3), Fillmore/Gatuni; O:9(D1), Gallinarum (Pullorum)/Enteritidis/Blegdam/Kimpese; O:18(K), Cerro. ***The differencee in prevalence rate of *S.* Pullorum between M-hatcheries and L-hatcheries was statistically significant ($p < 0.001$). ****The differencee in prevalence rate of *S.* Gallinarum between M-hatcheries and L-hatcheries was statistically highly significant ($p < 0.0001$).

TABLE 3 | Prevalence of Antimicrobial Resistance among *Salmonella* isolates.

Classes	Antimicrobials	concentrations (μg)	*n*	%Resistance
Aminoglycosides	KAN	30	40	7.94
	GEN	10	41	8.13
	AMK	30	41	8.13
Penicillin	AMP	10	280	55.56
β-lactams	AMC	20/10	16	3.17
Cephems	CRO	30	33	6.55
	CAZ	30	16	3.17
	CFZ	30	159	31.55
Carbapenems	MEM	10	0	0.00
	IPM	10	0	0.00
Monobactams	ATM	30	35	6.94
Tetracyclines	TET	30	181	35.91
	OTC	30	180	35.71
Polypeptide	CST	10	0	0.00
Phenicol	CHL	30	4	0.79
Quinolones	ENR	5	59	11.71
	CIP	5	389	77.18
Sulphonamides	SXT	23.75/1.25	103	20.44
	SIZ	250	367	72.82
Nitrofurans	NIT	300	47	9.33

were resistant to three or more antimicrobial agents. Moreover, we found that the penta-drug resistance pattern was the most represented (20.24%), while the most extensive resistance pattern was observed in one strain of *Salmonella* serovar Thompson (0.20%), which was resistant to 15 antimicrobials belonging to eight different classes (**Figure 6**).

MDR analysis showed the detection of tetra-, hexa-, hepta-, and octa-drug resistance patterns, which were presented in **Figure 7**. These MDR patterns were distributed in serovars or biovars Pullorum (382), Thompson (14), Enteritidis (36), and Gatuni (1) which were all recovered from the south of the Yellow River. Tetra-resistance pattern (CSACf, i.e., resistance to ciprofloxacin, sulfisoxazole, ampicillin, and cefazolin) was the most frequently observed (maximum of 14.48% for Pullorum) among the recorded serovars or biovars. Hexa-drug resistance (CSACfTO, i.e., CSACf with tetracycline and oxytetracycline) and hepta-drug resistance (CSACfTOE, i.e., CSACfTO with enrofloxacin) were both highest (11.51% and 0.40%) for *S.* Pullorum. Octa-drug resistance (CSACfTOEN, i.e., CSACfTOE with nitrofurantoin) was recorded only in *S.* Pullorum (0.20%).

The distribution of MDR among the isolates was analyzed and compared related to the scale of hatcheries. This analysis was limited only to the *Salmonella* Gallinarum biovar Pullorum, due to the low diversity of serovars isolated from L-hatcheries, and serovars Thompson, Enteritidis and Gatuni were not detected in L-hatcheries. The results showed that the MDR isolates of *S.* Pullorum isolated from M-hatcheries were higher than those reported in L-hatcheries ($p < 0.0001$) (**Figure 8**).

DISCUSSION

Salmonella is a common pathogen in poultry farms worldwide, which can be transmitted by horizontal and vertical ways causing

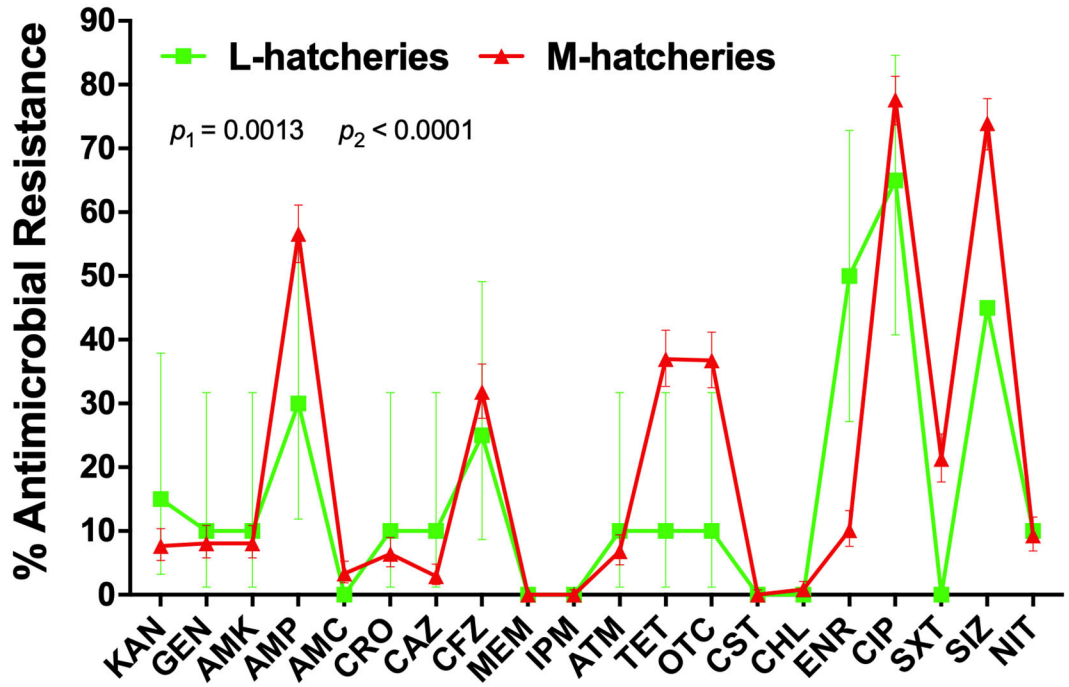

FIGURE 3 | Distribution of the average antimicrobial resistance (in percent) of individual antimicrobials in *Salmonella* isolates from samples collected across L-hatcheries, M-hatcheries. S-hatcheries have no positive samples. The overall average resistance of *Salmonella* isolated from M-hatcheries was higher than that of L-hatcheries ($p = 0.0013$), and the difference in resistance to the individual antimicrobial was statistically highly significant ($p < 0.0001$).

huge economic losses to the poultry industries every year. In fact, avian salmonellosis can be transmitted vertically through fertilized eggs and causes the death of hatching chicken embryos. In this study, we selected dead embryos instead of the normal embryo samples in order to reflect the actual disease burden of *Salmonella* in breeders.

In this study, the prevalence of *Salmonella* in dead embryos of breeder chickens in Henan province was around 23.56%. These results were similar to those reported in chicken farms in Shandong province, China (24.0%) (Zhao et al., 2017), higher than those reported in El-Menofia province in Egypt (14.3%) (Abdeen et al., 2018), and lower than those reported in the Thailand-Cambodia border provinces (35.75%) (Trongjit et al., 2017). This comparison should be made with suspicion because the prevalence of *Salmonella* may be influenced by several factors like the difference in economic development between countries, samples types, sampling locations, sampling seasons, hygienic quality of the production units, and isolation methods (Kuang et al., 2015; Ed-Dra et al., 2017). Moreover, our results showed that the prevalence of *Salmonella* varies depending on the hatcheries scale. In fact, the isolation rate of *Salmonella* from M-hatcheries was much higher than that of L-hatcheries ($p < 0.0001$), while *Salmonella* isolates were not detected in both S-hatcheries and the unknown-scale hatchery. These results can be explained by the implementation of biosecurity measures in the larger hatcheries which participate in minimizing the prevalence of pathogens. However, *Salmonella* was not detected in S-hatcheries, probably because the scale was very small, which might be conducive to the well-established management and control.

Serovars distribution was performed according to Kauffmann-White scheme and differentiated 12 different serotypes among the 504 *Salmonella* isolates. Two common biovars within serovar Gallinarum were identified. *Salmonella* Gallinarum biovar Pullorum was the most represented (75.79%), followed by Enteritidis (7.14%), Blegdam (3.57%), and Cerro (3.57%). These results demonstrated that there is serious *S.* Pullorum contamination in breeding chicken farms in Henan Province, which were consistent with the earlier studies about *Salmonella* serotypes of avian origin in 12 Chinese provinces (Gong et al., 2014). However, other studies have reported the dominance of other *Salmonella* serovars; *S.* Weltevreden and *S.* Agona in broiler farms (cloacal swab samples) in Guangdong Province (Ren et al., 2016), *S.* Enteritidis (rectal swab samples, chicken embryo samples) in Shandong Province (Zhao et al., 2017), *S.* Derby and *S.* Typhimurium (cloaca swab sampling) in Sichuan Province (Ma et al., 2017). Moreover, *S.* Corvallis and *S.* Typhimurium were described in Thailand (Trongjit et al., 2017), *S.* Typhimurium and *S.* Enteritidis in Egypt and America (Medalla et al., 2016; Mahmoud et al., 2018), *S.* Havana and *S.* Enteritidis in Portugal (Clemente et al., 2014). Therefore, the predominant serotypes of *Salmonella* may vary with time and region of sampling (Kuang et al., 2015). Importantly, the major serovar or biovar isolated in this study (Pullorum) has a major

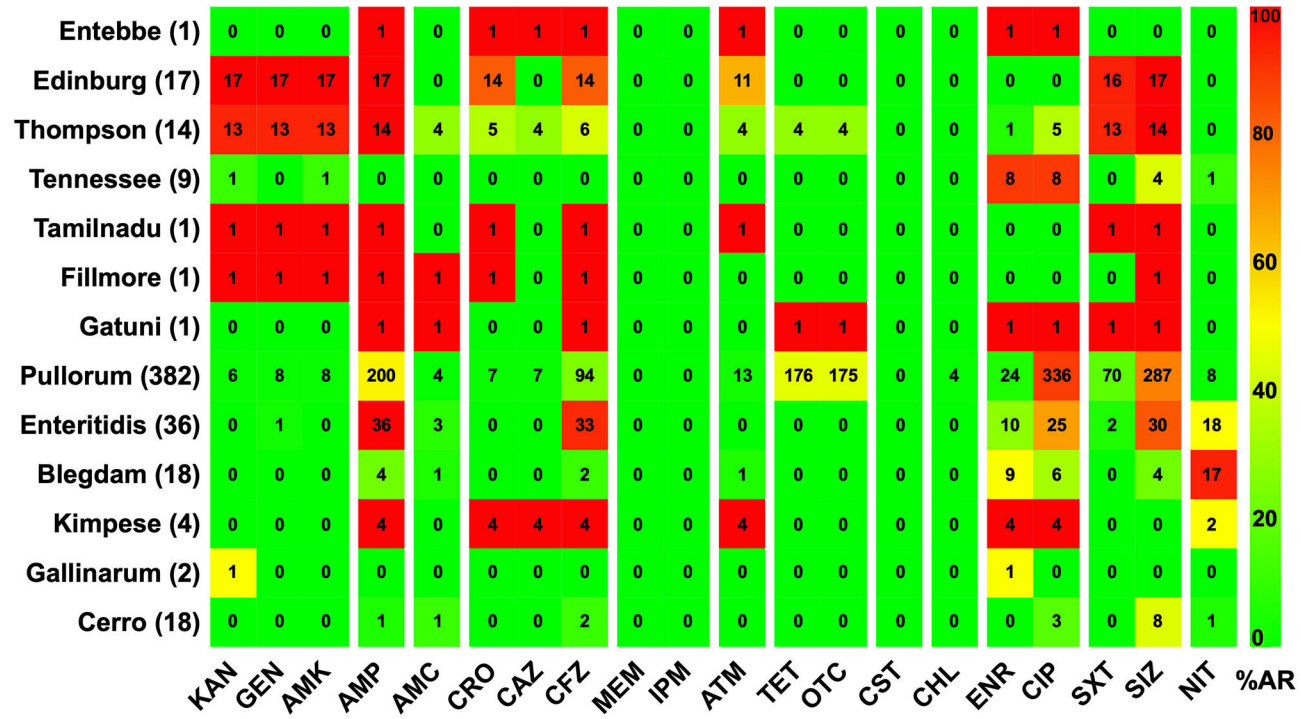

FIGURE 4 | The prevalence of resistance among *Salmonella* isolates against the different classes of antimicrobials. The distribution of the average antimicrobial resistance (in percent) of various serotypes to 20 antimicrobials of 12 different classes independent of the source of the strains. The color of individual cells varies with the percentage of antimicrobial resistance. The number in individual cells represents number of resistant strains (*n*). The names of the antimicrobials (XX') are abbreviated as KAN, kanamycin; GEN, gentamicin; AMK, amikacin; AMP, ampicillin; AMC, amoxicillin-clavulanic; CRO, ceftriaxone; CAZ, ceftazidime; CFZ, cefazolin; MEM, meropenem; IPM, imipenem; ATM, aztreonam; TET, tetracycline; OTC, oxytetracycline; CST, colistin; CHL, chloramphenicol; ENR, enrofloxacin; CIP, ciprofloxacin; SXT, sulfamethoxazole-trimethoprim; SIZ, sulfisoxazole; NIT, nitrofurantoin.

veterinary concern because it is the causative agent of Pullorum disease in chicken, resulting in considerable economic losses to the poultry industries (Geng et al., 2014). However, *S.* Enteritidis have been reported as responsible for many foodborne outbreaks in Henan province, China (Xia et al., 2009). Indeed, *Salmonella* can be transmitted from farms to humans through food products, including meat and eggs (Zhao et al., 2017). Therefore, it is necessary to continuously monitor the local serovar variation of *Salmonella* and formulate a reasonable prevention and control strategy accordingly.

In order to project the prevalence and the distribution of *Salmonella* among the breeder breeds, a total of six breeds were subjected to this study, including four imported breeds, namely Ross 308 (UK), Cobb (USA), Arbor Acres (USA) which are mainly used for meat production and Hyline (USA) mainly used for eggs production, and two local breeds, namely Ma and San huang which are both used for meat and eggs production. Our results showed that the prevalence of *Salmonella* in domestic breeds was much higher than that of foreign breeds ($p < 0.0001$). Moreover, the serovars distribution among domestic breeds were consistent with the dominance of *S.* Pullorum, while only one strain of *S.* Pullorum was detected in the imported breed. To a certain extent, it was indicated that *S.* Pullorum has been decontaminated thoroughly of foreign breed chicken (Lu et al., 2014), while the Chinese poultry industries still suffered from serious *S.* Pullorum contamination (Gong et al., 2014). Among these various breeds, the prevalence of *Salmonella* in San huang and Ma was the highest (48.60 and 34.60%, respectively) with additional serovars or biovar distribution ($n = 11$) in Ma chicken breed. However, only one *Salmonella* strain was isolated from Hyline and Cobb, respectively, while the prevalence of *Salmonella* in Ross 308 was more serious (11.48%) than the other imported breeds. Notably, we found that 34 of the 41 *Salmonella* isolates from Ross 308 were recovered from the same hatchery, with an isolation rate of 28.33%. However, the breeding farm corresponding to the hatchery was in a poor sanitary environment, which *S.* Enteritidis has been detected in this farm before entering the chicks. Therefore, the prevalence of *Salmonella* in different chicken farms was related to the chicken breed, feeding management and sanitation environment. The prevalence of *Salmonella* in LH-1, LH-2, and ZZ-6 hatcheries was 5.26, 15.00, and 8.26%, respectively, which is lower than that of other hatcheries of Ma and San huang breeds. After investigation, we found that varying degrees of *S.* Pullorum quarantine and purification had been carried out in these three hatcheries. It was showed that quarantine and purification had a significant effect on the control of *S.* Pullorum and suggested that the hatcheries and farms should pay particular attention to the introduction

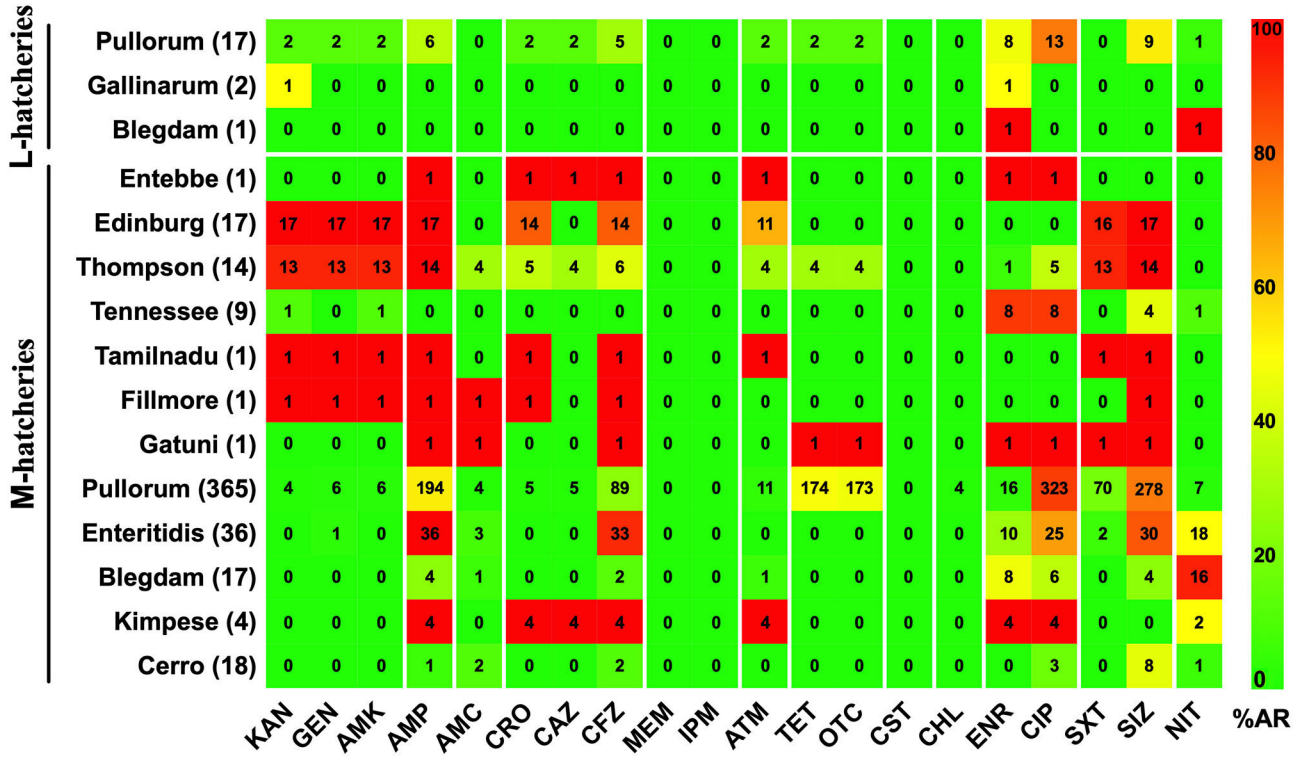

FIGURE 5 | Prevalence of Antimicrobial Resistance and Serovars between L-hatcheries and M-hatcheries. The upper 3 rows are the distribution in the large-scale hatcheries while the lower rows are the distribution in medium-scale hatcheries. The color of individual cells varies with the percentage of antimicrobial resistance. The number in individual cells represents number of resistant strains (*n*).

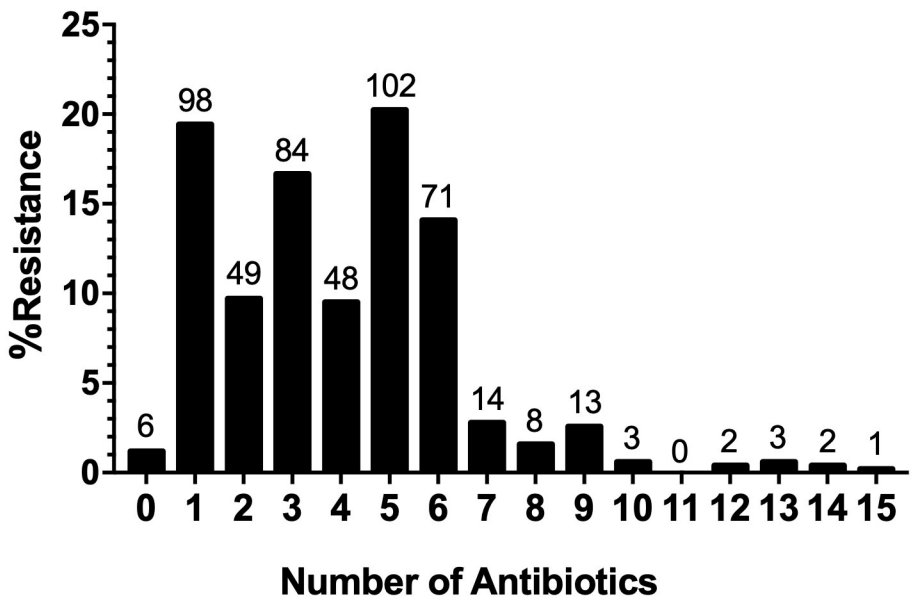

FIGURE 6 | Distribution of Multi-drug resistance (MDR) Strains. MDR patterns for the number of antimicrobials and the distribution of multidrug-resistant strains.

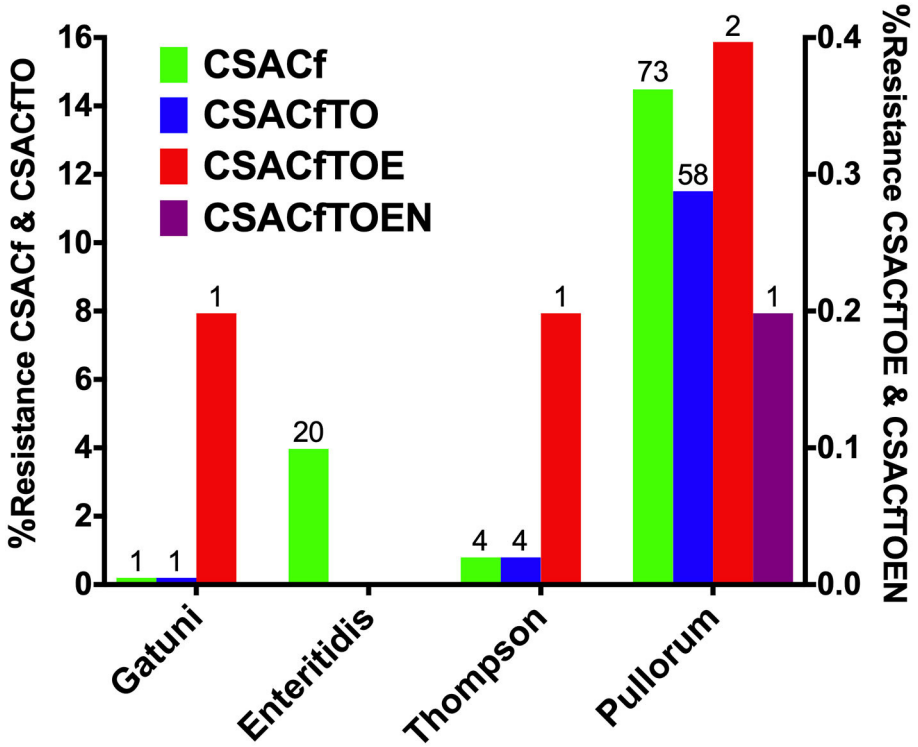

FIGURE 7 | Serovar distribution of Multi-drug resistance (MDR) Prevalence. The left Y-axis shows tetra- and hexa-drug resistance while the right Y-axis shows hepta-drug resistance, except in serovar Enteritidis and octa-drug resistance (see only in *S.* Pullorum).

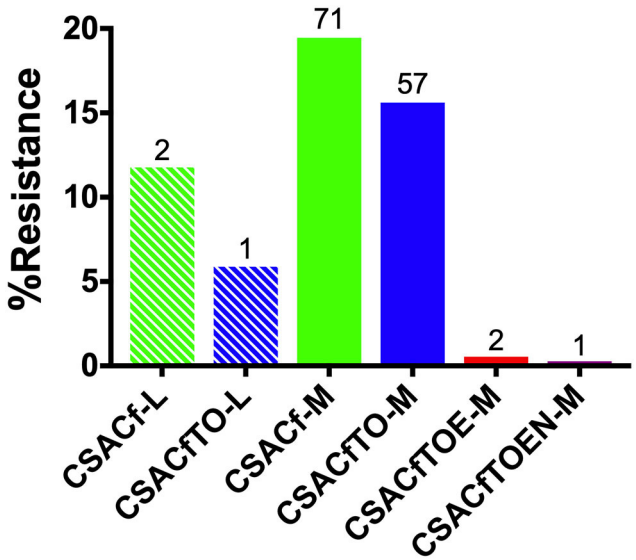

FIGURE 8 | Hatchery scale distribution of multi-drug resistance (MDR) prevalence of Pullorum. Hatchery scale are denoted as medium-scale hatcheries (M) and large-scale hatcheries (L). Due to the low diversity of serotypes isolated from L-hatcheries, there were no Thompson, Enteritidis, or Gatuni isolated from L-hatcheries, so here we only compared the MDR strains of Pullorum in L-hatcheries and M-hatcheries (S-hatcheries had no positive samples).

of healthy chicks, strict disinfection, and regular quarantine to eliminate positive eggs and chickens, so as to reduce the prevalence of *Salmonella*.

AR prevalence of *Salmonella* to ciprofloxacin (77.00%), sulfisoxazole (73.00%), ampicillin (55.60%), tetracycline (36.00%), and oxytetracycline (36.00%) exceeded the 36%, which was similar to the AR situation of Sichuan, Shandong, Guangdong, and Shanxi provinces (Li et al., 2013; Yang et al., 2014; Zhao et al., 2017). As well as some imported studies were reported the high resistance of *Salmonella* isolates to ampicillin, tetracycline, ciprofloxacin, and sulfonamides (Bacci et al., 2012; Clemente et al., 2014; Abdeen et al., 2018; Chuah et al., 2018), which indicate that *Salmonella* of poultry origin from several countries has developed a high resistance to traditional antimicrobials such as quinolones, penicillins, sulfonamides, and tetracyclines which are widely used in livestock and poultry farming to treat bacterial avian diseases and to promote growth (Mehdi et al., 2018). However, our findings showed that the *Salmonella* isolates were susceptible to meropenem, imipenem, and presented a low resistance to chloramphenicol, with only one strain of *S.* Pullorum, in fact, the use of these antimicrobials was banned by veterinarians, indicating that the strengthening of veterinary medicine management can effectively avoid the development of AR bacteria. Moreover, the low resistance of isolated strains to amoxicillin-clavulanic acid, ceftazidime, aztreonam, and nitrofurantoin may be related to the seldom use of these antimicrobials in animal production.

Different serovars and biovars of *Salmonella* showed different AR patterns. The antimicrobial resistance of serovar Edinburg and Thompson were alarming high, which showed high antimicrobial resistance rates, with a wide antimicrobial resistance spectrum. Although these two are not the dominant serovars, the prevalence of serovars may change over time and they are likely to be the dominant serovars under the multiple antimicrobial selection pressure (Rahmani et al., 2013). Pullorum as the dominant serovar showed very high resistance to quinolones (ciprofloxacin) and sulphonamides (sulfisoxazole), which were commonly used in farms. Similarly, Enteritidis as the second dominant serovar of these isolates, showed high resistance to penicillin (ampicillin), cephems (cefazolin), quinolones (ciprofloxacin), and sulphonamides (sulfisoxazole), which were frequently used in farms. These also supported that most of the *Salmonella* pathogens presented in farms have a common antimicrobial resistance pattern (a Tetra-resistance pattern toward ciprofloxacin, sulfisoxazole, ampicillin, and cefazolin), suggesting that veterinarians in farms need to improve the traditional medication regimen. Tennessee and Blegdam had high resistance to quinolones (enrofloxacin, ciprofloxacin) and nitrofurans (nitrofurantoin), respectively, but were sensitive to other antimicrobials. Compared with serovars discussed above, Cerro as a relatively uncommon serovar, was sensitive to various antimicrobials. Here, we also found a few minority serovars, including Entebbe, Tamilnadu, Fillmore, Gatuni, Kimpese, and Gallinarum biovar Gallinarum.

This study showed a high prevalence of MDR patterns among the isolated *Salmonella* strains. In fact, 351 (69.64%) *Salmonella* isolates present resistance to three or more antimicrobial agents. These results were in accordance with numerous worldwide studies reporting the widespread of MDR among the isolated *Salmonella* strains (Li et al., 2013; Lu et al., 2014; Moawad et al., 2017; Abdeen et al., 2018; Chuah et al., 2018). However, the intensive use of antimicrobials in both veterinary and medical fields has led to a quite high AR by exerting a selection pressure against the used antimicrobials. Face the issue of the spread of AR bacteria, the government of China has established the program "Pilot Programme for Reduction of Veterinary Antimicrobial Use (2018-2021)" in April 2018 mentioning all pharmaceutical feed additives which would be withdrawn in 2020 (MOA, 2018). Therefore, the use of pharmaceutical feed additives should be gradually reduced and stopped in clinical practice, and the administration of alternatives medication should be strengthened. Furthermore, the use of veterinary banned drugs should be strictly prohibited, and the rotation of medication, alternating medication, and combination of Chinese and Western medicines should be implemented to avoid severe drug resistance caused by prolonged use of the same drugs.

CONCLUSION

This study expands the knowledge of epidemiology and AR prevalence of *Salmonella* strains recovered from breeder chicken hatcheries in Henan province, China. Our findings indicate serious contamination of samples by *Salmonella* especially for domestic breeds from M-hatcheries. The dominance of *S*. Pullorum and *S*. Enteritidis is with a major concern for veterinary and food safety fields. Additionally, the detection of MDR *Salmonella* (tetra-, hexa-, hepta-, and octa-drug resistance patterns) is alarming, which requires the implementation of an antimicrobial management plan for rational uses of critical antimicrobials in chicken farms.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

YX and ZJ collected the samples. YX, ZJ, and YQ did the lab analysis. YX and XZ did the data analysis and prepared a draft. AE made the comments and corrections of the draft. MY conceived the project and provided critical comments for the draft. All authors have read and agreed to the manuscript.

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The Mycotoxin Deoxynivalenol (DON) Promotes *Campylobacter jejuni* Multiplication in the Intestine of Broiler Chickens With Consequences on Bacterial Translocation and Gut Integrity

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Deoxynivalenol (DON) is one of the major health concern in poultry production as it targets epithelial cells of the gastrointestinal tract and contributes to the loss of the epithelial barrier function. It is well-documented that DON severely compromises various important intestinal functions in coincidence with aggravated clinical symptoms in livestock. In addition, a prolonged persistence of intestinal pathogens (e.g., *Salmonella*, *Clostridium*) in the gut has also been reported in pigs and chickens, respectively. Similar to DON, recent studies demonstrated that an experimental *Campylobacter* infection has severe consequences on gut health. Through experimental infection, it was found that *Campylobacter (C.) jejuni* negatively affects the integrity of the intestine and promotes the translocation of bacteria from the gut to inner organs. So far, no data are available investigating the simultaneous exposure of DON and *C. jejuni* in broilers albeit both are widely distributed. Thus, the aim of the present study was to explore the interaction between DON and *C. jejuni* which is of a significant public and animal health concern as it may affect the prevalence and the ability to control this pathogen. Following oral infection of birds at 14 days of age with *C. jejuni* NCTC 12744, we show that the co-exposure to DON and *C. jejuni* has a considerable consequence on *C. jejuni* loads in chicken gut as well as on gut permeability of the birds. A reduced growth performance was found for DON and/or *C. jejuni* exposed birds. Furthermore, it was found that the co-exposure of DON and *C. jejuni* aggravated the negative effect on paracellular permeability of the intestine already noticed for the bacteria or the mycotoxin alone by the Ussing chamber technique at certain times or intestinal segments. Furthermore, the increased paracellular permeability promotes the translocation of *C. jejuni* and *E. coli* to inner organs, namely liver and spleen. Interestingly, *C. jejuni* loads in the intestine were higher in DON-fed groups indicating a supportive growth effect of the mycotoxin. The actual study demonstrates that co-exposure of broiler chickens to DON and *C. jejuni* has not

only considerable consequences on gut integrity but also on bacterial balance. These findings indicate that the co-exposure of broiler chickens to DON and *C. jejuni* could have a significant impact on gut health and bacteria translocation leading to an increased risk for public health.

Keywords: deoxynivalenol, *Campylobacter jejuni*, translocation, Ussing chamber, intestinal permeability, broiler chickens

INTRODUCTION

The global contamination of food and feed with mycotoxins is a crucial problem, and the presence of mycotoxins in poultry feeds represents a constant threat to the poultry industry and production losses. Deoxynivalenol (DON) is the most widespread trichothecene mycotoxin in feeds. The main mode of action of DON is the inhibition of protein synthesis, which primarily affects rapidly dividing cells, such as those of the gut and the immune system (1, 2). As a consequence, it was shown that DON increases the susceptibility to diseases (3, 4).

Likewise, *Campylobacter* (*C.*) *jejuni* is the most frequent cause of foodborne disease in humans. These bacteria are a major concern for the poultry industry as the prevalence of infected broiler flocks is reported with an average of 71.2% in EU countries (5). Recent studies also elucidated that *Campylobacter* can have a negative impact on broiler gut health (6–11). Mainly, it affects the integrity of the gut epithelium which results in reduction of villi height, decrease of crypt depth with negative consequences on nutrient transport and absorption. Furthermore, *C. jejuni* can spread to internal organs, because of increased intestinal permeability (10, 12, 13). In this context, it was also shown that *C. jejuni* facilitates not only the translocation of *C. jejuni* itself but also the spread of *Escherichia* (*E.*) *coli* to internal organs (14–17).

So far, only few studies have investigated the interaction between DON and enteric pathogens. It was found that the co-exposure of pigs to DON and *Salmonella* Typhimurium potentiated the inflammatory gut response, promoted *Salmonella* invasion and its translocation across the intestinal epithelium (3). Furthermore, it was shown *in vitro* that DON enhanced the translocation of a pathogenic *E. coli* strain over the intestinal epithelial cell monolayer (18). Recently, it was also demonstrated that feeding of DON is a predisposing factor for the development of necrotic enteritis in broiler chickens due to the negative influence of the mycotoxin on the epithelial barrier (4).

Consequently, the objective of the present study was to demonstrate the interaction between DON and *C. jejuni* in broiler chickens. For this purpose, the epithelial paracellular permeability on intestinal parts was determined by applying the Ussing chamber technique. In this context, Ussing chambers, an *ex vivo* technique, are increasingly being used to measure epithelial ion transport, epithelial barrier function and electrophysiological properties of living tissues (19–22). Furthermore, the colonization of *C. jejuni* in the gut as well as the translocation of *C. jejuni* and *E. coli* to inner organs were analyzed.

MATERIALS AND METHODS

Study Design, Sampling, and Performance Data

One hundred and twenty 1-day–old male and female broiler chicks were obtained from a commercial hatchery (Ross-308; Gefluegelhof Schulz, Lassnitzhoehe, Austria) and randomly allocated to four treatment groups (30 birds/ group, divided into 5 replicates (6 birds/replicate): group 1 (control); group 2 (DON 5 mg/kg); group 3 (DON 5 mg/kg + *C. jejuni*); and group 4 (*C. jejuni*). Upon arrival, birds were weighed and tagged (Swiftack, Heartland Animal Health Inc., Fair Play, MO). The broilers were kept in floor pens on wood shavings and light was set at a 12h:12h light/dark cycle. Temperature was maintained at 35°C during the first days of life and reduced to 25°C with the age of birds. Feed and water were provided *ad libitum*. The experiment was carried out for 5 consecutive weeks.

Birds in groups 1 and 4 were fed with control diet without DON (non-contaminated diet during the starter and grower periods). Birds in groups 2 and 3 received the same diet contaminated with DON (DON 5 mg/kg). Diet was supplied from the first day without adaption period, as there is no evidence of gender differences or diet adaption period affected results in the response of gut physiology to diet as previously described (23). The control diet was prepared with non-contaminated wheat. The mycotoxin contaminated diet was prepared by replacing "non-contaminated" control wheat with DON contaminated wheat. Moreover, the 5 mg DON/kg feed in this study is the currently applicable EU guidance value of DON contamination (5 mg DON/kg poultry feed) (24, 25). Consequently, the used feeding model is relevant to the field situation. The composition of the diet comprised maize, wheat, soy, soybean meal, soybean oil, and rapeseed oil. Additionally, a premix of vitamins, minerals, mono calcium phosphate, and salt was supplemented. Feeds were provided by Biomin Holding GmbH (Tulln, Austria). Starter diet was fed for 9 days, followed by the grower diet from day 10 until 35 days of age.

Cloacal swabs were taken on a weekly basis starting from 1st day of life until the end of the trial. At 7, 14, and 21 dpi, cloacal swabs were taken to monitor *C. jejuni* excretion and shedding. These swabs were streaked directly onto modified charcoal-cefoperazone-deoxycholate agar (CM0739, OXOID, Hampshire, UK) to determine the *Campylobacter* status. Plates were incubated under microaerophilic conditions at 41.5°C for 48 h (Genbox microaer, BioMerièux, Vienna, Austria).

C. jejuni reference strain NCTC 12744 was cultured in LB medium (Lennox L broth base, Thermo Fisher Scientific Inc., Invitrogen by Life Technologies Corporation, Waltham, MA) at

41.5°C for 48 h under microaerobic conditions using GENbox microaer bags (BioMerieux, Vienna, Austria). Thereafter, the bacteria were enumerated by preparing 10-fold dilutions in phosphate-buffered saline with a PH of 7.4 (PBS, Thermo Fisher Scientific Inc., Gibco Life Technologies Corporation) and plated on Campylosel agar (BioMerieux, Vienna, Austria), followed by microaerobic incubation at 41.5°C for 48 h.

At day 14, birds of groups 3 and 4 were orally inoculated via feeding tube (gavage) with 1-ml of a PBS suspension containing 1×10^8 CFU/ml of *C. jejuni* reference strain NCTC 12744. The group 1 (DON and pathogen free group) and group 2 (DON-only) received a sham infection with 1 ml of a PBS via feeding tube (gavage).

At 7, 14, and 21 days post infection five birds from each group (one bird/ replicate) were randomly selected and euthanized by intravenous application of Thiopental (20 mg/kg) into the wing vein followed by exsanguination via cutting of the jugular vein. Gross pathological examination was performed according to a standardized protocol (10). During post-mortem examination, samples in the following order: liver, spleen, duodenum, jejunum and caeca were aseptically collected and processed for bacteriological investigations. Furthermore, for Ussing chamber, intestinal segments were taken immediately after slaughter from the duodenum, mid-jejunum and cecum. The tissues were rinsed with ice-cooled PBS perfused with 95% O_2 and 5% CO_2. The underlying serosa was stripped off and the epithelial layers were mounted in Ussing chambers. The experimental procedures were performed as described below.

The following performance parameters were determined: (a) weekly individual body weight (BW) per bird; (b) weekly individual body weight gain (BWG) per bird calculated as the difference in body weight between the start and the end of each weighing period; (c) feed intake measured daily over the course of the feeding period at replicate level (5 replicates/group); (d) feed conversion ratio (FCR) calculated as relation of feed intake and body weight gain (5 replicates/group) at replicate level. The daily feed intake was calculated as the difference between the amount of feed supplied to the birds and the amount of feed left over in the morning. Until 21 days of age, the performance parameters of 30 birds/group were determined. Thereafter, 25 and 20 birds/group were used for measuring the performance parameters at 28 and 35 days of age, respectively.

Determination of Paracellular Permeability Applying Ussing Chambers

From five birds per group at each killing time point, segments of duodenum, jejunum and cecum (2 replicates/ segment/ bird) were harvested and prepared for Ussing chamber studies to measure the paracellular mannitol fluxes. Epithelial layers had an exposed serosal area of 1.1 cm^2 and were incubated with 12 mL of buffer solution on their mucosal and serosal sides under short-circuit conditions. Following tissue stabilization, ^{14}C-mannitol (0.1 mCi/ml; Hartmann Analytic) was added to the mucosal solution. After a 30-min equilibration period, at the beginning and at the end of the experiment, 100 µl were taken from the "hot side" of each chamber in order to calculate the specific activity of this Ussing chamber from the mean value of the samples. Thereafter, during the course of the experiment, 0.6-mL samples were taken from the serosal compartment (cold side) at defined time intervals (every 30-min). Manipulation of Ussing chambers and experimental procedures were performed as described by Awad et al. (26). Finally, after addition of a liquid scintillation fluid to all samples up to 5 mL, the presence of radioisotope in the samples was measured by a liquid scintillation counter (Aquasafe 300 Plus, Zinsser Analytic, Maidenhead, UK).

Bacterial Enumeration

For bacterial enumeration, 1 g of tissue samples from five birds per group (one bird/ replicate) at each killing time point was collected. The samples were homogenized (Ultra-Turrax IKA, Staufen, Germany) in 1:10 (wt:vol) phosphate-buffered saline (PBS). Afterwards serial ten-fold dilutions were prepared from the stock suspension. Serial dilutions varied for each organ as varying numbers of bacteria were expected (for liver and spleen from 10^0 up to10^2; duodenum from 10^0 up to 10^4; jejunum from 10^2 up to 10^4; and cecum from 10^4 up to 10^{10}).

C. jejuni reference strain NCTC 12744 was cultivated at 41.5°C for 48 h under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) on CASA agar (BioMerièux, Vienna, Austria) or modified charcoal-cefaprazone-deoxycholate (mCCD) agar (CM0739, OXOID, Hampshire, UK). *Escherichia coli* was aerobically grown at 37°C for 24 h on MacConkey agar (selective medium, Neogen, Heywood, UK) (16). Enumeration of *C. jejuni* (red colonies) and *E. coli.* (pink colonies) from intestinal samples was performed by plating 100 µl of each dilution on CASA agar (BioMerièux, Vienna, Austria) and MacConkey agar in duplicate, respectively. To determine the translocation of *C. jejuni* and *E. coli* in liver and spleen, 100 µl of each dilution was also plated in duplicate on either CASA or MacConkey agar, respectively. CFU counts were determined by calculating the mean value of the both plates. Generally, the suitable colony counting range was < 300 CFU on plate.

Statistical Analysis

All statistical analyses were performed using IBM SPSS statistics 24, SPSS software (Chicago, IL, USA). Data are presented as means with standard error of mean (SEM). To evaluate the normality Kolmogorov-Smirnov's test was utilized. A multivariate general linear model, ANOVA, Duncan's multiple range test and LSD were performed to analyze performance, bacterial translocation and mannitol flux data. Furthermore, statistical analysis of *in vitro* bacterial growth data for significant differences between the two groups was performed using Student's *t*-test. *P*-values of ≤ 0.05 were considered significant.

RESULTS

Birds' Performance Parameters

Results of the average body weights (BW, means of all individual birds) and body weight gains (BWG) of the broilers fed with either control diets or diets contaminated with DON are presented in **Figures 1A,B**. In general, a tendency of decreased BW was found in broilers when fed with 5 ppm DON compared to the controls ($P < 0.1$), but this effect did not reach statistical significance. However, at wk 2 and wk 5 of age, a significant decreased in BW ($P = 0.029$ and $P = 0.050$, respectively) was

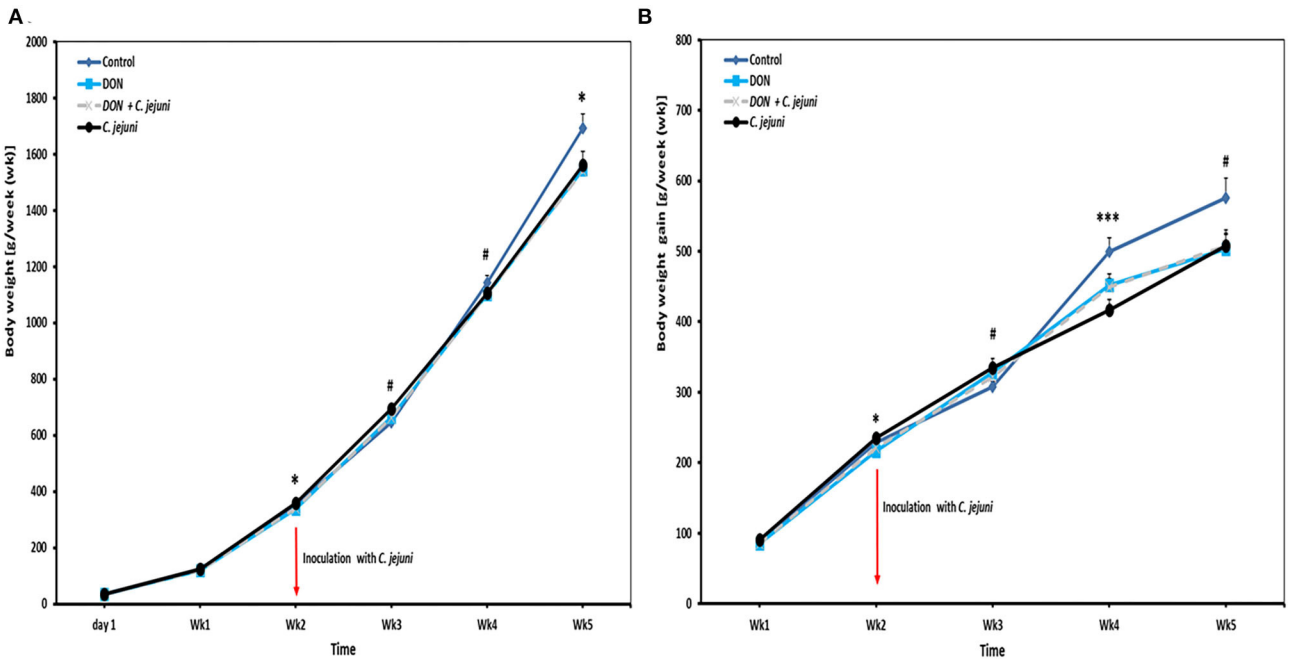

FIGURE 1 | Effect of DON and/or *C. jejuni* exposure on **(A)** body weight (BW) and **(B)** body weight gain (BWG). Results are presented as mean and standard error of mean (SEM). Asterisks mark differences with $^{\#}P \leq 0.1$, $^{*}P \leq 0.05$, or $^{***}P \leq 0.001$.

found in the DON-only group compared to the control group. Furthermore, at 2 and weeks 4 of age, a significantly lower BWG was seen in birds fed with DON contaminated diet with or without *C. jejuni* compared to the control group ($P < 0.05$ and $P < 0.001$). But this effect did not reach statistical significance at week 3 and 5. The overall body weight was significantly ($P = 0.052$) lower (1,509 ± 33 g) in the DON group compared with the control group (1,659 ± 48 g).

Throughout the whole experiment the feed intake was numerically lower in DON-only group compared to the other three groups (**Figure 2A**). In addition, slight differences were observed in the average daily feed intake between control birds (80 g/bird/d) and infected birds (73 g/bird/d). At week 2 of age, a significant decreased in feed intake ($P = 0.041$) was found in DON-only group compared to the control group. However, an increased feed intake from week 4 onwards was recorded in birds from groups 3 and 4 infected with *C. jejuni* compared to the other groups. In this context, the feed conversion rate (FCR) at wk 5 of infected birds with or without DON was higher (1.8 ± 0.10) compared with control birds (1.5 ± 0.07). Throughout the whole trial the average feed conversion ratio was lower in the group in which birds received DON contaminated diet only compared with the other three groups, but this effect did not reach statistical significance (**Figure 2B**).

Intestinal Permeability

The unidirectional flux of ^{14}C-mannitol (paracellular marker) in duodenum, jejunum and cecum is shown in **Figures 3A–C**. During the baseline period (30–60 min), at 7, 14, and 21 dpi, there were significant differences ($P < 0.05$) in the flux of mannitol in the duodenum among the different groups. Moreover, the co-exposure to DON and *C. jejuni* potentiates this negative effect on the permeability of the duodenum in the second (60–90 min) and the third (90–120 min) flux periods, which is indicated by a higher mannitol flux at 14 dpi ($P < 0.05$) and 21 dpi ($P < 0.01$) compared to the controls. Similarly, the results revealed that DON exposure induces an increased mannitol flux in the jejunum and cecum at 14 and 21 dpi especially during the second flux period (60–90 min). This indicated that the increased luminal mannitol concentration from 10 to 20 mM resulted in an increase in mannitol flux most probably due to passive diffusion. The results provided evidence that DON could increase the paracellular intestinal permeability at certain time points or intestinal segments, which might explain the elevated *E. coli* translocation to the liver and spleen. Data showed that DON impact on intestinal permeability varies highly between different gut sites, which likely reflects different mechanisms for the alterations of intestinal permeability of each intestinal segment. The results also revealed that *C. jejuni* as such induces an increase in the flux of ^{14}C mannitol in the jejunum and cecum at 14 and 21 dpi, and in the duodenum at 21 dpi.

Influence of DON on Colonization and Translocation of *E. coli* and *C. jejuni*

Higher numbers of *E. coli* were detected in the duodenum, jejunum and cecum at 14 dpi in both DON-fed groups either with or without *C. jejuni* compared to the control group (**Figure 4**). A significant increase in bacterial counts was only found in cecum,

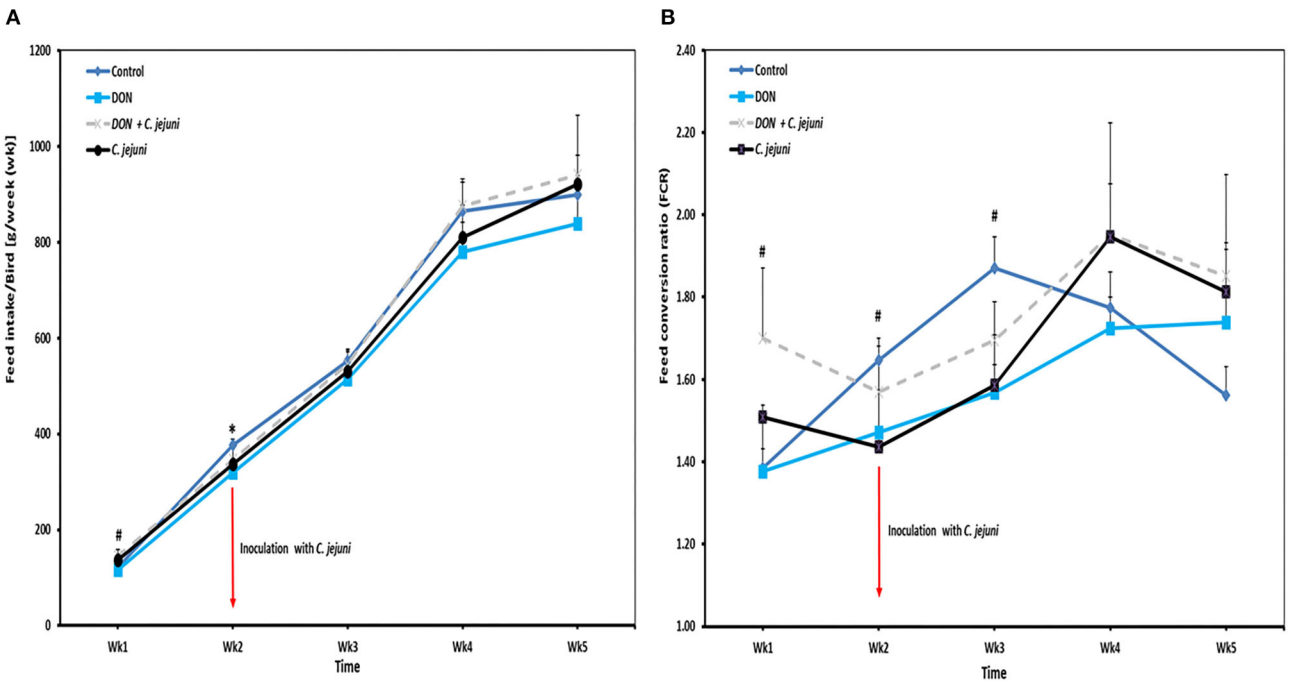

FIGURE 2 | Effect of DON and/or *C. jejuni* exposure on **(A)** feed intake (FI) and **(B)** feed conversion rate (FCR). Results are presented as mean and SEM. Asterisks mark differences with $^{\#}P \leq 0.1$, or $^{*}P \leq 0.05$.

while the counts for the other locations were higher as well but the statistical significance was not reached. Interestingly, at 7 dpi the translocation (translocation in all birds present) of *E. coli* to the liver and spleen was higher in group 3 (DON diet + *C. jejuni*) coinciding with the highest number of *C. jejuni* in the duodenum. However, at 14 and 21 dpi this effect could only be attributed to the presence of DON.

No *Campylobacter jejuni* were detected in swab samples taken from day-old birds and prior to infection at 14 days of age. Non-infected birds stayed *Campylobacter jejuni* -negative throughout the experiment. In infected groups, shedding of *Campylobacter* was detected in almost all infected birds (90%). Fecal droppings remained normal in both control and infected birds, with no signs of diarrhea over the course of the animal trial. A tendency of increased *Campylobacter* load in the jejunum at 7 dpi and 14 dpi as well as in cecum at 7 dpi was recognized in birds fed with DON contaminated diet (**Figure 5**). This increase was more pronounced in the ceca 21 dpi ($P < 0.01$). Furthermore, the presence of DON in the diet led to an increase in *Campylobacter* translocation to liver and spleen at 7 dpi, but this effect did not reach statistical significance. The results demonstrated that the translocation of *C. jejuni* to liver and spleen varied depending on sampling time point postinfection, as indicated by the detection of *C. jejuni* in 3/5 infected birds at 7 and 21 dpi but not at 14 dpi.

DISCUSSION

Both, mycotoxin contamination of feed and *C. jejuni* prevalence in broilers have an increasing global health and economic impact on poultry production. The *Fusarium* mycotoxin DON is a ubiquitous mycotoxin with negative effects on the growth performance of broiler chickens (26, 27). As an inhibitor of protein synthesis DON mainly affects cells with a high-protein turnover, such as intestinal epithelial and immune cells (28). The intestinal mucosa acts as an effective defensive barrier against the invasion of harmful substances and counteracts the occurrence of intestinal diseases (29). The negative impact of DON on the gut barrier has been associated with destruction of intestinal architecture, inhibition of activity of intestinal stem cells and changes in the modulation of tight junction protein expression (2, 18, 26, 30–35). Similarly, effects of *C. jejuni* on the gut physiology of chickens have already been reported. It was shown that these bacteria have a negative impact on the nutrient absorption indicating that a lower slaughter weight might probably be due to the reduction in feed efficiency (13). Furthermore, the occurrence of a leaky gut syndrome caused by *C. jejuni* is known by enhancing bacterial translocation from the gut to internal organs (16).

Vanderbroucke et al. (3) reported that the exposure of pigs to DON and *S.* Typhimurium promoted *Salmonella* invasion in the gut and its translocation across the intestinal epithelium. Similarly, an increased translocation of *E. coli* following DON exposure was reported in human and porcine cell monolayers *in vitro* and in chickens *in vivo* (18, 26, 36). So far, no data are available on the co-exposure of DON and *C. jejuni* in broiler chickens, the purpose of the actual study.

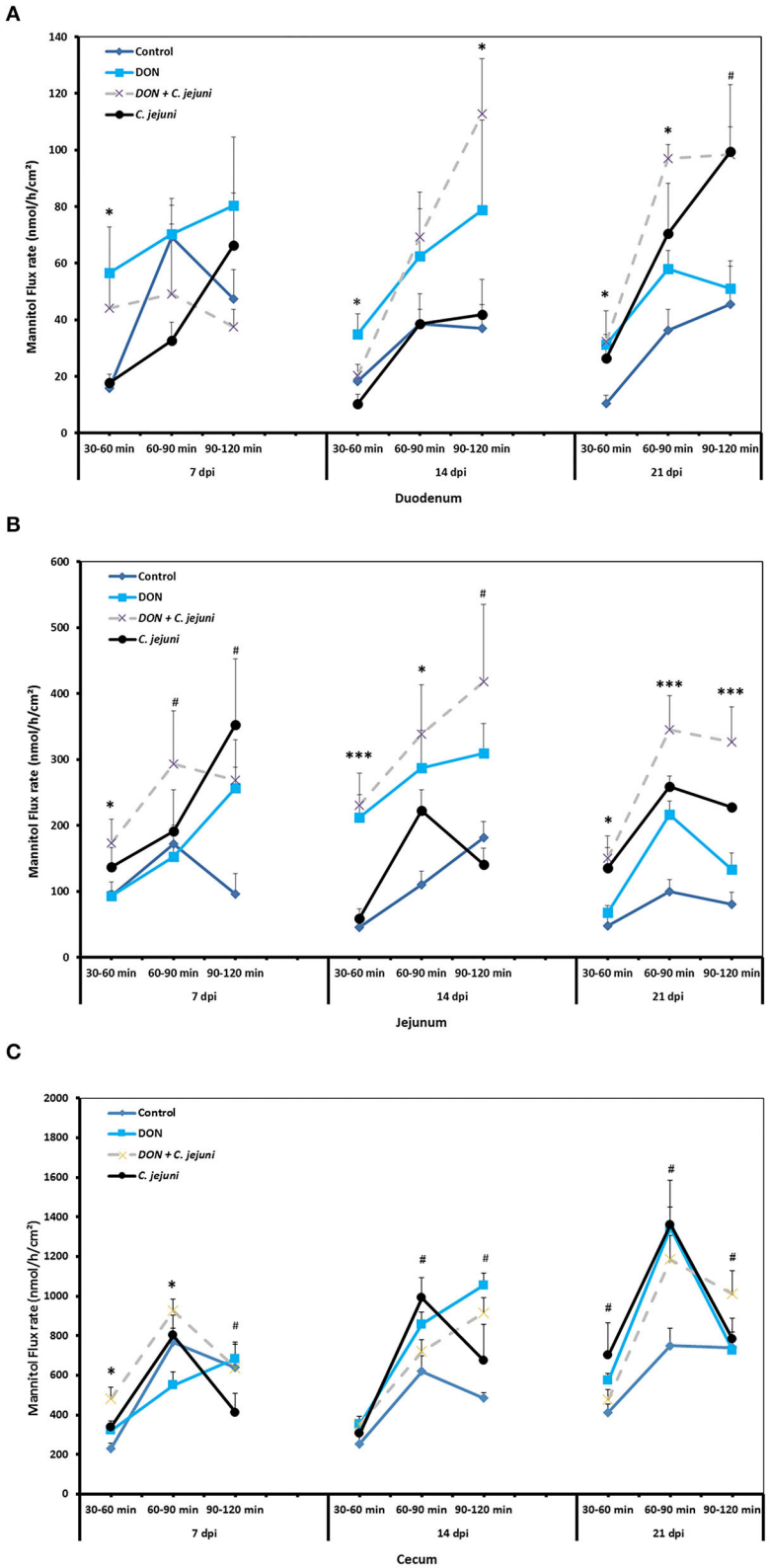

FIGURE 3 | Effect of DON and/or *C. jejuni* exposure on paracellular permeability in duodenum **(A)**, jejunum **(B)**, and cecum **(C)** at different times post infection. Mucosal to serosal flux (J_{ms}) of the permeability marker ^{14}C-mannitol were performed in Ussing chambers. Data are presented as the mean values and SEM ($n = 5$). Asterisks mark differences with $^{\#}P \leq 0.1$, $^{*}P \leq 0.05$, or $^{***}P \leq 0.001$.

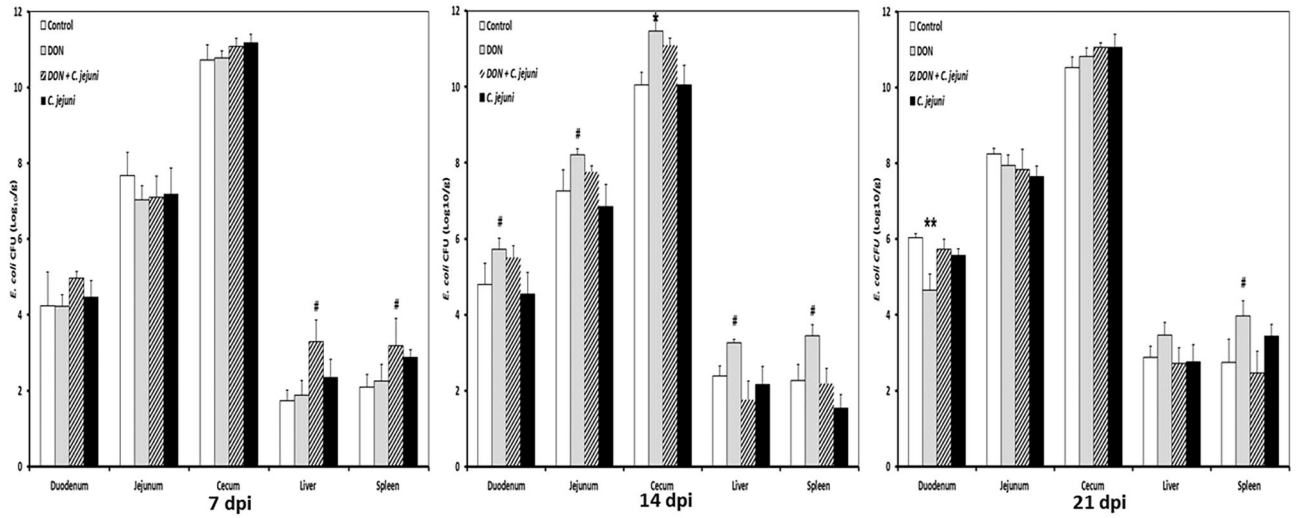

FIGURE 4 | *E. coli* counts at different time points post infection from duodenum, jejunum, cecum, liver, and spleen in birds fed with either control or DON contaminated diet with or without *C. jejuni* infection. Results are presented as mean values and SEM ($n = 5$). Number of bacteria are expressed in logarithmic form of colony forming units (log CFU/g). Asterisks mark differences with $^{\#}P \leq 0.1$, $^{*}P \leq 0.05$, or $^{**}P \leq 0.01$.

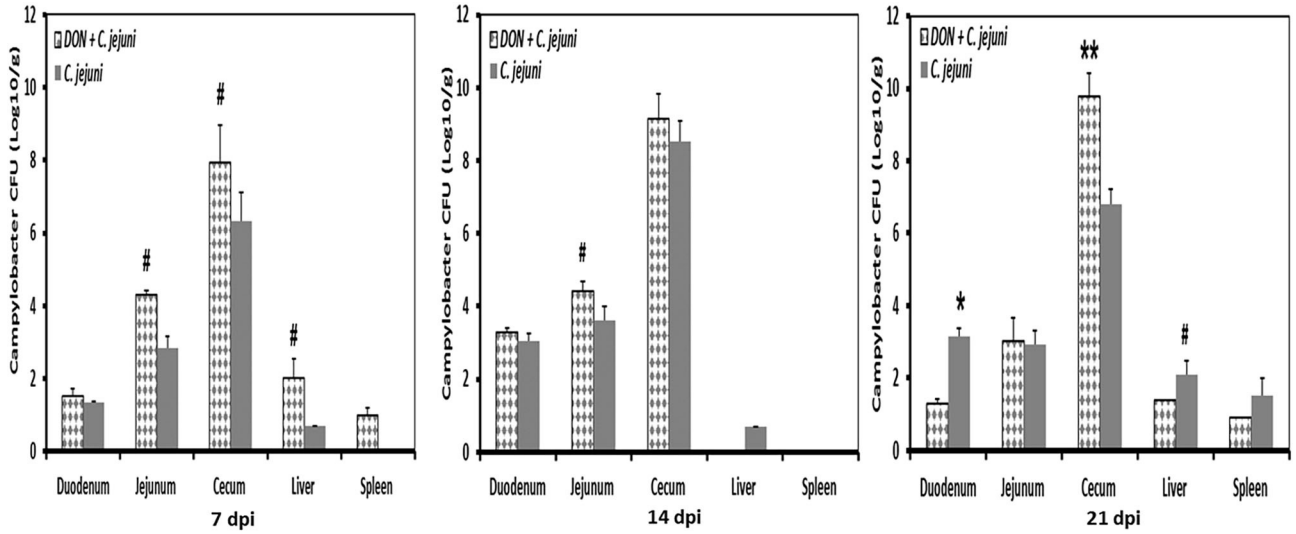

FIGURE 5 | *C. jejuni* counts at different time points post infection from duodenum, jejunum, cecum, liver, and spleen of infected birds fed with either control or DON contaminated diet. Results are presented as mean values and SEM ($n = 5$). Numbers of bacteria are expressed in logarithmic form of colony forming units (log CFU/g). Asterisks mark differences with $^{\#}P \leq 0.1$, or $^{*}P \leq 0.05$.

The study indicates that the co-exposure of broiler chickens to DON and *C. jejuni* supported the *C. jejuni* colonization in the gut at certain time points post-infection, revealing that DON might provide a favorable condition for *Campylobacter* growth. Furthermore, an indirect mechanism should not be excluded as DON can induce a leakage of plasma amino acids or proteins into the intestinal lumen (4, 20). The mode of action, either direct or indirect, needs to be determined in future studies to elucidate whether such substances provide the necessary growth substrate for extensive proliferation of *Campylobacter*. Understanding how mycotoxins influence prokaryotes began to emerge as an important area of future research perspectives (37, 38). Recently presented evidence indicates that DON can negatively affect the gut microbiota of either humans or animals (39, 40). This, in turn, has led to a greater interest in understanding bacterial responses toward DON.

Independent of this, the co-exposure promotes the translocation of *C. jejuni* and *E. coli* to the liver and spleen at certain time points post infection. In this context, the results also indicated that DON increased the intestinal paracellular

permeability as reported in our previous study (26). However, in the current study, in addition to jejunum and cecum, the permeability of the duodenum was impaired at different time points demonstrating a detrimental effect of DON on the barrier function of the entire intestine.

The study also revealed that the co-exposure to DON and *C. jejuni* potentiates a significant increase in paracellular permeability as both, pathogen and mycotoxin, affected barrier functions of the intestine. Thus, the presented data indicated that increased gut paracellular permeability could be associated with a higher translocation of *C. jejuni* and *E. coli* at certain time points. In context with the previous results (26), the present study showed that DON in a concentration of 5 mg/kg could have a negative effect on the growth performance of chickens. Those results are of practical relevance as the current guidance for the tolerated value of DON in poultry diets is set at 5 mg/kg feed (European Commission No. 1881/2006).

Taken altogether, results of the actual studies strengthen the hypothesis that DON could influence the infection profile of *C. jejuni*, a subject not demonstrated so far. Indeed, it was found that the co-exposure of broilers to DON and *C. jejuni* can potentiate gut permeability, promote *Campylobacter* colonization and its translocation across the intestinal epithelium. This in turn will lead to a greater interest in understanding bacterial responses toward DON, and the involved mechanisms in order to build a more comprehensive picture of DON-induced changes in prokaryotes, highlighting the need for further investigations of DON effects on prokaryotes.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the institutional ethics committee of the University of Veterinary Medicine and the Ministry of Research and Science under the license number GZ -68.205/0179-V/3b/2018.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Enteropathogenic *Escherichia coli* Infection Induces Diarrhea, Intestinal Damage, Metabolic Alterations, and Increased Intestinal Permeability in a Murine Model

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Enteropathogenic *E. coli* (EPEC) are recognized as one of the leading bacterial causes of infantile diarrhea worldwide. Weaned C57BL/6 mice pretreated with antibiotics were challenged orally with wild-type EPEC or *escN* mutant (lacking type 3 secretion system) to determine colonization, inflammatory responses and clinical outcomes during infection. Antibiotic disruption of intestinal microbiota enabled efficient colonization by wild-type EPEC resulting in growth impairment and diarrhea. Increase in inflammatory biomarkers, chemokines, cellular recruitment and pro-inflammatory cytokines were observed in intestinal tissues. Metabolomic changes were also observed in EPEC infected mice with changes in tricarboxylic acid (TCA) cycle intermediates, increased creatine excretion and shifts in gut microbial metabolite levels. In addition, by 7 days after infection, although weights were recovering, EPEC-infected mice had increased intestinal permeability and decreased colonic claudin-1 levels. The *escN* mutant colonized the mice with no weight loss or increased inflammatory biomarkers, showing the importance of the T3SS in EPEC virulence in this model. In conclusion, a murine infection model treated with antibiotics has been developed to mimic clinical outcomes seen in children with EPEC infection and to examine potential roles of selected virulence traits. This model can help in further understanding mechanisms involved in the pathogenesis of EPEC infections and potential outcomes and thus assist in the development of potential preventive or therapeutic interventions.

Keywords: enteropathogenic *Escherichia coli*, murine model, diarrhea, enteropathy, antibiotics, inflammation

INTRODUCTION

Gastroenteritis remains a major cause of morbidity and mortality in young children especially in developing countries (Liu et al., 2015). Enteropathogenic *E. coli* (EPEC) has been recognized by the Global Enteric Multicenter Study (GEMS) and Malnutrition and Enteric Disease (MAL-ED) studies as one of the major causes of moderate to severe diarrhea in children (Kotloff et al., 2013; Platts-Mills et al., 2015). Infection results in acute watery diarrhea accompanied by fever, vomiting and dehydration (Kaper et al., 2004; Guerrant et al., 2011).

EPEC contains the locus of enterocyte effacement regulator (*Ler*) gene, a major transcriptional activator of LEE pathogenicity island, comprising around 41 open reading frames (Frankel et al., 1998; Friedberg et al., 1999). EPEC virulence is mediated by the Type 3 secretion system (T3SS), characterized by attaching and effacing (A/E) lesions (Frankel et al., 1998). The T3SS consists of EPEC secreted components (Esc) and EPEC secretion proteins (Esp), encoded in the LEE pathogenicity island. In addition, *EscN* is the main driving force assisting in the ATPase response to enable activation of the T3SS, for efficient transportation of effector proteins into the enterocytes of the host (Andrade et al., 2007). During infection, EPEC attaches to epithelial cells *via* bundle forming pili (*bfp*) (Giron et al., 1991) followed by intimate adherence with the aid of the translocated intimin receptor (*tir*) and intimin (*eae*), which results in actin accumulation and formation of pedestal structures (Knutton et al., 1989; Guerrant et al., 2011). EPEC is characterized by the presence (typical EPEC) or absence (atypical EPEC) of *bfp*. Typical EPEC are characterized by Localized Adherence (LA) *in vitro* (Kaper et al., 2004) and have been reported to cause severe diarrhea in children under 12 months of age and in certain cases results in death (Kotloff et al., 2013; Platts-Mills et al., 2015). Atypical EPEC is characterized by LA-like (Scaletsky et al., 2010), aggregative adherence or diffuse adherence *in vitro* (Pelayo et al., 1999; Mora et al., 2009) and are increasingly being detected in children worldwide (Abe et al., 2009; Hu and Torres, 2015).

Pathogens such as EPEC compete with the resident microbiota for nutrients in order to colonize the intestinal environment. According to Freter's nutrient niche, in order for microbes to be successful, they must have the capacity to grow fast in the intestine compared to its competitors (Freter et al., 1983). These pathogens require the same carbon pathways which commensal *E. coli* uses, such as mannose and galactose *in vivo* (Fabich et al., 2008).

EPEC have been studied extensively *in vitro*, which enables studies of localization traits, A/E lesions and expression of the T3SS effector proteins (Giron et al., 1991; Knutton et al., 1999; Leverton and Kaper, 2005). *In vivo* studies have shown that a complete intestinal environment helps further determine the specific roles of EPEC traits involved in infections in animals and humans (Law et al., 2013). Animal models such as *Caenorhabditis elegans*, rabbits, pigs, and cattle have been used to study EPEC infections (Moon et al., 1983; Larsen et al., 1995; Dean-Nystrom et al., 2002; Misyurina et al., 2010). Infections induced by EPEC in C57BL/6 mice have also been reported (Savkovic et al., 2005; Royan et al., 2010; Zhang et al., 2010; Rhee et al., 2011; Manthey et al., 2014; Dupont et al., 2016), showing colonization of EPEC in the intestinal epithelial microvilli (Savkovic et al., 2005), changes in tight junction morphology and epithelial barrier function accompanied by inflammatory responses (Zhang et al., 2010; Zhang et al., 2012).

Most of the previous EPEC infection murine models have used streptomycin in order to disrupt the intestinal microbiota and promote colonization in mice (Mundy et al., 2006; Zhang et al., 2010; Rhee et al., 2011; Zhang et al., 2012; Dupont et al., 2016). Recently, a study demonstrated that mice are susceptible to colonization with EPEC in an age and microbiota disruption-dependent manner, with infant (preweaning) mice being more susceptible (Dupont et al., 2016). These animal models have provided insights into the understanding of potential pathogenetic mechanisms of EPEC infection in humans. However, these models have not been able to replicate clinical outcomes observed in humans (growth decrement and diarrhea).

A murine EPEC infection model able to induce changes in body weight and diarrhea, which are important outcomes in children infected by EPEC, had been needed and was the focus of our current study (Law et al., 2013). We have previously shown that disruption of intestinal microbiota using a broad-spectrum antibiotic cocktail enabled colonization of bacterial pathogens such as Enterotoxigenic *E. coli* (Bolick et al., 2018), *Campylobacter jejuni* (Giallourou et al., 2018) and *Shigella flexneri* (Medeiros PH et al., 2019) resulting in diarrhea in C57BL/6 mice. This current study also used the same antibiotic cocktail to enable the assessment of disease outcomes associated with EPEC infection. We therefore, describe a weaned murine model in which the microbiota have been disrupted *via* broad-spectrum antibiotics to enable efficient colonization and clinical outcomes of EPEC infection in mice resulting in growth impairment, diarrhea, intestinal damage, metabolic alterations, and increased intestinal permeability.

MATERIALS AND METHODS

Ethics Statement

The mice used in the study have been handled with strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol has been approved by the Committee on the Ethics of Animal Experiments of the University of Virginia (Protocol Number: 3315). All efforts were made to minimize suffering. This is also in accordance with the Institutional Animal Care and Use Committee policies of the University of Virginia. The University of Virginia is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International (AAALAC).

Mice

Mice used in this study were male, 22 days old, C57BL/6 strain, ordered from Jackson Laboratories (Bar Harbor, ME). Mice weighed approximately 10 grams on arrival and were co-housed in groups of up to 4 animals per cage. The vivarium was kept at a temperature of between 20 and 23°C with a 14-h light and 10-h dark cycle. Mice were allowed to acclimate for

3 days upon arrival. Mice were fed standard rodent house chow diet from arrival and throughout the infection challenge.

EPEC Inoculum Preparation

Bacterial strains used included: wild type EPEC E2348/69 [(serotype O127:H6) first isolated in 1969 during infantile diarrhea outbreak in Taunton, United Kingdom, it belongs to phylogroup B2 with full length chromosomal nucleotide (Accession number: FM180568)] (Levine et al., 1985; Iguchi et al., 2009) and EPEC E2348/69 Δ*escN* CVD425 (Jarvis et al., 1995). Bacterial cultures were prepared from glycerol stocks maintained at -80°C. Cultures were grown in 20 ml Dulbecco's modified Eagle's medium containing phenol red (DMEM) at 37°C in a shaking incubator until cultures turned orange indicating optimal growth, OD_{600} ~ 0.6. Cultures were centrifuged at 3500 x g for 10 min at 4°C. The bacterial pellet was resuspended in DMEM high glucose in order to obtain 10^{10} CFU/ml.

Enteropathogenic *Escherichia coli* Infection Model

Four days prior to challenge with EPEC, mice were given an antibiotic cocktail of gentamicin (35 mg/L), vancomycin (45 mg/L), metronidazole (215 mg/L), and colistin (850 U/ml) in drinking water for 3 days in order to disrupt resident microbiota, followed by 1 day on normal water in order to clear antibiotics (Bolick et al., 2018). Then, mice were administered 100 µl of 10^{10} CFU/ml (10^{10} bacteria per mouse) bacterial culture in DMEM high glucose orally using 22-gauge feeding needles. Uninfected control mice were administered only 100 µl DMEM high glucose.

After infection, all mice were weighed and stools were collected daily until 8-days post infection (p.i.). Mice were euthanized on days 3, 7, and 8 p.i. **Figure 1A** shows the schematic presentation summarizing the experimental procedure.

Analysis of Clinical Outcomes

The clinical outcomes, body weight and diarrhea were assessed daily. Body weight was measured for 9 days (starting before infection, day 0) and percentage of changes in body weight was measured based on each individual mouse weight from day 0 before infection. Diarrhea scores were measured until day 7 p.i. Diarrhea score were based on the following 0–4: 0-well-formed pellets; 1- stick stools adhering in microtubes wall; 2-pasty stools with or without mucus; 3-watery stools with or without mucus; and 4-Stools with blood.

Tissue Burden and Stool Shedding

For stool shedding, DNA was extracted from stools using the QIAamp DNA Stool Mini kit (Qiagen) according to the manufacturer's instructions. For tissue burden, tissues were homogenized using the beat-beater and DNA was extracted using DNeasy Kit (Qiagen) according to the manufacturer's instructions. The *eae* (intimin) gene was used as a specific target for detecting EPEC in stools and tissues. Primer sequences included *eae* 5'-CCCGAATTCGGCACAAGCATAAGC-3' (sense) and 5'-CCCGGATCCGTCTCGCCAGTATTCG-3' (antisense) (Zhang et al., 2002). Real-time PCR was performed using Bio-Rad CFX under the following conditions: 95°C for 3 min, followed by 40 cycles of 15 s at 95°C, 60 s at 55°C, and lastly 20 s at 72°C.

EPEC Adherence on the Intestine

Ileal tissue segments from EPEC-infected mice on day 3 p.i. were fixed in 4% formalin, embedded in paraffin, the slides were stained with rabbit anti-intimin at the University of Virginia Histology core, and viewed using light microscope.

Transmission Electron Microscopy

For TEM, the ileal tissues from EPEC-infected mice on day 3 p.i. and uninfected (control) were fixed with 4% glutaraldehyde. The samples were washed with 1X cacodylate buffer for 10 min and placed in 2% osmium tetroxide for 1 h. Then washed for 10 min with cacodylate buffer and distilled water. Followed by dehydration with 30% ethanol for 10 min and concentrations of 50%, 70%, 95%, and 100% ethanol all for 10 min each. About 1:1 ethanol/propylene oxide was used for 10 min followed by 100% propylene oxide (PO) for 10 min. The samples were then placed in 1:1 of PO/epoxy resin (EPON) overnight followed by 1:2 PO/EPON for 2 h, then 1:4 PO/EPON for 4 h, and lastly 100% EPON overnight. The samples were then embedded in fresh 100% EPON and allowed to bake in a 65°C oven. Ultra-thin sections were cut at 75 nm and picked up on 200 mesh copper grids. Sections were stained with 0.25% lead citrate and 2% uranyl acetate. The slides were viewed using JEOL 1230 microscope, with 4k x 4k CCD camera.

Histology Analysis

Mice intestinal samples (ileum and colon) from day 3 or day 7 p.i. were fixed in 10% neutral buffered formalin for 20 h, dehydrated and embedded in paraffin. Ileal and colonic sections (5 µm) were stained with hematoxylin and eosin staining (H&E) and were examined using light microscopy. Histopathological scores were performed by a blinded investigator, using previously described methods (Wong et al., 2015). Histological damage scores were determined by quantifying the intensity of epithelial tissue damage (0–3, 0-no damage, 1-mild, 2-moderate, 3-extensive), edema in submucosa layer (0-3), and neutrophil infiltration (0-3).

Intestinal Inflammatory Response by Enzyme-Linked Immunosorbent Assay

Protein lysates were extracted from the stools, ileum, colon and cecal contents using radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS) containing protease inhibitor cocktail (Roche) and phosphatase inhibitors (1 mM sodium orthovanadate, 5 mM sodium fluoride, 1 mM microcystin LR, and 5 mM β-glycerophosphate). Lysates were centrifuged at 13,000 rpm for 15 min and the supernatant was used to perform the protein assay using the bicinchoninic acid assay (Thermo Fisher Scientific). Inflammatory biomarkers (LCN-2, MPO, IL-23, IL-22, IL-17, GMCSF, IL-33, and IL-10) were measured using a commercial ELISA kit (R&D Systems) according to the manufacturer instructions. Interleukin-6 (IL-6), IL-1β, interferon gamma

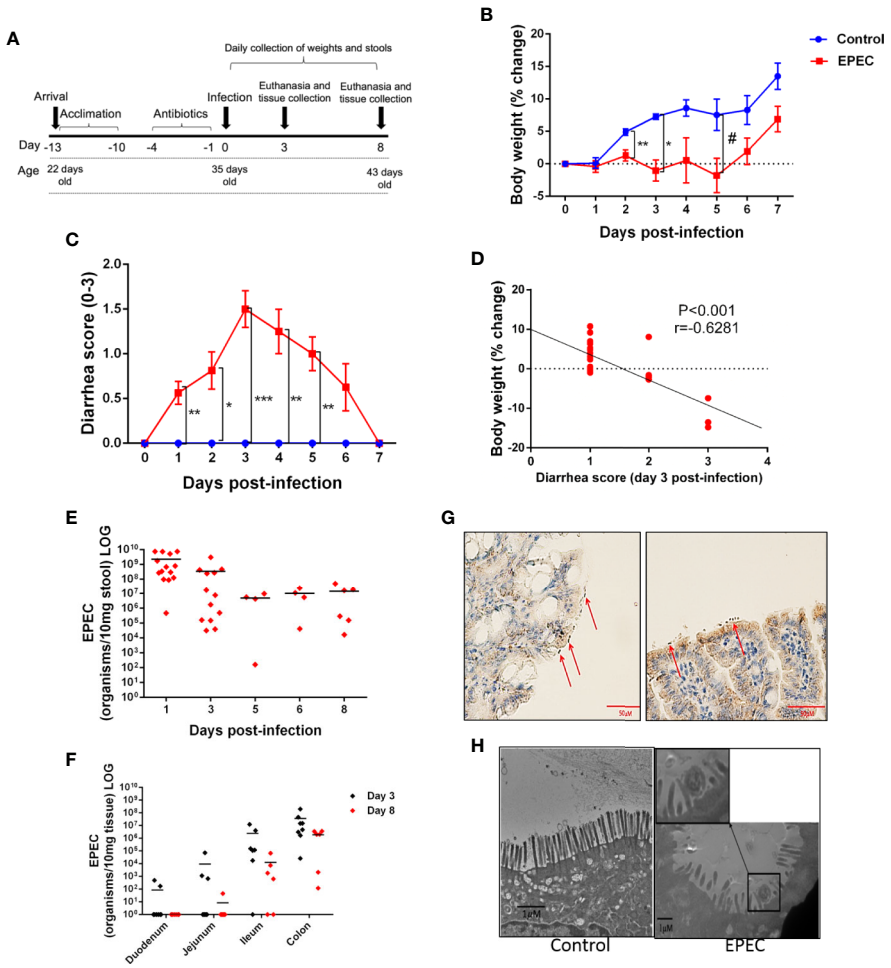

FIGURE 1 | EPEC infection impairs weight gain, induces diarrhea and colon colonization in C57BL/6 mice. **(A)** Experimental timeline of EPEC infection model. Weaned C57BL/6 mice pretreated with antibiotic cocktail were orally infected with EPEC (10^{10} CFU). **(B)** Change in body weight of C57BL/6 mice infected with EPEC (EPEC) or uninfected (Control) (n=12/group). Line graphs represents mean ± SEM. $^{**}p< 0.009$, $^{*}p<0.001$ and $^{\#}p< 0.04$ using multiple Student's *t-test*. **(C)** Diarrhea score of EPEC and control mice. Line graphs represent median ± SEM. $^{*}p<0.01$, $^{**}p<0.006$ and $^{***}p<0.0001$ using Kruskal-Wallis test followed by Dunn's test. **(D)** Significant negative correlation between diarrhea score and change in body weight at day 3 p.i (Spearman rank test). **(E)**. Quantification of EPEC shedding in stools. **(F)** Quantification of EPEC tissue burden in the intestinal tissues (duodenum, jejunum, ileum and colon) at days 3 and 8 p.i. **(G)** Immunohistochemistry staining of intimin (red arrows), in ileal tissue of EPEC infected mice at day 3 p.i. **(H)** TEM at day 3 p.i., showing disruption of the microvilli of mice infected with EPEC.

(INF-γ), TNF-α, KC (analogue to human IL-8), and IL-18 and CRP levels were measured using a ProcartPlex multiplex immunoassay (Invitrogen) by Luminex (Biorad). Biomarkers levels were measured as picograms per milligram of protein.

Phosphorylated-Signal Transducer *and* Activator *of* Transcription-3, Phosphorylated-Cyclic AMP Response Element-Binding Protein and Cleaved Caspase-3 Measurement

Colonic samples from EPEC-infected and control mice collected at day 3 p.i. were homogenized in ice-cold using RIPA buffer containing protease and phosphatase inhibitors. The colonic levels of phosphorylated STAT3 3 (pSTAT3), phosphorylated CREB (pCREB) and cleaved caspase-3 were measured using ELISA kits (R&D Systems) according to the manufacturer's instructions.

TaqMan- Real Time Polymerase Chain Reaction

The isolation of total RNA from colon tissues of EPEC-infected (presenting moderate or severe diarrhea) and control mice were performed by using a Qiagen RNeasy mini kit and QIAcube. cDNA was synthetized from 1μg of total RNA, quantified by Qubit 3 fluorometer 3000 (Invitrogen) and purified by deoxyribonuclease I (Invitrogen) treatment, with the iScript cDNA (Bio-Rad) as described by manufacturer instructions. qPCR was performed with 50 ng of cDNA in each well and SensiFAST probe no-ROX mix (Bioline) using a CFX Connect system (Bio-Rad) with the following conditions: 95°C for 2 min, 40 cycles of 95°C for 10 s and

60°C for 50 s. A pre-designed TaqMan array mouse immune fast 96-well plates (Applied Biosystems) was used to assess the expression of 92 genes listed in **Supplementary Table 1**. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene. All fold changes were determined using the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001).

In Vivo Intestinal Permeability Assay

For assessing *in vivo* intestinal permeability fluorescein isothiocyanate (FITC)-labeled dextran assay (4kDa, Sigma Aldrich) was used. Mice were deprived food, with free access to water, for 4 h. Then, 200 µl of FITC-dextran solution (80 mg/ml in water) was administered by oral gavage for each mouse. After 4 h of FITC administration, mice were anesthetized to collect blood using cardiac puncture. Then, the blood samples were centrifuged (5 min, 8,000 rpm, 4°C) and plasma was obtained. Fluorescence intensity in 100 µl of plasma placed on Qubit 0.5 ml-microtubes (Life Technologies) was measured using Qubit 3fluorometer (Life Technologies) using an excitation wavelength of 470 nm. A plasma sample from mice not receiving FITC-dextran solution was used as a blank.

^{1}H Nuclear Magnetic Resonance Spectroscopy-Based Metabolic Profiling

Urine specimen were collected in a sterile 1.5 ml eppendorf tube and placed at -80°C until further analysis. The metabolic profiling was performed on all urine samples using ^{1}H nuclear magnetic resonance (NMR) spectroscopy. A 30 µl urine aliquot was combined with 30 µl of phosphate buffer (pH 7.4, 100% D_2O, 0.2 M Na_2HPO_4/NaH_2PO_4) containing 1 mM of the internal standard, 3-(trimethylsilyl)-[2,2,3,3-$^2H_4$]-propionic acid (TSP), and 2 mM sodium azide (NaN_3) as a bacteriocide. Samples were vortexed and spun at 13,000 x g for 10 min and 50 µl of the supernatant was then transferred to 1.7 mm NMR tubes. Spectroscopic analysis was performed on a 600 MHz Bruker Avance™ NMR spectrometer at 300 K using a Bruker BBI probe and an automated SampleJet for tube handling (Bruker, Germany). ^{1}H NMR spectra of the urine samples were acquired using a standard one-dimensional pulse sequence [recycle delay (RD) -90°-t_1–90°-t_m-90°-acquire free induction decay (FID)]. The water signal was suppressed through irradiation during the RD of 4 s and a mixing time of (t_m) 100 ms was used. For each spectrum, 64 scans were obtained into 64 K data points using a spectral width of 12.001 ppm. The NMR spectra were calibrated to the TSP resonance at 0 ppm using TopSpin 3.5 NMR software (Bruker, Germany) and imported into MATLAB (R2018a, Mathworks Inc, Natwick, MA) using in-house scripts. Regions containing the TSP, water and urea resonances were removed from the urinary spectra. ^{1}H NMR spectra were manually aligned and normalized to the unit area.

Western Blotting

Colon tissues from EPEC-infected and control mice at day 7 p.i. were collected, lysed using RIPA lysis buffer containing complete EDTA-free protease inhibitor cocktail (Roche) and phosStop (Roche) and centrifuged (17 min, 4°C, 13,000 rpm). Then the supernatant was collected for extracting protein. Protein concentrations were determined through the bicinchoninic acid assay according to the manufacturer`s protocol (Thermo Fisher Scientific). Reduced 60 µg protein samples (previously prepared with sample reducing agent- Invitrogen- and protein loading buffer-LI-COR) were denatured at 95°C for 5 min, separated on NuPAGE 4%–12% BIS-Tris gel (Invitrogen) and transferred to nitrocellulose membranes (Life Technologies) for 2 h. The membranes were then immersed in iBind fluorescent detection solution (Life technologies) and placed in a iBIND automated Western device (Life Technologies) overnight at 4°C for blocking, incubating with primary antibodies (rabbit anti-β-actin, 1:1000, Thermo Fisher Scientific, PA1-183; mouse anti-claudin-2, 3:500, Invitrogen, 325600; rabbit anti-claudin-1, 1:1000, Novus biological, NBP1-77036) and secondary antibodies (Cy3-conjugated AffiniPure donkey anti-rabbit, 711-165-152, 1:1000, Jackson Immuno-Research and Cy5-conjugated AffiniPure donkey anti-mouse, 715-175-150, 1:1000, Jackson ImmunoResearch). Then, the membranes were immersed in ultrapure water and fluorescent signal was detected using the Typhoon system (GE healthcare). Densitometric quantification of bands was performed using ImageJ software (NIH, Bethesda, MD, USA).

Systemic Inflammation Analysis

Blood collected at day 3 p.i. was centrifuged at 8,000 rpm for 5 min at 4°C in order to obtain the plasma, to measure the levels of SAA as a marker of systemic inflammation. The levels of SAA were measured using a commercial ELISA kit (R&D Systems) according to the manufacturer's instructions. The results were expressed as picograms per milliliter.

Statistical Analysis

All data were analyzed using GraphPad Prism 7 software (GraphPad Software). Data are presented as the mean ± standard error of the mean (SEM) or as medians when appropriate. Student's t test and one-way Analysis of Variance (ANOVA) followed by Tukey's *test* were used to compare means, and the Kruskal–Wallis and Dunn tests were used to compare medians. Spearman rank test was used to correlation analyses. Differences were considered significant when $p< 0.05$. Experiments were repeated at least two times.

For metabolomics data analysis multivariate statistical modelling was performed including principal component analysis (PCA) using the Imperial Metabolic Profiling and Chemometrics Toolbox (https://github.com/csmsoftware/IMPaCTS) in MATLAB (Version 2018a, Mathworks Inc) and unsupervised hierarchical clustering analysis (HCA) to unveil metabolic differences between EPEC infected and control mice. Unsupervised clustering for all samples was done with the use of the normalized abundance of metabolites that were identified through the PCA models. Hierarchical clusters were generated with the use of an average-linkage method by means of the pdist and linkage functions in the MATLAB bioinformatics toolbox. Heat maps and dendrograms after the HCA were generated with MATLAB imagesc and dendrogram functions, respectively. Pathway analysis was performed using the MetaboAnalyst 4.0 platform (https://www.metaboanalyst.ca/). The raw data supporting the conclusions of this

article will be made available by the authors, without undue reservation.

RESULTS

EPEC Infection Leads to Growth Impairment and Diarrhea

Depletion of intestinal microbiota by antibiotics has been shown to be effective in promoting colonization by bacterial pathogens (Bolick et al., 2018; Giallourou et al., 2018; Medeiros PH et al., 2019). We therefore, tested whether pretreatment with antibiotics could enable the study of body weight and diarrhea in mice infected with EPEC (10^{10} CFU) (**Figure 1A**). EPEC inhibited the growth of mice when compared to the control group from days 2 to 5 post infection (p.i.) ($p<0.05$, **Figure 1B**). EPEC infection also induced moderate (days 1, 2, and 6 p.i.) to severe diarrhea at days 3 to 5 p.i. ($p<0.0001$, $p<0.006$) (**Figure 1C**). The changes in body weight exhibited by individual EPEC-infected mice were correlated with their individual diarrhea scores at the overall peak of diarrhea and weight decrements on day 3 (**Figure 1D**), showing that higher diarrhea scores tended to associate with greater weight shortfalls.

EPEC Colonizes the Ileum and Colon in Mice

In order to confirm that growth impairment and diarrhea were promoted by EPEC infection, DNA was extracted from stools of EPEC-infected mice and quantitative PCR was used to measure shedding. As shown in **Figure 1E**, most of the EPEC-infected mice exhibited 10^8-10^{10} organisms/10 mg stool at day 1 p.i. and less shedding was observed from days 5 to 8 p.i.

Given that EPEC is an intestinal pathogen, we further investigated which intestinal sections were predominantly colonized by EPEC using quantitative PCR to measure tissue burdens. EPEC was found to predominantly colonize the ileum ($<10^7$ organisms/mg tissue) and colon ($<10^8$ organisms/mg tissue) of mice at day 3 pi (**Figure 1F**), and the same trend was also observed at day 8 p.i. **Figure 1G** shows intimin-stained EPEC adherence on blunted ileal mucosa, with disruption of the microvilli shown by TEM (**Figure 1H**).

These findings indicate that EPEC infection promotes a self-limited symptomatic acute disease in antibiotic-pretreated mice. Fecal shedding of EPEC, and tissue burdens were detected up to day 8 p.i. in infected mice.

EPEC Infection Promotes Acute Intestinal Tissue Damage and Inflammation

Given that our EPEC infection model resulted in a significant colonization in the ileum and colon, we next investigated whether EPEC infection promotes ileal and colonic damage. The ileal and colonic histological damage in EPEC-infected mice was characterized by the loss of epithelial integrity, moderate edema in the submucosa and infiltration of inflammatory cells into the lamina propria and submucosa, with significant histology score differences from controls in both ileum and colon at day 3, and persistent significant differences in the ileum extending to day 7. The damage was found to be greater in colon compared to control mice (**Figure 2A**) at day 3 p.i., as confirmed by measurement of histologic damage score ($p<0.0001$, **Figure 2B**). On day 7 p.i., the damage in the ileum of EPEC-infected mice was higher when compared to the control mice ($p<0.0001$, **Figures 2A, C**).

Myeloperoxidase (MPO), a marker of neutrophil activity in intestinal mucosa, and lipocalin-2 (LCN-2), a glycoprotein upregulated in tissue damage under infection conditions, have been considered as biomarkers of environmental enteric dysfunction, including EPEC infection in children (Prata et al., 2016; Kosek et al., 2017). To ensure that our EPEC infection model mimics the alterations of these biomarkers as observed in children, we measured MPO and LCN-2 in the ileal and colonic tissues, as well as in cecal contents and stools. We found increased MPO levels in ileum and colon tissues of EPEC-infected mice at day 3 p.i. when compared to the control group ($p<0.05$ and $p<0.03$ respectively, **Figure 2D**). Of note, a trend of increase in MPO levels was also observed in the cecal contents and stools at day 3p.i. (**Supplementary Figure 1**), however no statistical significance was found. On day 7 p.i., MPO levels were reduced in the intestinal (ileum and colon), cecal contents and stools of EPEC infected mice compared to controls (**Figure 2D**, **Supplementary Figure 1**). However, increased LCN-2 levels were found in cecal contents (day 3 p.i.) and stools (days 3 and 7 p.i.) of EPEC-infected mice when compared to control mice ($p<0.05$-day 3 p.i. or $p<0.03$-day 7 p.i., **Figure 2E**).

We found that the diarrhea score correlated with MPO or LCN-2 levels in stools samples of EPEC-infected mice at day 3 p.i., when mice exhibited higher diarrhea score, a positive correlation was found between diarrhea score and MPO levels in stools ($p<0.0001$, r=0.7014, **Figure 2F**). Positive correlation was also seen of diarrhea score with LCN-2 levels at day 3 p.i. ($p<0.006$, r=0.5406, **Figure 2G**). These data indicated that high diarrhea scores are associated with increased MPO and LCN-2 levels.

EPEC Infection Alters Pro-Inflammatory and Anti-Inflammatory Cytokine Synthesis in Ileum and Colon in a Stage Disease-Dependent Manner in Mice

Next, we further analyzed which pro-inflammatory [IL-6, IL-1β, INF-γ, IL-23, tumor necrosis factor-α (TNF-α), IL-17, IL-18] and anti-inflammatory (IL-22, IL-33, IL-10) cytokines were affected by EPEC infection during acute (day 3 p.i.) and later (day 7 p.i.) stage of the disease. As shown in **Figure 3**, higher levels of IL-6 ($p<0.05$), IL-1β ($p<0.05$), INF-γ ($p<0.05$), IL-23 ($p<0.01$), and IL-22 ($p<0.05$) were detected in colonic tissues of EPEC-infected mice at day 3 p.i. and were significantly different when compared to controls (**Figures 3A–D, F**). EPEC infection in the ileal tissue resulted in significant increase in IL-6, IL-1β, and IL-22 at day 3 p.i. when compared to control mice ($p<0.05$, **Figures 3A, B, F**). In the later stage of disease (day 7 p.i.), TNF-α (ileal and colonic tissues) and IL-22 (colonic tissues) were

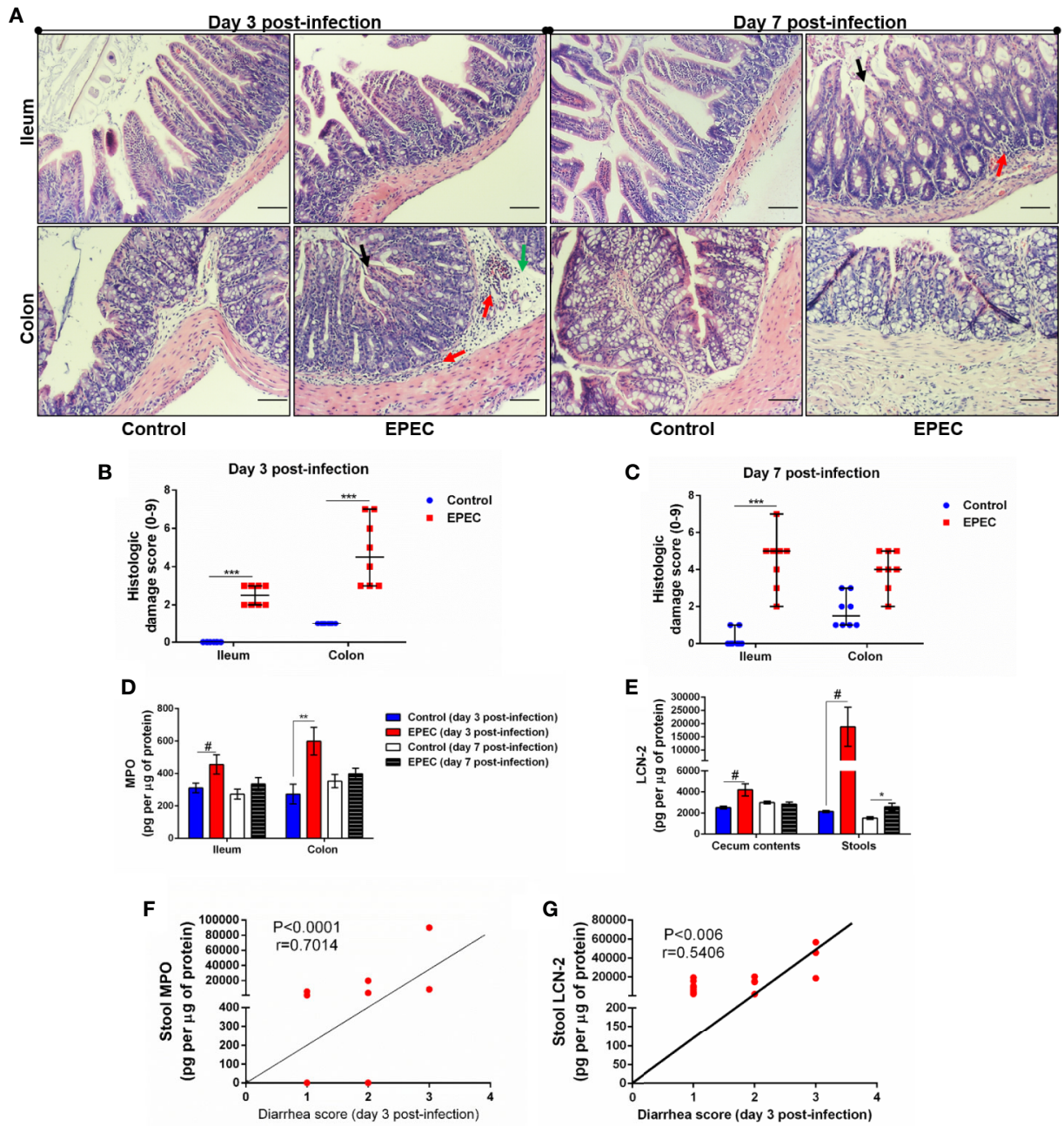

FIGURE 2 | EPEC infection model induces acute colonic damage and inflammation followed by a positive correlation between diarrhea and inflammation markers in stools. **(A)** Representative H&E stains of ileal and colonic tissue collected from control and EPEC infected mice at days 3 and 7 p.i. Scale bars, 100 µm. **(B, C)** Histologic damage score based on epithelial damage (black arrow), mucosal edema (green arrow) and inflammatory cell infiltrate (red arrow) in the ileal and colonic tissue of uninfected (control group) and EPEC-infected (EPEC group) **(B)** mice at day 3 and **(C)** mice at day 7 p.i. Bars represent median ± SEM (n=8). ***$p<0.0001$ using Kruskal-Wallis test followed by Dunn's test. **(D)** MPO levels measured in intestinal tissue lysates (ileum and colon) collected at days 3 and 7 p.i. **(E)** LCN-2 levels measured in the cecal and stool lysates collected at days 3 and 7 p.i. Bars represent mean ± SEM (n=8). $^{\#}p<0.05$, *$p<0.03$ and **$p<0.007$ using multiple Student's *t-test*. **(F)** Significant positive correlation between diarrhea score and MPO levels in stools of EPEC-infected mice at day 3 p.i (Spearman rank test). **(G)** Significant positive correlation between diarrhea score and LCN-2 levels in stools of EPEC-infected mice at day 3 p.i (Spearman rank test).

significantly increased in EPEC-infected mice when compared to control mice ($p<0.01$, **Figures 3E, G**). In addition, increase of KC in the ileal and colonic tissues was observed, however, not statistically significant (**Figure 3F**).

We analyzed the correlation of diarrhea severity with the levels of INF-γ in colonic tissues of EPEC-infected mice and found a strong positive correlation between diarrhea score and colonic INF-γ levels ($p<0.03$, r=0.9258, **Figure 3H**). We did not

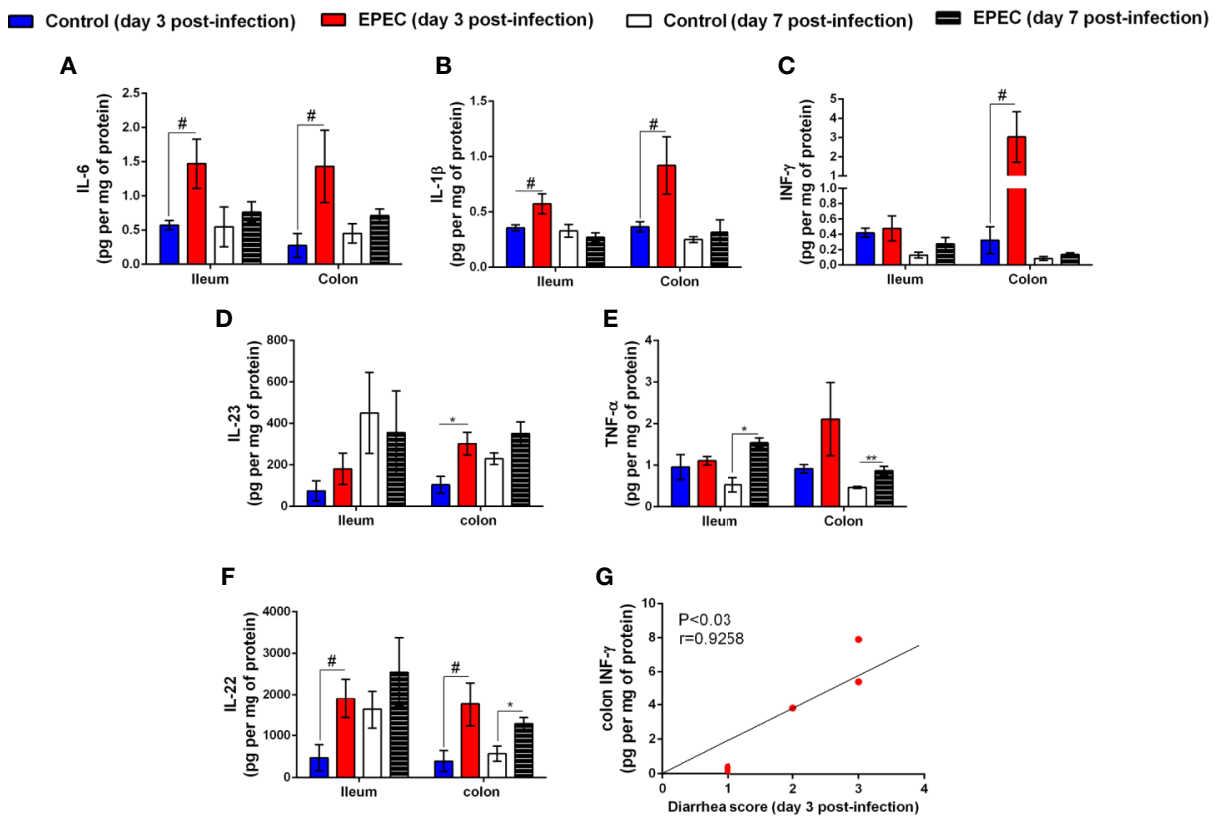

FIGURE 3 | EPEC infection increases pro-inflammatory mediators, and diarrhea scores correlates positively with INF-γ and KC levels in colonic tissues of EPEC infected mice. **(A)** Levels of IL-6, **(B)** IL-1β, **(C)** INF-γ, **(D)** IL-23, **(E)** TNF-α, and **(F)** IL-22 in the ileal and colonic tissues of control and EPEC infected mice at days 3 and 7 p.i. were measured by ELISA. Bars represent mean ± SEM (n=8). $^{\#}p<0.05$, $^{*}p<0.01$ and $^{**}p<0.001$ using multiple Student's *t-test.* **(G)** Significant positive correlation between diarrhea score and INF-γ levels in colonic tissues of EPEC infected mice at day 3 p.i (Spearman rank test).

find any change in the levels of IL-17, IL-18, granulocyte-macrophage colony–stimulating factor (GMCSF), IL-33, and IL-10 in ileal and colonic tissues samples of EPEC-infected mice when compared to the controls (**Supplementary Figures 2A–E**).

Taken together, these data indicate that EPEC infection promotes two-phase of disease in mice; an acute stage characterized by growth impairment, intestinal damage, presence of diarrhea and increased of MPO, LCN-2, IL-6, IL-1β, INF-γ, IL-23, and IL-22; and a later stage with an increase in LCN-2 and TNF-α levels in an absence of diarrhea. In addition, the data suggests that the colon is more affected by EPEC infection in mice.

EPEC-Infected Mice With Diarrhea Demonstrates Upregulation of Pro-Inflammatory Cytokines, Inflammatory Markers, STAT, and Apoptosis Markers in Colon

Due to an increase in diarrhea severity and colonic INF-γ levels that were positively correlated on day 3 p.i., we evaluated the profile of gene expression from the colonic tissues of EPEC-infected mice with diarrhea and controls using Taqman assay. In total, 92 genes were evaluated, among these 37 were upregulated and four were downregulated ($p<0.05$, **Figure 4A**). *INF-γ, GZMB*, *CXCL10*, *IL-6*, and *IL-1β* were the most upregulated genes, showing approximately 85, 30, 27, 23, and 16-fold-change in relation to controls ($p<0.05$, **Figure 4A**). In mice with diarrhea, EPEC infection resulted in upregulation of pro-inflammatory mediators (*INF-γ, TNF-α, TNFRS, IL-1α, IL-1β, IL-2Rα, IL-5, IL-6, and IL-12b*, **Figure 4B**), chemokines (*CCL2, CCL5, CCL19, CXCL10*, and *CXCL11*, **Figure 4C**), chemokine receptors (*CCR2* and *CCR7*, **Figure 4C**), cellular recruitment (*VCAM1*, *ICAM1*, and *SELP*, **Figure 4D**), *CD68* (a macrophage marker, **Figure 4E**), *CD3ϵ* (marker of T-cell activation, **Figure 4E**), inflammation markers (*C3*, *CD38*, *CD40*, *CSF2*, *GZMB*, and *MD2*, **Figure 4F**), transcription factors [phosphorylated-signal transducer and activator of transcription-1 (*STAT1*), *STAT3*, and *STAT4*, **Figure 4G**] and apoptosis markers (*Fas* and *Bax*, **Figure 4H**) in colon of mice at day 3 p.i. In addition, EPEC-infection also upregulated gene expression of anti-inflammatory mediators, such as *TGF-β1*, *HMOX1*, *PTPRC*, *SOCS1*, and *LIF* when compared to control mice ($p<0.05$, **Figure 4I**).

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor involved in response of cytokines such

as IL-6, and its activation results in expression of target genes involved in inflammatory and anti-inflammatory responses (Griesinger et al., 2015; Aden et al., 2016). To investigate the levels of phosphorylated STAT3 (pSTAT3), its active form, we found increased levels of pSTAT3 in the colon of mice infected with EPEC on day 3 p.i. (p<0.05, **Figure 4J**). Cyclic AMP Response Element-Binding protein (CREB) is another transcription factor involved on the transcription of inflammatory (such as IL-6, IL-2, and TNF-α) and anti-inflammatory (IL-4, IL-10 and IL-13) mediators (Westbom et al., 2014; Zhang et al., 2019). To determine the levels of phosphorylated CREB (pCREB), its active form, we examined, but did not find a difference between EPEC-infected mice and controls on day 3 p.i. (**Figure 4K**).

Given that EPEC infection increased the gene expression of apoptosis markers, we further evaluated the levels of cleaved caspase-3 using ELISA. We found that EPEC infection induced an increase in cleaved caspase-3 in the colonic tissues when compared to control mice at day 3 p.i. (p<0.05, **Figure 4L**), confirming an apoptosis process during EPEC infection.

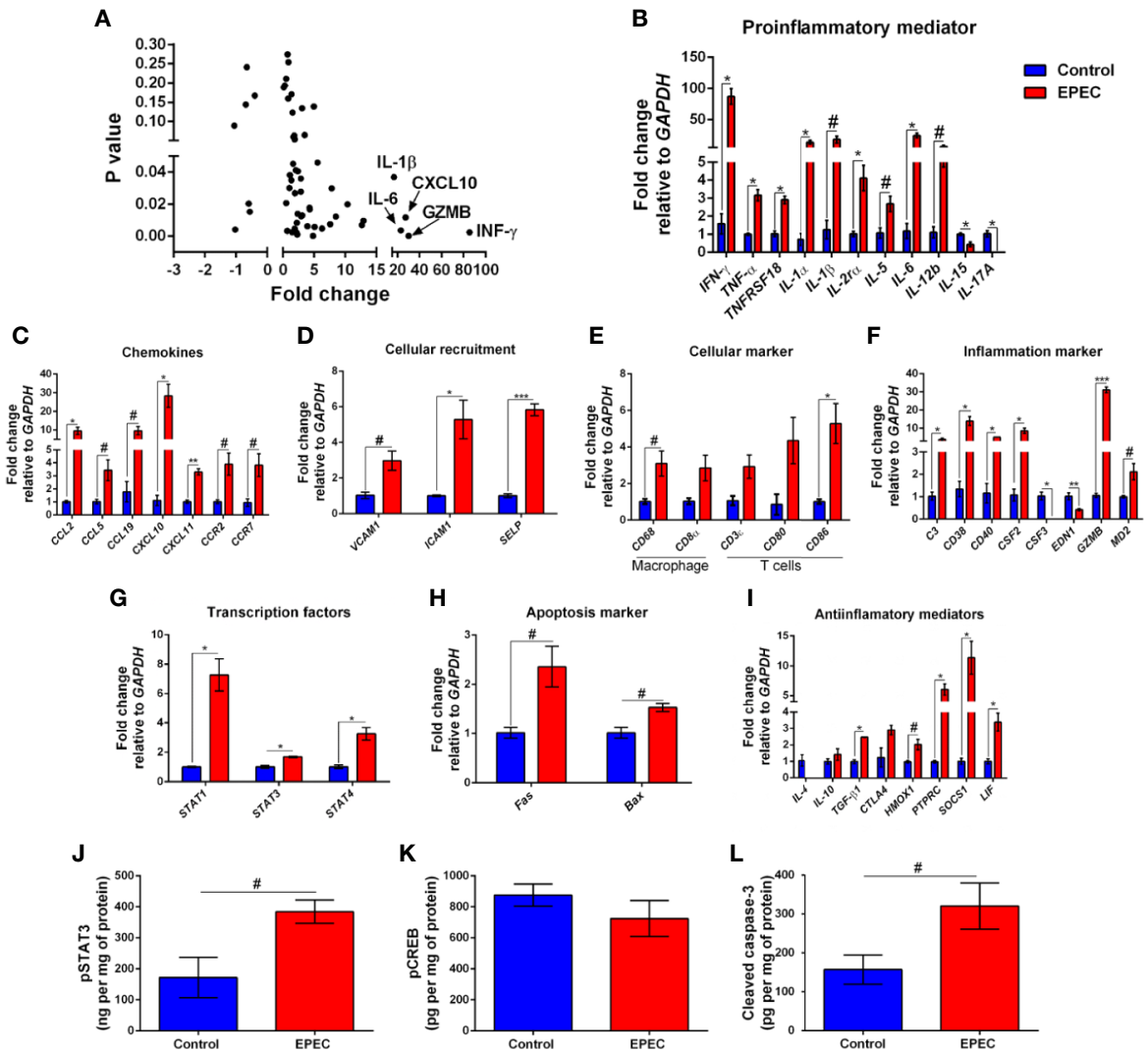

FIGURE 4 | EPEC-infected mice with soft or unformed stools exhibit higher expression of mRNA for pro-inflammatory mediators, chemokines, and other inflammatory markers. **(A)** mRNA expression fold changes (on the x axis) and the corresponding p-values (on the y axis). Gene names are labeled next to highlighted significant results. Taqman qPCR analysis of **(B)** pro-inflammatory, **(C)** chemokines, **(D)** cellular recruitment, **(E)** cellular marker, **(F)** inflammation marker, **(G)** transcription factors, **(H)** apoptosis marker, and **(I)** anti-inflammatory genes in the colonic tissues of control and EPEC-infected mice with moderate (score=2) or severe (score=3) diarrhea scores at day 3 p.i. **(A–I)** Expression levels were normalized with GAPDH, as an internal housekeeping gene. Bars represent mean ± SEM (n=3). $^{\#}p$<0.05, $^{*}p$<0.01, $^{**}p$<0.001 and $^{***}p$<0.0001 using multiple Student's *t-test*. Levels of **(J)** pSTAT3, **(K)** pCREB and **(L)** cleaved caspase-3 in colonic tissue lysates of control and EPEC-infected mice at day 3 p.i. measured using ELISA. Bars represent mean ± SEM (n=8). $^{\#}p$<0.05 using Student's *t-test*.

EPEC Infection Model Induces Metabolic Perturbations

Metabolic perturbations following EPEC infection were further analyzed using Principal Component analysis (PCA). Urinary metabolic profiles of each of the mice infected with EPEC were compared to the age-matched uninfected mice at days 1 and 3 p.i. No differences were observed between the controls and EPEC infected mice on the day 1 p.i. (**Figure 5A**).

EPEC infection-driven metabolic variation was, however, observed at day 3 pi (PCA model: $Q^2 = 0.25$, $R^2 = 0.59$ (1000 permutations). EPEC infection resulted in reduced excretion of TCA cycle metabolites (succinate, cis-aconitate, citrate, 2-oxoglutarate and fumarate) and choline related metabolite, dimethylglycine (DMG) and increased trimethylamine (TMA) (**Figure 5B**). Lower urinary excretion of the tryptophan catabolite N-methylnicotinic acid (NMNA), the creatinine precursor guanidinoacetic acid (GAA) and the amino acid catabolites 2-oxoisocaproate, 2-oxoadipate were also observed. Isethionate, formate, pantothenate, and sucrose were also excreted in lower amounts by EPEC infected mice. Increases in the excretion of gut microbial-derived metabolites [acetate, phenylacetylglycine (PAG), m-hydroxyphenylpropionylsulfate (m-HPPS)], were observed in infected mice. Urinary excretion of taurine, creatine, and b-oxidation product hexanoylglycine were also elevated at day 3 p.i (**Figure 5B**).

Pathway analysis revealed that the TCA cycle was the biochemical pathway most influenced by the infection (Impact: 0.26, *p*-value: 2.5E-6, FDR adjusted *p*-value: 2.1E-4) (**Figure 5C**).

EPEC Infection Increases Intestinal Permeability and Decreases Colonic Claudin-1 Expression in Mice

Alteration on intestinal permeability related to EPEC infection has been reported in children (Johansen et al., 1989). Given that the intestinal tissues of EPEC-infected mice showed increase in inflammatory markers on later stage of disease, we investigated whether intestinal permeability was altered by using a FITC dextran assay in our experimental model. As shown in **Figure 6A**, EPEC infection resulted in increased levels of plasma 4kDa FITC dextran, indicating higher intestinal permeability when compared to control mice at day 7 p.i. ($p<0.006$). A strong positive correlation was found between the levels of plasma 4kDa FITC dextran and colonic INF-γ at day 7 p.i. in EPEC-infected mice ($p<0.003$, r=0.9219, **Figure 6B**).

Tight junctions play a crucial role in regulating intestinal permeability, we therefore, investigated if EPEC infection alters claudin-1 and claudin-2 expression in the colon of mice, and found that EPEC infection decreased claudin-1 ($p<0.03$, **Figures 6C, D**), but not claudin 2 (**Figures 6E, F**) in the colon when compared to control mice. Uncropped versions of **Figures 6C, E** are available in **Supplementary Data** (**Supplementary Figures 3A, B**).

Loss of escN Expression in EPEC Inhibits Intestinal and Systemic Inflammation Induced by Wild-Type Enteropathogenic *Escherichia coli* Infection Model Without Changes in Stools Shedding in Mice

The T3SS is essential for EPEC pathogenesis, and disruption of the *escN* gene (ATPase energizer), can lead to inefficient injection of EPEC effectors into the host cell (Andrade et al., 2007). We therefore, tested whether inactivation of *escN* in EPEC strain could affect changes in body weight, intestinal and systemic inflammation in our EPEC infection model. The Δ*escN* EPEC-infected mice exhibited weight gain when compared to wild-type (WT) EPEC-infected mice on days 1 and 3 p.i ($p<0.05$ and $p<0.001$, respectively, **Supplementary Figure 4**). Deletion of Δ*escN* did not affect EPEC shedding in the stools (**Figure 7A**) and colonization in the intestinal tissues (**Figure 7B**). However, at day 8 p.i., tissue burden of Δ*escN* EPEC infected mice was detected only in the colonic tissue ($p<0.01$ **Figure 7C**). No histological changes were observed in the colon of mice infected with Δ*escN* EPEC when compared to mice infected with WT EPEC (**Figure 7D**). Furthermore, Δ*escN* EPEC infection resulted in decreased LCN-2 in the stools (days 2 and 7 p.i., $p<0.01$ and $p<0.05$, respectively) and cecal contents (day 3 p.i., $p<0.05$) (**Figure 7E**); and MPO in stools (day 2 p.i., $p<0.01$) (**Figure 7F**), as well as IL-6 in ileum and colon (day 3 p.i., $p<0.01$) (**Figure 7G**).

To assess whether WT EPEC infection induces systemic inflammation, as well as the role of the T3SS using Δ*escN* during infection, C-reactive protein (CRP) in intestinal tissues (ileum and colon) and serum amyloid A (SAA) in plasma of mice collected at day 3 p.i. were measured. WT EPEC infection resulted in increased the levels of CRP in the intestinal tissues ($p<0.0001$, **Figure 7H**) and increased SAA ($p<0.0001$, **Figure 7I**) when compared to control mice. Whereas deletion of Δ*escN* in EPEC led to a significant decrease in levels of these markers of systemic inflammation at day 3 p.i. when compared to WT EPEC-infected mice ($p<0.0001$, **Figures 7H, I**).

Taken together, these findings showed that EPEC induces later colonic colonization, intestinal and systemic inflammation, in an *escN*-encoded T3SS-dependent manner, confirming the effectivity of this new EPEC infection model in mice.

DISCUSSION

Typical EPEC infections have been suggested to be associated with inflammatory enteropathy and/or diarrhea in resource-limited populations (Platts-Mills et al., 2015; Rogawski et al., 2018). Infections caused by EPEC have been widely reported in *in vitro* studies to demonstrate the effects of adherence traits (Knutton et al., 1999) and the T3SS (Andrade et al., 2007). However, there was still a need for a suitable *in vivo* model that clearly shows the effects of human EPEC infection in an intestinal environment. *Citrobacter rodentium* is a natural murine pathogen that has also been shown to cause A/E lesions, with the formation of pedestal structures and polarized actin accumulation at the site of infection analogous to that seen in human EPEC and Enterohemorrhagic *E. coli* (EHEC) infections (Luperchio and Schauer, 2001; Mundy et al., 2006).

Herein an EPEC murine infection model is reported using mice pretreated with antibiotic cocktail (vancomycin, gentamicin, metronidazole and colistin) to enable colonization, and induce growth impairment, acute diarrhea, intestinal damage and inflammation, as well as metabolomic perturbations and intestinal permeability alterations. Efforts to develop an EPEC

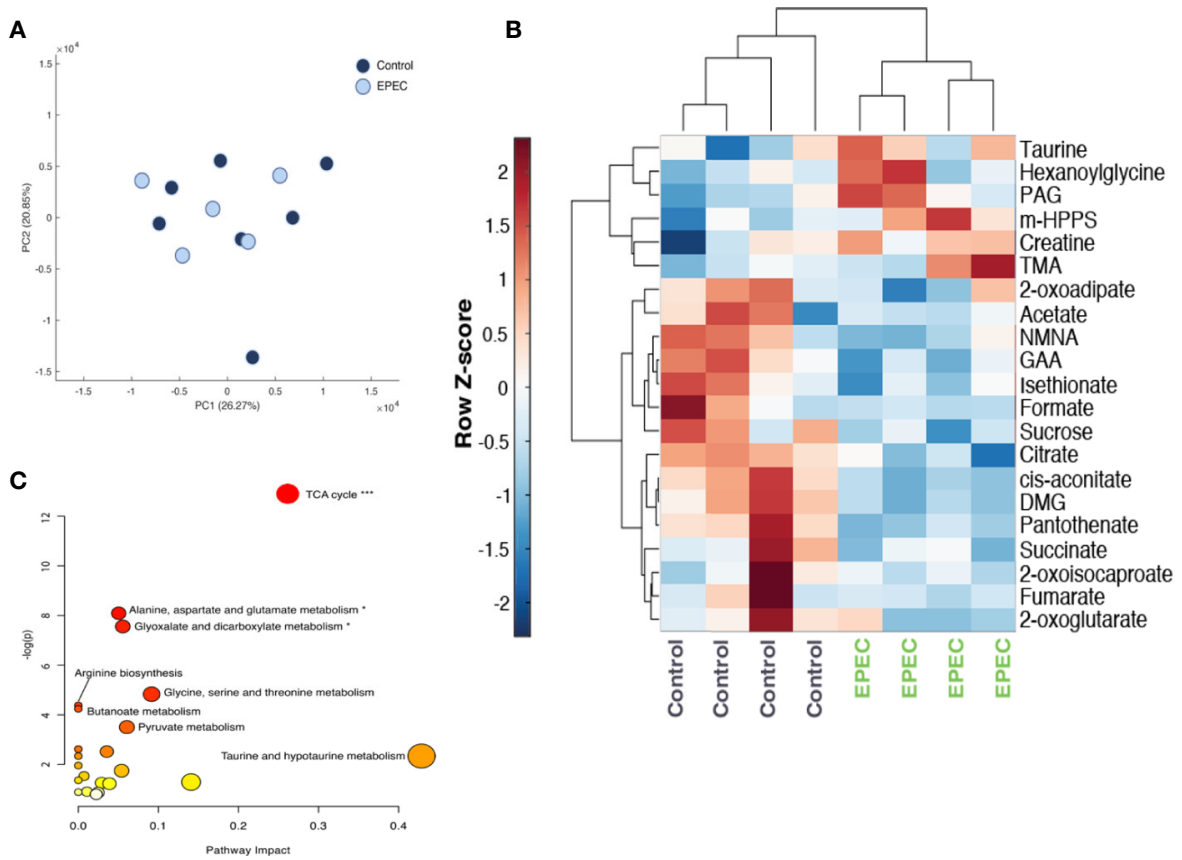

FIGURE 5 | Metabolomic analysis of urinary specimens collected from age-matched, control, and EPEC infected mice. **(A)** Principal component analysis **(**PCA) scores plot at day 1 p.i. showing no difference in the metabolic profiles of between infected and uninfected mice (control n=7, EPEC n=5). **(B)** Unsupervised hierarchical clustering and heat-map of urinary metabolites from control and EPEC-infected mice at day 3 p.i (n=4). Each row represents a metabolite and each column represents a mouse sample. The row Z-score (scaled expression value) of each metabolite is plotted in red-blue color. The red color indicates metabolites, which are high in abundance and blue indicates metabolites low in abundance. DMG, dimethylglycine; GAA, guanidinoacetic acid; m-HPPS, m-hydroxyphenylpropionylsulfate; NMNA, N-methyl-nicotinic acid; PAG, phenylacetylglycine; TMA, trimethylamine. **(C)** Pathway analysis plot created using MetaboAnalyst 4.0 showing metabolic pathway alterations induced by EPEC. Each pathway is represented as a circle. Darker colors indicate more significant changes of metabolites annotated on the corresponding pathway. The x-axis represents the pathway impact value computed from pathway topological analysis, and the y-axis is the-log of the *p*-value obtained from pathway enrichment analysis. The pathways that were most significantly changed are characterized by both a high-log(*p*) value and high impact value (top right region). FDR adjusted *p*-values, **p*<0.05, ****p*<0.0001.

model that mimics clinical outcomes, such as growth impairment and diarrhea as observed in humans have been a challenge for *in vivo* studies (Johansen et al., 1989; Savkovic et al., 2005; Law et al., 2013; Dupont et al., 2016). We also identified a two-phase disease promoted by the EPEC infection model, an acute symptomatic and a later asymptomatic phase. In the acute symptomatic phase of the disease, EPEC-infected mice had growth impairment and diarrhea as clinical outcomes, and this was accompanied with ileal and colonic damage (loss of integrity of epithelial cells, edema in submucosa and intense infiltrate of inflammatory cells) and intense inflammation. Similar findings showing disruption of colonic damage following EPEC infection has also been previously reported (Zhang et al., 2012).

In humans, EPEC infection promotes watery diarrhea and dehydration (Guerrant et al., 2011). Most of the EPEC infected mice developed moderate to severe diarrhea at day 3 p.i. Previously, C57BL/6 mice infected with EPEC have been reported to develop semi-solid stools in the proximal colon with no apparent diarrhea (Savkovic et al., 2005).

MPO has been used as biomarker of enteropathy in clinical studies (Guerrant et al., 2016; McCormick et al., 2017), also exhibiting inflammation, growth and development decrements in children infected with different enteropathogens in low-income countries. In our present study, MPO was increased during acute phase of disease, in the intestinal tissues (ileum and colon); MPO was detected mainly in stools of mice that developed soft or unformed stools or diarrhea. In addition, LCN-2 which is known as a neutrophil gelatinase-associated protein expressed by intestinal epithelial cells (Makhezer et al., 2019), was detected in higher concentrations in stools of all the mice that were infected with EPEC. Particularly impressive in our model was the striking inflammatory enteropathy (as evidenced by fecal LCN-2 and

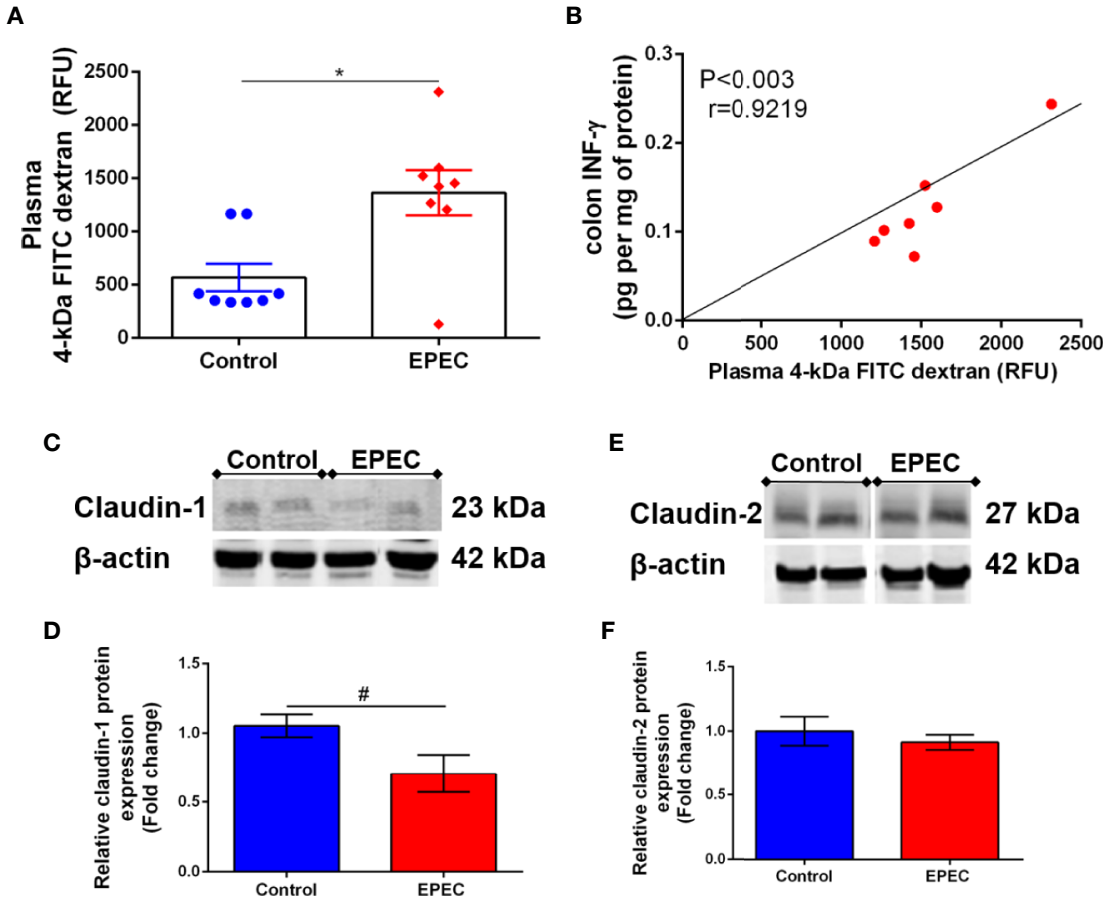

FIGURE 6 | EPEC infection model increases intestinal permeability and decreases claudin-1 expression in colon of mice. **(A)** Intestinal permeability was determined in the plasma of control and EPEC-infected mice at day 7 p.i. using FITC dextran assay. Each point represents a mouse. Lines represent mean ± SEM (n=8). $^{*}p < 0.006$ using Student's *t-test.* **(B)** Significant positive correlation between diarrhea score and INF-γ levels in colon tissues of EPEC-infected mice at day 7 p.i (Spearman rank test). **(C)**. Representative western blots of levels of claudin-1 and β-actin in colonic tissues of control and EPEC-infected mice at day 7 p.i. **(D)** Quantification of western blot bands of claudin-1 and β-actin. Bars represent mean ± SEM (n=3). $^{\#}p < 0.03$ using Student's *t-test.* **(E)** Representative western blots of levels of claudin-2 and β-actin in colonic tissues of control and EPEC-infected mice at day 7 p.i. **(F)** Quantification of western blot bands of Claudin-2 and β-actin. Bars represent mean ± SEM (n=3).

MPO during acute infection) with EPEC infected mice developing watery mucoid stools.

EPEC infection in infant C57BL/6 mice has been previously reported to colonize the small intestine and colon for 3 days as a result of human milk oligosaccharides administration (Manthey et al., 2014). Here, the ileum and colon were markedly colonized by EPEC during infection challenge. Similar to our findings, EPEC infection in other mouse models have also been reported to colonize the ileal and colonic tissues (Savkovic et al., 2005; Dupont et al., 2016). Moreover, the colon in our model was most affected by EPEC infection with higher colonization by EPEC; and similar high EPEC colonization has been previously reported in the colon of germ-free mice albeit without mention of diarrhea (Dupont et al., 2016).

The pro-inflammatory cytokines such as IL-6, IL-1β, IL-23, IL-22, and INF-γwere increased in colon of mice infected with EPEC. IL-6 is a pleiotropic cytokine showing a pro-inflammatory phenotype and is protective against infection. For instance, deficiency of IL-6 in C57BL/6 mice has been reported to cause colonic damage, increase infiltrate of inflammatory cells and apoptosis during infection with *C. rodentium* (Dann et al., 2008). Mice lacking IL-1β are more susceptible to *C. rodentium*-induced colonic inflammation (Liu et al., 2012), in contrast blocking IL-1β in EPEC-infected mice with persistent IL-1β response decreased the colonic damage (Sham et al., 2013), suggesting the role of IL-1β during intestinal infection in a concentration- and timing-dependent manner. Binding of IL-1β, to IL-1 receptor type I (IL-1RI) and activation of nuclear factor κB (NF-κB), promotes the recruitment of inflammatory cells at the site of inflammation by inducing the expression of adhesion molecules on endothelial cells and the release of chemokines (Bolick et al., 2007; Gabay et al., 2010). In our model, EPEC infection increased the expression of adhesion molecules (VCAM1, ICAM1, and SELP), as well as chemokines (CCL2, CCL5, CCL19, CXCL10, and CXCL11) and the chemokine receptors CCR2 (activated by CCL2 and expressed by macrophage and lymphocytes) and CCR7 (activated by CCL19, promoting migration of dendritic cells, monocytes and T cells) (Pezoldt et al.,

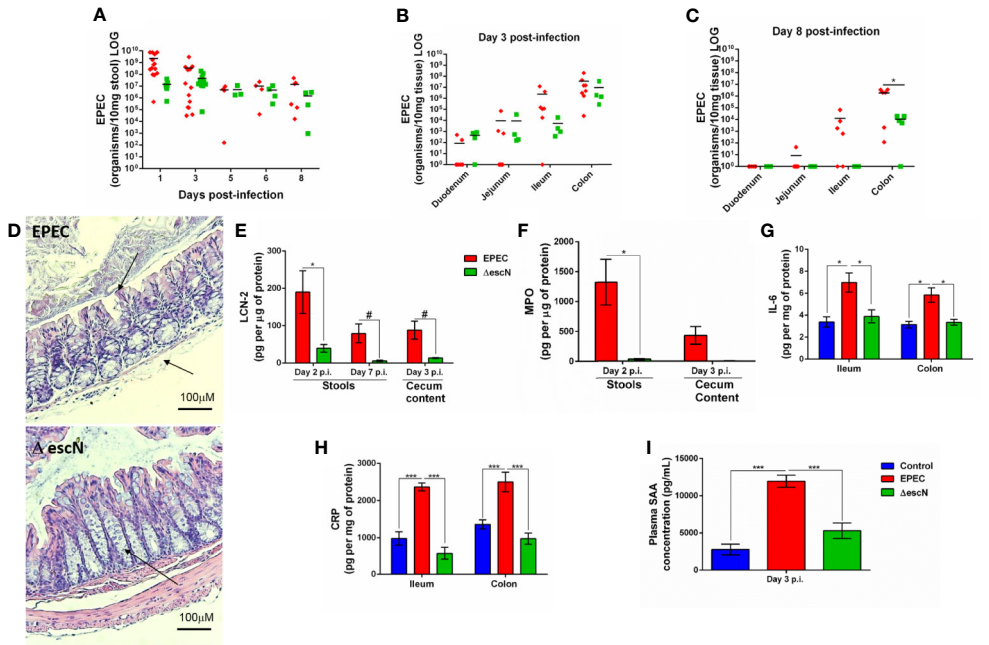

FIGURE 7 | Inactivation of *escN* in EPEC decreases colonization in colon and reduces intestinal and systemic inflammation promoted by EPEC infection in mice. **(A)** Quantification of EPEC shedding in stools of WT EPEC (EPEC) or Δ*escN* EPEC infected mice. **(B, C)** Quantification of EPEC tissue burden in intestinal tissues (duodenum, jejunum, ileum and colon) at day 3 and 8 p.i. Bars represent mean ± SEM. *$p < 0.01$ using multiple Student's *t-test*. **(D)** Representative H&E stains of colon from WT EPEC (EPEC) or Δ*escN* infected mice at day 3 p.i. Scale bars, 100 μm. **(E)** LCN-2 levels measured in stools (days 2 and 7 p.i.) and cecal contents (day 3 p.i.) of WT EPEC (EPEC) or Δ*escN* infected mice. **(F)** MPO levels measured in stools (day 2 p.i.) and cecal contents (day 3 p.i.). **(G)** IL-6 levels measured in the ileal and colonic tissue lysates at day 3 p.i. using ELISA **(H)** CRP levels measured in the ileal and colonic tissue lysates of uninfected (control, in blue), WT EPEC (in red) or Δ*escN* EPEC (in green) infected mice at day 3 p.i (**I**). Concentration of SAA in plasma at day 3 p.i. was determined using ELISA. Bars represent mean ± SEM (n=8). #$p < 0.05$, *$p < 0.01$ and ***$p < 0.0001$ using multiple Student's *t-test* **(E, F)** or One-way ANOVA followed by Turkey's test **(G–I).**

2017), contributing to the intense recruitment of inflammatory cells in the colon, but not in the ileum, of EPEC-infected mice.

Similar to IL-6, INF-γ is a pleiotropic protein that promotes the transcription of pro-inflammatory mediators and CXCL10 (by binding to CXCR3 in order to promote the recruitment of monocytes/macrophages and T cells at the site of infection) (Lee et al., 2017), and were both upregulated in the colon of mice following challenge with EPEC, likely activating STAT1 by binding to INF-γ receptor (INFGR) (Green et al., 2017). In fecal samples from children with symptomatically EPEC infection, intermediate levels of INF-γ have been associated with an increase in infection duration (Long et al., 2010). INF-γ levels were increased in colonic tissues in our study, and severity of diarrhea was associated with higher levels of INF-γ in EPEC-infected mice at day 3 p.i.

IL-23, was also increased following EPEC infection, and has been shown to be required to promote IL-22 expression, a cytokine involved in promoting tissue regeneration and regulating inflammation, and also to negatively control the potentially deleterious production of IL-12 (Aychek et al., 2015). The data therefore, suggests that an increase in these cytokines during EPEC-infection in our model is protective, but not enough to prevent the intestinal damage promoted by EPEC. In *C. rodentium* infection, lack of IL-23 in macrophages led to increased mortality in mice (Aychek et al., 2015). Here, we also provided data suggesting that our EPEC infection model was able to activate NF-κB *via* IL-1β, STAT1 *via* INF-γ, and STAT3 *via* IL-22 and IL-6, but not CREB. STAT1 and STAT3 contribute to the expression of pro-apoptotic and anti-apoptotic genes respectively (Wittkopf et al., 2015; Lee et al., 2017). However, in the present study, it seemed that the response mediated by STAT1 (whose expression was higher than STAT3 in colon of EPEC-infected mice) prevailed over anti-apoptotic response promoted by STAT3, once increased cleaved caspase, a marker of apoptosis, were detected. Moreover, because our EPEC infection model exhibits evident diarrhea more investigation of how these cytokines contribute to its pathogeneses is needed.

Furthermore, during acute phase of infection, EPEC infection resulted in perturbations of multiple biochemical pathways, with the TCA cycle intermediates appearing to be the most sensitive to EPEC infection. The TCA cycle in *E. coli* is linked to energy metabolism in which CO_2 concomitant is oxidized from pyruvate leading to production of NADH and $FADH_2$ (Alteri and Mobley, 2012). In our model, the TCA cycle metabolites were excreted in lower quantities following EPEC infection, suggesting that energy production was reduced or conserved in the infected host. A shut-down of the TCA cycle during infection suggests that the energy requirements of the host were not met, potentially explaining in part the significant weight loss in the infected mice. *C. jejuni* infection in zinc deficient mice have also been reported to perturb the TCA cycle, affecting amino acid and muscle catabolism as a

result of increased creatine excretion (Giallourou et al., 2018). Pantothenate is the key precursor of the fundamental TCA cycle cofactor, coenzyme A (Leonardi et al., 2007). Reduced pantothenate excretion following EPEC colonization further adds to the TCA cycle disruption by infection. Interestingly, excretion of creatine which is a source for energy production in the form of ATP was also increased during infection. Sugiharto and colleagues reported on post-weaning pigs infected with *E. coli* F18 and found that there was a reduction in creatine and betaine which was due to inhibition of antioxidant system that resulted in piglets developing diarrhea (Sugiharto et al., 2014). Taurine has been shown to possess antioxidant properties and its concentrations are elevated in inflamed tissues where oxidants are abundant (Jeon et al., 2009; Oliveira et al., 2010). EPEC infection in this study was characterized by elevated urinary taurine excretion. As we have previously observed (Swann et al., 2011), treating rodents with antibiotics suppresses the bacterial metabolism of taurine thus increasing taurine bioavailability and uptake in the host reflected by greater urinary taurine excretion. Metabolites derived from bacteria in the gut were excreted in greater amounts following infection suggesting gut microbial metabolism was altered by EPEC infection. These findings help to understand host metabolism during infection, suggesting potential pathways to be further explored and targeted in future studies.

In relation to later phase of EPEC infection, we observed an increase in TNF-α in the colon and ileum, as well as increased LCN-2 in stools samples and increased intestinal permeability and decreased claudin-1. Despite TNF-α gene expression, as well as its receptor TNFRS being increased during the acute phase of disease, the TNF-α protein levels were increased in intestinal tissues of EPEC-infected mice only during the later phase. TNF-α synthesis is promoted by NF-κB activation which in turn can be promoted by IL-1β (Kalliolias and Ivashkiv, 2016). The biological effects of TNF-α mediated by binding to TNFRS include inflammation, apoptosis and tissue regeneration *via* activation of NF-κB, caspase-8 and AKT respectively

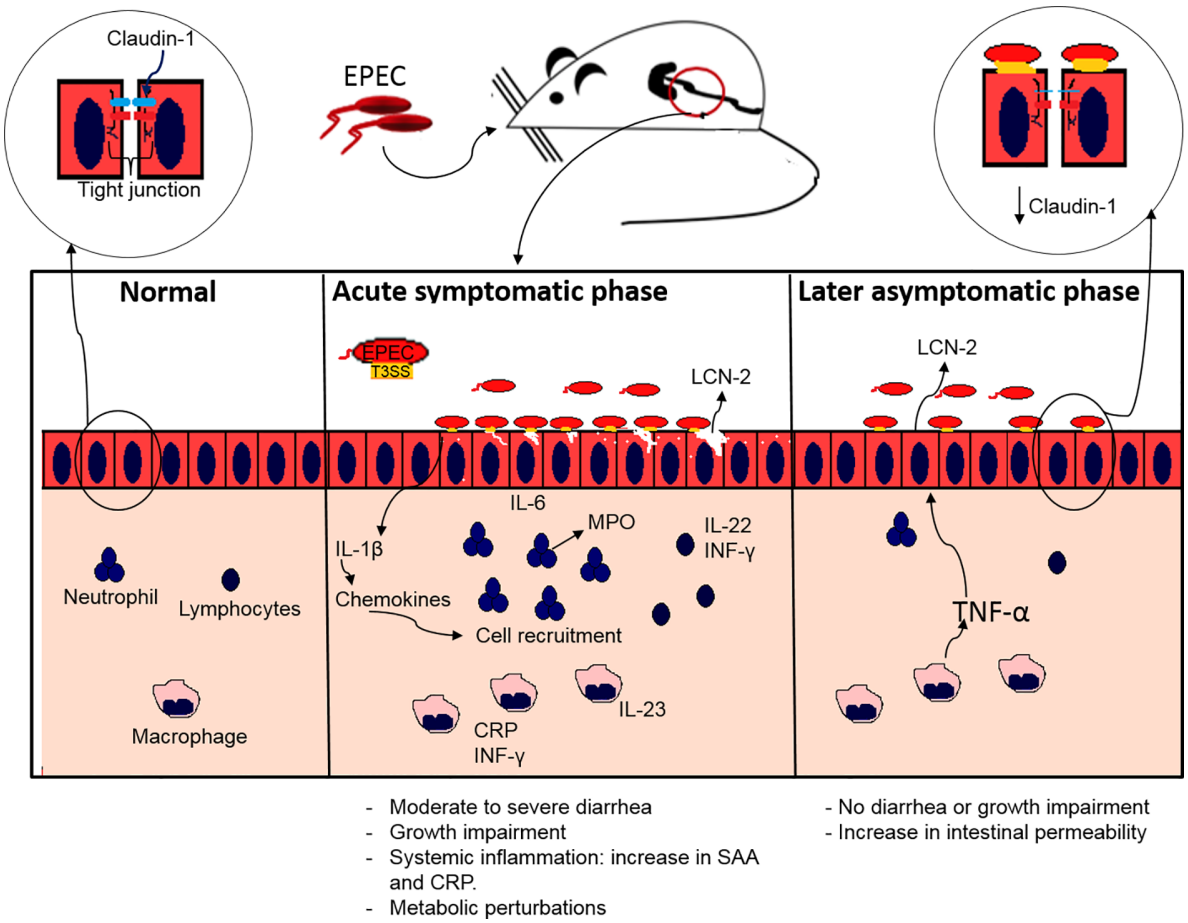

FIGURE 8 | Proposed model of EPEC infection antibiotic pretreated mice displaying acute symptomatic and late asymptomatic phase. During acute symptomatic phase, colonization by EPEC leads to growth impairment, accompanied with moderate to severe diarrhea. Adherence of EPEC on the intestinal epithelial cells leads to increased IL-1β, which in turn stimulates chemokines synthesis, and consequently recruitment of neutrophils, macrophages and lymphocytes, resulting in increased release of MPO by neutrophils and LCN-2 by epithelial cells, as well as pro-inflammatory (IL-6, IL-23, INF-γ) and IL-22 cytokines indicating intestinal inflammation. A further increase in CRP and SAA indicates acute systemic inflammation. During the later asymptomatic phase an increase in LCN-2 inflammatory biomarker and an increase in TNF-α leads to increased intestinal permeability affecting the tight junction integrity by decreasing claudin-1 with no signs of diarrhea and growth impairment.

(Kalliolias and Ivashkiv, 2016). Similar to our findings, later increases of TNF-α in the ileum and colon has been observed by others in EPEC-infected mice at day 5 p.i (Shifflett et al., 2005), findings that we showed at day 7 p.i. This increase in TNF-α was associated with increased LCN-2 in stools, indicating the presence of intestinal damage and inflammation, despite partial recovery from the acute phase of disease. TNF-α has been shown to induce LCN-2 expression by activating NF-κB (Makhezer et al., 2019). TNF-α and INF-γ have been associated with a loss of integrity of the intestinal epithelial barrier (Chelakkot et al., 2018). Despite TNF-α, but not INF-γ, being increased in colonic tissues in EPEC-infected mice, only INF-γ levels were associated positively with an increase in intestinal permeability; a similar association has also been reported in an *in vitro* study using T84 epithelial cell monolayer (Smyth et al., 2011). The permeability of the intestinal barrier is regulated by the tight junction proteins (Wang et al., 2017). EPEC infection has been reported to impair tight junction barrier function of ileal and colonic mucosa (Mundy et al., 2006; Guttman et al., 2006; Zhang et al., 2010; Zhang et al., 2012; Ugalde-Silva et al., 2016). Claudin-1, a component of tight junction expressed by epithelial cells from small and large intestine, is responsible for increasing barrier tightness (Luissint et al., 2016). In our model, a decrease of claudin-1 in the colon of EPEC-infected mice was associated with an increase in intestinal permeability. Although we detected alterations in intestinal permeability at the later stage of EPEC infection, this might have been due in part to an increase in systemic markers (SAA and CRP) that were detected at day 3 p.i. during the acute phase leading to disruption of intestinal tight junctions. SAA has been a biomarker of enteropathy in clinical studies also associated with inflammation, and with growth and developmental impairment in children infected with multiple enteropathogens in low-income countries (Guerrant et al., 2016).

The T3SS is essential for EPEC pathogenesis and requires an effective ATPase energizer, *escN* (Gauthier et al., 2003; Andrade et al., 2007; Zarivach et al., 2007). Here, we also demonstrated that mice infected with *escN* deletion mutant resulted in diminished growth impairment and inflammation. Even without an effective T3SS, *escN* mutant was able to colonize all sections of the intestinal tissue (due to the presence of functional bfp), albeit at much lower levels to day 8 p.i., as shown by our results. These findings reinforce the importance of a functional T3SS in the virulence of EPEC in this model (Frankel et al., 1998; Andrade et al., 2007).

In conclusion, our findings showed that EPEC infection causes growth impairment, diarrhea and increased inflammatory responses in weaned antibiotic pretreated mice. These effects were also dependent on an intact EPEC T3SS. In addition, metabolic perturbations and intestinal permeability were also observed in mice with EPEC infection, suggesting relevant biochemical pathways involved. Further, the findings presented here suggest that EPEC infections leads to an increase in intestinal and systemic inflammatory responses and transient overt diarrhea and growth impairment, as is often seen in children with EPEC infections. This EPEC infection model also presents two phases of diseases: an acute symptomatic and a later asymptomatic phase (**Figure 8**). This model can help further explore mechanisms involved in EPEC pathogenesis and perhaps facilitate the development of vaccines or therapeutic interventions.

AUTHOR'S NOTE

This manuscript has been released as a pre-print at bioRxiv 2020.06.12.148593 (Ledwaba et al., 2020).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Committee on the Ethics of Animal Experiments of the University of Virginia (Protocol Number: 3315). The mice used in the study have been handled with strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All efforts were made to minimize suffering. This is also in accordance with the Institutional Animal Care and Use Committee policies of the University of Virginia. The University of Virginia is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International (AAALAC).

AUTHOR CONTRIBUTIONS

SL, DB, RG, JN, NP, and AT designed the project. SL, DC, and DB performed the experiments. SL, DC, DB, and RG analyzed the data and wrote the manuscript. PM assisted in analysis of results. NG and JS performed and assisted with metabolome analysis. All authors revised the manuscript. NP, AT, RG, and JN supervised the project. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.595266/full#supplementary-material

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MicroRNAomes of Cattle Intestinal Tissues Revealed Possible miRNA Regulated Mechanisms Involved in *Escherichia coli* O157 Fecal Shedding

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Cattle have been suggested as the primary reservoirs of *E. coli* O157 mainly as a result of colonization of the recto-anal junction (RAJ) and subsequent shedding into the environment. Although a recent study reported different gene expression at RAJ between super-shedders (SS) and non-shedders (NS), the regulatory mechanisms of altered gene expression is unknown. This study aimed to investigate whether bovine non-coding RNAs play a role in regulating the differentially expressed (DE) genes between SS and NS, thus further influencing *E. coli* O157 shedding behavior in the animals through studying miRNAomes of the whole gastrointestinal tract including duodenum, proximal jejunum, distal jejunum, cecum, spiral colon, descending colon and rectum. The number of miRNAs detected in each intestinal region ranged from 390 ± 13 (duodenum) to 413 ± 49 (descending colon). Comparison between SS and NS revealed the number of differentially expressed (DE) miRNAs ranged from one (in descending colon) to eight (in distal jejunum), and through the whole gut, seven miRNAs were up-regulated and seven were down-regulated in SS. The distal jejunum and rectum were the regions where the most DE miRNAs were identified (eight and seven, respectively). The miRNAs, bta-miR-378b, bta-miR-2284j, and bta-miR-2284d were down-regulated in both distal jejunum and rectum of SS (log_2fold-change: –2.7 to –3.8), bta-miR-2887 was down-regulated in the rectum of SS (log_2fold-change: –3.2), and bta-miR-211 and bta-miR-29d-3p were up-regulated in the rectum of SS (log_2fold-change: 4.5 and 2.2). Functional analysis of these miRNAs indicated their potential regulatory role in host immune functions, including hematological system development and immune cell trafficking. Our findings suggest that altered expression of miRNA in the gut of SS may lead to differential regulation of immune functions involved in *E. coli* O157 super-shedding in cattle.

Keywords: cattle, microRNA, gut, *E. coli* O157, super-shedder

INTRODUCTION

Escherichia coli (*E. coli*) O157 is a foodborne pathogen that causes severe human disease, such as hemolytic uremic syndrome, bloody diarrhea, and even death (Pennington, 2010). The *E. coli* O157 in the feces of cattle shed into the farm environment can survive in the soil and water for extended periods of time (Maule, 2000) and can cause contamination of vegetables and fruits during planting and irrigation. Furthermore, fecal contamination in the food processing and preparation chain can also lead to the adulteration of meat (Ferens and Hovde, 2011). Cattle shedding $>10^4$ CFU *E. coli* O157 per gram of feces are often referred as super-shedders (SS), which have been reported to be responsible for most of the *E. coli* O157 spread into the farm environment (Chase-Topping et al., 2008). However, only a small portion (<10%) of the cattle in a herd have been reported to be SS in a number of studies (Chase-Topping et al., 2008; Munns et al., 2014), suggesting certain individuals have a propensity to become SS. The recto-anal junction (RAJ) of cattle has been suggested to be the primary colonization site of *E. coli* O157 (Naylor et al., 2003), and our recent study revealed that expression of genes involved in immune functions at the RAJs differed between natural SS and non-shedders (NS, cattle negative for *E. coli* O157) (Wang O. et al., 2016). However, the regulatory mechanisms responsible for this differential gene expression in the RAJ of SS *vs* NS remains unclear.

One possibility is that the non-LEE-encoded type III secretive proteins and Shiga toxins produced by *E. coli* O157 may suppress host lymphocyte responses, as suggested by both *in vitro* and *in vivo* studies (Menge et al., 2003; Hoffman et al., 2006; Walle et al., 2013). Another possibility is that the regulatory mechanisms of the transcriptome that lead to immune responses in the gut may differ between SS and NS. One form of transcriptome regulation occurs through non-coding RNAs, especially microRNAs (miRNAs), which have been reported to regulate gene expression in many biological processes (Wahid et al., 2010). In addition, detection of miRNAs in extracellular spaces, such as milk, serum, urine and feces (Weber et al., 2010; Liu et al., 2016), suggests that the functions of miRNAs are not restricted to within cells, but also with extracellular functions associated with functions including immune responses, cell communication and even shaping the gut microbiota (Turchinovich et al., 2012; Liu et al., 2016).

Gene expression is regulated by miRNA through recognition of complementary sequences on transcripts, commonly through binding to the 3′-UTR region (Bartel, 2009; Fang and Rajewsky et al., 2011; Allantaz et al., 2012). Many studies have reported that miRNA expression was associated with host–microbial interaction. Bao et al. (2015) studied whole blood miRNAomes of *Salmonella*- challenged pigs, and suggested that miR-214 and miR-331-3p may regulate host immune responses associated with persistent shedding of *Salmonella*. Others have reported that miRNA-155 influences the presence of *Helicobacter pylori* in mouse gastrointestinal tract by mediating T-cell responses (Oertli et al., 2011). *Mycobacterium tuberculosis* has been reported to interfere with the expression of miR-125b and miR-155 blocking the expression of Tumor Necrosis Factor (TNF) responses in macrophages (Rajaram et al., 2011). Moreover, a recent effort revealed that the expression of miRNAs involved in B-cell functions in the small intestine were associated with beneficial bacterial (*Lactobacilli* and *Bifidobacterium*) populations in the gut of young calves (Liang et al., 2014). Therefore, we hypothesize that the expression of gut miRNAs differs between SS and NS and that it is one of the key regulatory mechanisms of the reported differential gene expression through the gut in response to the *E. coli* O157 colonization and shedding in cattle (Wang O. et al., 2016). We performed miRNA expression profiling on the whole gut of cattle, including the duodenum, proximal jejunum, distal jejunum, cecum, spiral colon, and descending colon using miRNA sequencing to examine whether miRNAs may play a regulatory role in host immune functions that may be associated with the presence of *E. coli* O157 within the digestive tract.

MATERIALS AND METHODS

The steers used in this experiment followed the Canadian Council of Animal Care Guidelines, and the protocol was reviewed and approved by the Animal Care Committee of Lethbridge Research Centre, Agriculture Agri-food Canada (Animal Care Committee protocol number: 1120; approved June 2011).

Super-Shedder Cattle Identification and Intestinal Tissue Collection

The s for identification of SS and tissue collection were as detailed previously (Zaheer et al., 2017). Briefly, fecal samples (50 g each) were collected from RAJ of 400 British × Continental feedlot steers for *E. coli* O157 identification and quantification using plate culturing methods on sorbitol MacConkey agar with 2.5 mg/L potassium tellurite and 0.05 mg/L cefixime. In total, 11 SS were identified with fecal *E. coli* O157 ≥104 CFU per gram of feces. The *E. coli* O157 isolates from CT-SMAC were confirmed using an *E. coli* O157 Latex Test kit (Oxoid Ltd, Basingstoke, Hampshire, UK) and the serotype (*E. coli* O157:H7) was further confirmed with multiplex PCR targeting verotoxin (vt), intimine (eaeA), and H7 flagellin (fliC) following protocols described previously (Gannon et al., 1997). Based on fecal *E. coli* O157 numbers (Wang O. et al., 2016), five SS (out of 11 identified) and five control pen-mates (NS, negative for fecal *E. coli* O157) were used for sampling of intestinal tissue (**Supplementary Table S1**). The fecal *E. coli* O157 of SS were monitored for 4 to 10 days prior to slaughter. All SS were positive for fecal *E. coli* O157 before slaughter (Munns et al., 2014), among them one animal remained SS at slaughter, while the other four were tested positive but not at SS level. NS animals on the other hand, were tested negative for fecal *E. coli* O157 at slaughter. From the slaughtered NS and SS, two 2 cm^2 of tissues were collected from duodenum, proximal jejunum, distal jejunum, cecum, spiral colon, descending colon, and RAJ (one RAJ sample from a NS was lost during storage) and immediately snap-frozen in liquid nitrogen and stored at −80°C.

RNA Extraction and miRNA Sequencing

The tissue samples were ground into fine powders in liquid nitrogen prior to RNA extraction. Approximately 100 mg of tissue was used for RNA extraction using a mirVana total RNA Isolation Kit (Ambion, Carlsbad, CA, USA) per manufacturer's instructions. The RNA integrity was measured using an Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA, USA), and the RNA concentration was measured using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). The RNA samples with an integrity number (RIN) ≥7.0 and ratio of 28S/18S ≥1.7 were used for miRNA-Seq library construction. For each sample, 1 μg of total RNA was used for library preparation using a TruSeq Small RNA sample preparation kit (Illumina, San Diego, CA) following the manufacturer's instruction. RNA sequencing was performed using an Illumina HiSeq 2000 (illumina, San Diego, CA, USA) sequencing platform (single end, 1 × 50 bp) at the Genome Quebec Innovation Centre (Montreal, PQ, Canada).

miRNA Sequencing Data Processing, Functional Analysis, Differential Analysis

All the data were presented as mean ± standard deviation. The miRNA sequencing reads were trimmed, quality filtered, and processed using a web based analysis tool, "sRNAtoolbox" (using default parameters) (Rueda et al., 2015). The read number of detected miRNAs was normalized as counts per million (cpm), and the miRNAs with cpm >1 were defined as expressed miRNAs. The miRNA family conservation was defined according to miRNA family information from TargetScan (release 7.0, 2016) (Agarwal et al., 2015). The miRNA target prediction was performed using PITA (Probability of Interaction by Target Accessibility) (Kertesz et al., 2007) and miRanda (Betel et al., 2008). The targets predicted by both miRanda (matching score > 145, free energy < −10) and PITA (free energy < −10) were used for functional analysis for selected miRNA. The Ingenuity Pathway Analysis® (IPA, IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) was used for function enrichment, and the Fisher's exact text implemented by internal functions of IPA was used for the association analysis of miRNA targets and functions, with significance level 5% (Fisher's exact test p-value 0.05) for selection of enriched functions. The Bioconductor edgeR (Robinson et al., 2010), which fits the read counts to a binomial distribution and estimates the dispersion and determines differential expression by exact test, was used to identify differentially expressed (DE) miRNA. Only miRNAs expressed in >2 of either NS or SS group were used for differential expression analysis, and FDR (false discovery rate) of 10% was used as a cut-off for DE miRNA identification.

Identification of Potential miRNA Regulation on Target mRNA Expression

To assess the potential for interactions between identified DE miRNAs and predicted target genes, a Spearman's rank-order correlation analysis between expression of DE miRNAs and all genes that encode predicted target transcripts (gene expression levels are unpublished data, manuscript under review). The correlation analysis was performed using R (version 3.3.2), and only miRNA/transcripts with cpm >1 in at least five animals were used for correlation analysis. The correlation coefficients, ρ (rho)-value < −0.6 and p-value <0.05 were considered as significant negative correlation.

Availability of microRNAome Data

The miRNA-Seq data are available at NCBI Gene Expression Omnibus (GEO) database under accession number GSE96973.

RESULTS

miRNAome Profiling of Intestinal Tissues Collected From Beef Steers

The sequencing reads were generated from 69 miRNA libraries prepared from intestinal tissues collected at seven different locations, including duodenum, proximal jejunum, distal jejunum, cecum, spiral colon, descending colon, and RAJ. The preliminary miRNA data of RAJ were reported in our previous publication (Wang O. et al., 2016), and we have included in-depth analysis in the current study. The number of sequencing reads ranged from 30 to 75 million for each region (**Table 1**), and the read number for each tissue ranged from 0.9 to 22 million (**Figure 1A**). The number of reads mapped to miRNA ranged from 0.5 to 19 million per sample (**Figure 1B**), and the number of miRNAs detected in each region ranged from 390 ± 13 (duodenum) to 413 ± 49 (descending colon) (**Table 1** and **Figure 1C**). In total, 464 miRNAs were shared by all the intestinal regions (seven regions), while 39 miRNAs were only detected in 1 region (**Figure 1D**). Among the identified miRNAs in each region, the number of highly conserved miRNAs ranged from 151 (spiral colon) to 157 (descending colon), and the number of conserved miRNAs ranged from 83 (duodenum and RAJ) to 88 (cecum and descending colon), while 280 (RAJ) to 307 (descending colon) of detected miRNAs were poorly conserved (defined by TargetScan 7.0 which also claims that certain poorly conserved miRNAs may be sequences misannotated as miRNAs) (**Figure 2**).

The bta-miR-143 (48.8%), bta-miR-192 (10.8%) and bta-miR-148a (3.29%) were the three most abundant miRNAs expressed in duodenum, and bta-miR-143 (44.3%), bta-miR-192 (14.1%), and bta-miR-10a (2.7%) were the three most abundant miRNAs expressed in the proximal jejunum. In the distal jejunum, cecum, spiral colon, descending colon, and RAJ,

TABLE 1 | Number of reads generated by Illumina HiSeq 2000 system for miRNA sequencing and average number of miRNAs detected in each intestinal region of beef steers.

Tissue	Number of reads, Million (M)	# identified miRNA
Duodenum	30.0	390 ± 13
Proximal jejunum	41.1	393 ± 20
Distal jejunum	37.7	395 ± 19
Cecum	39.9	411 ± 16
Spiral colon	38.2	409 ± 15
Descending colon	75.4	413 ± 49
Rectum[a]	33.5	398 ± 15

[a]*Data of rectal tissue were also reported in previous publication (Wang O. et al., 2016).*

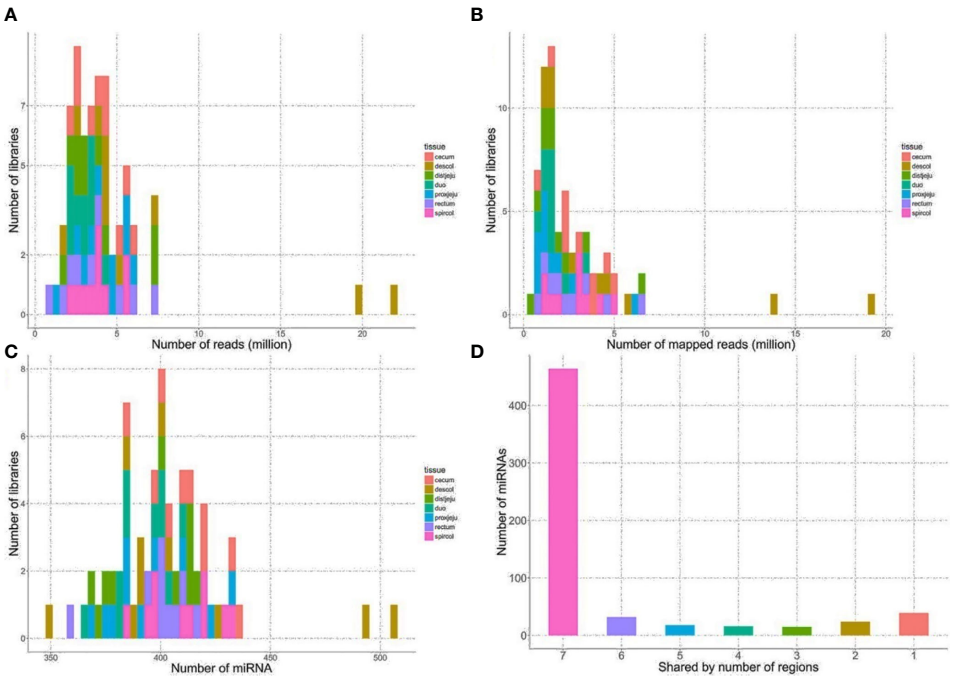

FIGURE 1 | General sequencing results and miRNA mapping results. **(A)** Distribution of numbers of reads generated from HiSeq 2000 sequencing platform. **(B)** Distribution of numbers of reads mapped to known bovine miRNA after quality filtering. **(C)** Distribution of numbers of detected miRNA from the intestinal regions. **(D)** Number of miRNA shared by the intestinal regions, the number on x-axis represents the number of intestinal regions a miRNA is shared by, seven, all intestinal regions; six, six out of seven regions, five, five out of 7 regions, four, four out of seven regions; three, three out of seven regions; two, 2 out of seven regions; one, only detected in one of 7 regions.

the bta-miR-143 (16.2% in cecum to 59.3% in descending colon), bta-miR-192 (5.4% in spiral colon to 12.5% in cecum), and bta-miR-10b (4.7% in descending colon to 14.6% in cecum) are the three most abundant miRNAs (**Supplementary Table S2**). The relative abundance of bta-miR-143 and bta-miR-192 miRNA accounted for more than 50% of the miRNAs detected in each intestinal region, with the abundance of top 15 miRNAs of each region accounting for >83% of total expressed miRNAs (**Supplementary Table S2**). Among the top 15 most abundant expressed miRNAs, 10 were shared by all seven regions, including bta-miR-143, bta-miR-192, bta-miR-10b, bta-miR-27b, bta-miR-26a, bta-miR-26c, bta-miR-21-5p, bta-miR-148a, bta-miR-22-3p, and bta-let-7f. Functional analysis of these miRNAs indicated that vascular system functions (cardiovascular system development and function, p-values ranged from 4.2E-07 to 3.3E-05) and organismal survival (p-value ranged from 8.9E-07 to 1.6E-04) were frequently enriched (**Table 2**). Also, bta-miR-10b was potentially associated with digestive system development and function (p-value: 1.4E-04), with the bta-miR-148a, bta-miR-192 and bta-miR-22-3p being associated with nervous system development and function (p-value ranged from 2.9E-06 to 3.4E-5) (**Table 2**). In addition, Euclidean distance based hierarchical clustering and PCA analysis indicated that the miRNA expression profiles were not distinct for each of the intestinal regions (**Supplementary Figure S1**).

Differentially Expressed miRNAs Between Non-Shedders and Super-Shedders in Each Intestinal Region

Comparing SS and NS for each region, the number of differentially expressed (DE) miRNAs ranged from one (in descending colon) to eight (in distal jejunum) in the gut tissues, and the $\log_2$fold-change ranged from −4.5 to 4.5 (**Table 3**). For duodenum (three DE miRNAs), cecum (three DE miRNAs), spiral colon (four DE miRNAs), and descending colon (one DE miRNAs), all identified DE miRNAs were down-regulated in SS. For proximal jejunum (two DE miRNAs) and distal jejunum (eight DE miRNAs), equal numbers of DE miRNAs were up-regulated and down-regulated in SS. For DE miRNAs identified in RAJ, four were down-regulated and three were up-regulated. Four DE miRNAs were shared by more than one intestinal region (**Table 3**): the bta-miR-378b was down-regulated in all regions (log2fold-change ranged from -3.3 to -4.5); bta-miR-2284j was down-regulated (log2fold-change ranged from −2.9 to −4.1) in duodenum, distal jejunum, cecum, spiral colon, and RAJ; bta-miR-2284d (log2fold-change ranged from −2.7 to −2.9) was down-regulated in the duodenum, distal jejunum, spiral colon and RAJ; and bta-miR-99a-5p was up-regulated in the proximal and distal jejunum (log2fold-change were 3.3 and 2.7, respectively) (**Table 3**).

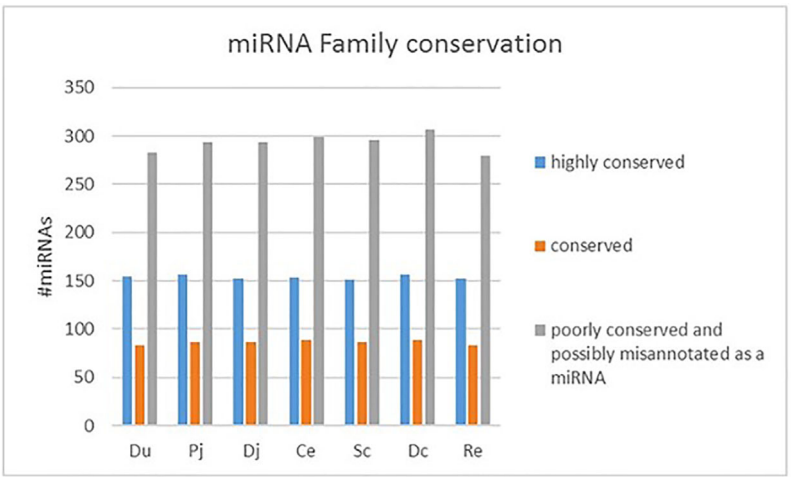

FIGURE 2 | Family conservation of detected bovine miRNAs in each region. Du, duodenum; pj, proximal jejunum; Dj, distal jejunum; Ce, cecum; Sc, spiral colon; Dc, descending colon; Re, rectum.

TABLE 2 | Functions enriched for predicted target genes of top 10 most abundant miRNAs that are shared by all the intestinal regions.

miRNA	Function categories[a]	p-value[b]
bta-let-7f	organismal development	7.89E-6
	tissue development	7.89E-6
bta-miR-10b	cardiovascular system development and function	1.45E-4
	digestive system development and function	1.45E-4
bta-miR-143	organ development	1.28E-5
	organismal survival	1.01E-5
bta-miR-148a	cardiovascular system development and function	3.31E-5
	nervous system development and function	4.44E-6
bta-miR-192	nervous system development and function	3.45E-5
	organismal development	1.61E-4
bta-miR-21-5p	cardiovascular system development and function	4.24E-7
	organismal survival	2.59E-9
bta-miR-22-3p	nervous system development and function	2.91E-6
	organismal development	2.22E-6
bta-miR-26a	cardiovascular system development and function	8.93E-7
	organismal development	8.93E-7
bta-miR-26c	cardiovascular system development and function	1.58E-4
	organismal development	1.58E-4
bta-miR-27b	organismal survival	1.96E-9
	tissue development	2.54E-8

(Only the two functions with smallest p-values were shown).
[a]*Functions enriched for the predicted mRNA genes using Ingenuity Pathway Analysis.*
[b]*The Fisher's exact test p-value calculated by Ingenuity Pathway Analysis, and the top two functions with the smallest p-values were shown.*

TABLE 3 | Differentially expressed miRNAs detected in each region of intestinal tract of beef steers.

Tissue	Differentially expressed miRNA	Log-2-(fold-change)	FDR
	bta-miR-378b	–4.37	<0.01
Duodenum	bta-miR-2284j	–3.28	0.02
	bta-miR-2284d	–2.90	0.02
Proximal jejunum	bta-miR-378b	–4.50	<0.01
	bta-miR-99a-5p	3.30	0.04
	bta-miR-378b	–3.74	<0.01
	bta-miR-2284j	–4.11	<0.01
	bta-miR-100	3.77	0.01
Distal jejunum	bta-miR-2284d	–2.92	0.04
	bta-miR-99b	2.53	0.09
	bta-miR-409a	2.51	0.09
	bta-miR-99a-5p	2.69	0.09
	bta-miR-18a	–1.58	0.09
	bta-miR-378b	–3.92	<0.01
Cecum	bta-miR-2284j	–3.36	0.02
	bta-miR-2904	–2.72	0.02
	bta-miR-378b	–3.95	<0.01
Spiral colon	bta-miR-451	–1.77	0.01
	bta-miR-2284j	–2.92	0.01
	bta-miR-2284d	–2.95	0.01
Descending colon	bta-miR-378b	–3.30	0.09
	bta-miR-2284j	–3.76	0.01
	bta-miR-1271	2.59	0.02
	bta-miR-378b	–3.42	0.02
Rectum	bta-miR-2887	–3.20	0.05
	bta-miR-211	4.51	0.05
	bta-miR-2284d	–2.72	0.07
	bta-miR-29d-3p	2.23	0.09

(10 tissue samples for each region, including 5 collected from non-shedders and 5 from super-shedders, but only 4 rectal tissues from non-shedders).

For the family conservation of detected DE miRNAs, five miRNAs were defined as poorly conserved (or misannotated as miRNAs), one was conserved, and seven were highly conserved (**Table 4**). Ten of the DE miRNAs were in intergenic regions of the genome, and four of the DE miRNAs were in the intragenic regions (introns) (**Table 4**). Two intronic miRNAs including bta-miR-2284j and bta-miR-2284d, which were likely bovine specific miRNAs, were present in the introns of Glutamate Ionotropic Receptor Kainate Type Subunit 4 (GRIK4) and SWAP Switching B-Cell Complex 70 kDa Subunit (SWAP70), respectively (**Table 4**). The other two intronic miRNAs were highly conserved including bta-miR-1271 and bta-miR-211, which were associated with ADP Ribosylation Factor Like GTPase 10

TABLE 4 | Differentially expressed miRNA, family conservation, and genomic locations.

miRNAs	Conservation[a]	Chromosome	Location
bta-miR-29d-3p	2	Chr16	intergenic
bta-miR-99a-5p	2	Chr1	intergenic
bta-miR-100	2	Chr15	intergenic
bta-miR-99b	2	Chr18	intergenic
bta-miR-18a	2	Chr12	intergenic
bta-miR-451	2	Chr19	intergenic
bta-miR-211	2	Chr21	TRPM1[b], intron 7
bta-miR-1271	2	Chr7	ARL10[c], intron 2
bta-miR-409a	1	Chr21	intergenic
bta-miR-378b	−1	Chr15	intergenic
bta-miR-2904	−1	Chr2, Chr3	intergenic
bta-miR-2887	−1	Chr2, Chr3	intergenic
bta-miR-2284j	−1	Chr15	GRIK4[d], intron 3
bta-miR-2284d	−1	Chr15	SWAP70[e], intron 2

[a]*Defined by TargetScan (release 7, 2016). 2 = highly conserved across human, mouse, rat, dog, and chicken; 1 = conserved across human, mouse, rat and dog; −1 = poorly conserved across species, potentially bovine specific miRNAs.*
[b]*Transient receptor potential cation channel subfamily M member 1.*
[c]*ADP ribosylation factor like GTPase 10.*
[d]*Glutamate ionotropic receptor kainate type subunit 4.*
[e]*SWAP switching B-cell complex 70 kDa subunit.*

(ARL10) and Transient Receptor Potential Cation Channel Subfamily M Member 1 (TRRM1), respectively (**Table 4**).

Target Gene Prediction and Functional Enrichment for Differentially Expressed miRNAs

The number of predicted targets of DE miRNAs ranged from 37 (bta-miR-99b) to 803 (bta-miR-211) (**Tables 5** and **6**). For the up-regulated DE miRNAs in SS, functions of tissue development (p-value ranged from 5.73E-06 to 1.83E-5), organism development (p-value ranged from 4.42E-07 to 4.64E-06), and nervous system development and functions (p-value ranged from 5.73E-06 to 9.01E-04) were the most frequently enriched (**Table 5**). For down-regulated miRNAs in SS, functions of organism development (p-value ranged from 4.87E-07 to 1.12E-04) and cardiovascular system development (p-value ranged from 1.73E-06 to 2.0E-05) were the most frequently enriched (**Table 6**). In addition, the down-regulated bta-miR-451(log2fold-change: −1.77 in spiral colon) and bta-miR-2284j (log2fold-change ranged from −2.9 to −4.1), as well as up-regulated bta-miR-211 (log2fold-change: 4.5 in RAJ) in SS, were associated with immune functions, such as development of the hematological system (p-value ranged from 1.8E-05 to 1.8E-05) (**Tables 5** and **6**).

miRNA and mRNA Correlation and Functional Analyses

For each intestinal region, the correlation analysis between expression of DE miRNAs and expression of all the computationally predicted target genes indicated that only a small number of predicted target genes were negatively correlated with the miRNAs: one (bta-miR-99a-5p, bta-miR-100 and bta-miR-99b in distal jejunum) to 74 (bta-miR-29d-3p in RAJ) of predicted target genes showed correlation with miRNAs (Spearman's $\rho < -0.6$, and p-value < 0.05) (**Table 7**). The functional enrichment of negatively correlated target transcripts of each miRNA indicated association between the DE miRNAs and immune functions. As shown in **Table 7**, with bta-miR-378b, the number of negatively correlated transcripts ranged from two (in cecum) to 38 (in spiral colon), and in duodenum, proximal

TABLE 5 | Up-regulated miRNAs in super-shedders and the functions enriched for their predicted target genes.

miRNA	#Target[a]	Regions[b]	Enriched functions[c]	P-value[d]
bta-miR-99a-5p	40	Pj, Dj	organ development	3.92E-04
			organismal development	3.32E-06
			tissue development	3.32E-06
bta-miR-100	47	Dj	nervous system development and function	9.01E-04
			organismal development	4.64E-06
			tissue development	4.64E-06
bta-miR-409a	175	Dj	cardiovascular system development and function	4.35E-06
			organismal development	4.35E-06
			tissue development	1.83E-05
bta-miR-99b	37	Dj	organismal development	2.67E-06
			tissue development	2.67E-06
			tissue morphology	2.63E-05
bta-miR-1271	443	Re	nervous system development and function	5.73E-06
			skeletal and muscular system development and function	5.07E-06
			tissue development	5.73E-06
bta-miR-211	803	Re	hematological system development and function	1.78E-05
			nervous system development and function	3.73E-06
			tissue development	3.73E-06
bta-miR-29d-3p	521	Re	organ morphology	2.17E-05
			organismal development	4.42E-07
			organismal survival	8.16E-07

[a]*Genes encodes target mRNAs predicted by PITA and miRanda.*
[b]*Intestinal regions where the differentially expressed miRNAs were identified. Pj, proximal jejunum; Dj, middle jejunum; Re, rectum.*
[c]*Functions enriched for the predicted mRNA genes using Ingenuity Pathway Analysis.*
[d]*The Fisher's exact test p-value calculated by Ingenuity Pathway Analysis, and the top three functions with the smallest p-values were shown.*

TABLE 6 | Down-regulated miRNAs in super-shedders, and the functions enriched for their predicted target genes.

miRNA	#Target[a]	Tissues[b]	Enriched functions[c]	P-value[d]
bta-miR-378b	514	Du, Pj, Dj, Ce, Sc, Dc, Re	cardiovascular system development and function	1.73E-06
			connective tissue development and function	5.47E-06
			tissue morphology	1.73E-06
bta-miR-2284j	486	Du, Dj, Ce, Sc, Re	cardiovascular system development and function	2.00E-05
			organismal development	1.12E-04
			tissue development	3.07E-05
bta-miR-2284d	696	Du, Dj, Sc, Re	cardiovascular system development and function	4.87E-07
			organ morphology	4.87E-07
			organismal development	4.87E-07
bta-miR-18a	559	Dj	cardiovascular system development and function	1.33E-06
			organ morphology	4.83E-06
			organismal survival	1.54E-07
bta-miR-2904	678	Ce	digestive system development and function	4.12E-05
			hematological system development and function	4.31E-05
			organismal development	3.48E-06
bta-miR-451	120	Sc	nervous system development and function	3.75E-06
			skeletal and muscular system development and function	2.64E-05
			tissue development	3.75E-06
bta-miR-2887	226	Re	hematological system development and function	1.76E-04
			nervous system development and function	6.50E-05
			organismal development	2.28E-05

[a]*Genes encodes target mRNAs predicted by PITA and miRanda.*

[b]*Intestinal regions where the differentially expressed miRNAs were identified. Du, duodenum; Pj, proximal jejunum; Dj, middle jejunum; Ce, cecum; Sc, spiral colon; Dc, Descending colon; Re, rectum.*

[c]*Functions enriched for the predicted mRNA genes using Ingenuity Pathway Analysis.*

[d]*The Fisher's exact test p-value calculated by Ingenuity Pathway Analysis, and the top three functions with the smallest p-values were shown.*

jejunum and distal jejunum, the top enriched functions (smallest p-value) were associated with immune functions, including cell-mediated immune responses and hematological system development (p-values < 0.05). In RAJ, two transcripts were negatively correlated with bta-miR-378b, PRDM1 (PR/SET Domain 1) (ρ = −0.68, p-value < 0.05) and CYLD (CYLD Lysine 63 Deubiquitinase) (ρ = −0.81, p-value < 0.05), which were also associated with humoral and cell-mediated immune responses (**Supplementary Table S3**). For the bta-miR-2284d in distal jejunum (down-regulated in SS, log2fold-change: −2.92), hematological system development and function (p-value < 0.05) were enriched for its negatively correlated target transcripts. Also in the duodenum, two target transcripts negatively correlated with bta-miR-2284d, MYC (MYC proto-oncogene) (ρ = −0.78, p-value < 0.05) and NLK (Nemo Like Kinase) (ρ = −0.85, p-value < 0.05), were associated with lymphoid tissue structure and development (**Supplementary Table S3**). For the bta-miR-211 (log2fold-change: 4.51) and bta-miR-29d-3p (log2fold-change: 2.23) that were up-regulated in the RAJ of SS, hematological system development and function (p-value < 0.05), immune cell trafficking (p-value < 0.05) and hematopoiesis (p-value < 0.05) were enriched for their negatively correlated target transcripts (**Table 7**). The bta-miR-18a (log2fold-change: −1.58) in the distal jejunum was negatively correlated with ITCH (Itchy E3 Ubiquitin Protein Ligase), GJA1 (Gap Junction Protein Alpha 1), IL4R (Interleukin 4 Receptor), F3 (Coagulation Factor III, Tissue Factor) and CD7 (CD7 Molecule) (ρ-values < −0.6, p-values < 0.05), which were associated with immune cell trafficking and humoral immune response (p-values < 0.05) (**Supplementary Table S3**). In the cecum, the negatively correlated target transcripts of bta-miR-2094 (log2fold-change: −2.72), SYK (Spleen Associated Tyrosine Kinase) and CD22 (CD22 Molecule) (ρ-values < −0.6, p-values < 0.05), were associated with humoral and cell-mediated immune response (p-values < 0.05) (**Supplementary Table S3**). In RAJ, the bta-miR-1271 (log2fold-change: 2.59) was negatively correlated with SCARB1 (Scavenger Receptor Class B Member 1), CNR2 (Cannabinoid Receptor 2), RALB (RAS Like Proto-Oncogene B), HBEGF (Heparin Binding EGF Like Growth Factor), DSC1 (Desmocollin 1) and SIT1 (Signaling Threshold Regulating Transmembrane Adaptor 1) (ρ-values < −0.6, p-values < 0.05), which were associated with humoral immune response and lymphoid tissue structure and development (p-values < 0.05) (**Supplementary Table S3**).

In the distal jejunum, several negatively correlated transcripts with bta-miR-18a, bta-miR-378b, and bta-miR-2284d were also identified as DE genes between NS and SS, including the F3 (log2fold-change: 2.23) with bta-miR-18a (ρ = −0.67, p-value < 0.05) and with bta-miR-2284d (ρ = −0.85, p-value < 0.05); PTGS2 (Prostaglandin-Endoperoxide Synthase 2) (log2fold-change: 1.3) with bta-miR-2284d (ρ = −0.78, p-value < 0.05); THEMIS (Thymocyte Selection Associated) (log2-fold-change: 1.4), ITK (IL2 Inducible T-Cell Kinase) (log2fold-change: 1.1) and TIFA (TRAF Interacting Protein With Forkhead Associated Domain) (log2fold-change: 1.2) with bta-miR-378b (ρ-values were −0.73, −0.71, and −0.78 respectively, and p-values < 0.05). Also, in RAJ, the DE genes SIT1 (log2fold-change, −2.3) and RGS13 (Regulator Of G-Protein Signaling 13) (log2-fold-change, −2.2) [also reported in previous publication Wang O. et al. (2016)] were predicted as targets of bta-miR-1271 and bta-miR-29d-3p, respectively, with expression being negatively correlated with these miRNAs (ρ-values were −0.67 and −0.83 respectively, and p-values < 0.05).

TABLE 7 | Differentially expressed miRNAs and number of target genes negatively correlated with miRNA expression in different intestinal regions.

miRNA	# predicted targets[a]	# negatively correlated target genes	Enriched functions[b]
		Duodenum:23	hematological system development and function; hematopoiesis
		Proximal jejunum:8	cell-mediated immune response; hematological system development and function
		Distal jejunum:36	hematological system development and function; immune cell trafficking
bta-miR-378b	514	Cecum:2	nervous system development and function: tissue morphology
		Spiral colon:38	connective tissue development and function; organismal development
		Descending colon:3	tissue morphology; connective tissue development and function
		Rectum:5	organismal development; tissue morphology
		Duodenum: N/A[c]	n/a
		Distal jejunum: N/A	n/a
bta-miR-2284j	486	Cecum: N/A	n/a
		Spiral colon: N/A	n/a
		Rectum: N/A	n/a
		Duodenum:21	tissue morphology; cardiovascular system development and function
bta-miR-2284d	696	Distal jejunum:65	hematological system development and function; tissue morphology
		Spiral colon: N/A	n/a
		Rectum: N/A	n/a
bta-miR-99a-5p	40	Proximal jejunum:1	not enriched
		Distal jejunum:1	not enriched
bta-miR-100	47	Distal jejunum:1	not enriched
bta-miR-99b	37	Distal jejunum:1	not enriched
bta-miR-409a	175	Distal jejunum:5	organismal development; digestive system development and function
bta-miR-18a	559	Distal jejunum:16	cardiovascular system development and function; skeletal and muscular system development and function
bta-miR-2904	678	Cecum:7	hematopoiesis; humoral immune response
bta-miR-451	120	Spiral colon:3	cardiovascular system development and function; tissue development
bta-miR-1271	443	Rectum:8	cardiovascular system development and function; connective tissue development and function
bta-miR-2887	226	Rectum: N/A	n/a
bta-miR-211	803	Rectum:69	hematological system development and function; immune cell trafficking
bta-miR-29d-3p	521	Rectum:74	hematological system development and function; hematopoiesis

[a]*Number of genes encoding miRNA target transcripts, predicted by miRanda and PITA.*
[b]*The Fisher's exact test p-value < 0.05 calculated by Ingenuity Pathway Analysis, and the top two functions with the smallest p-values were shown. Function enrichment was not performed if the negatively correlated miRNA-mRNA pairs were less than 2.*
[c]*The correlation analysis not performed because the miRNA expressed (cpm > 1) in less than five animals.*

DISCUSSION

This is the first study to investigate the potential role of miRNA in shedding of *E. coli* O157 in cattle through comparison of miRNAomes of the whole intestinal tract of SS and NS beef steers. Our findings indicated that different regions of the gut tended to share the same expressed miRNAs, and the hierarchical cluster and PCA analyses suggested similar expression patterns throughout the regions of the bovine intestinal tract. Similar results have previously been reported for miRNA expression patterns in the duodenum and jejunum of dairy cattle (Wang D. et al., 2016). The observation of high abundance of bta-miR-143 and bta-miRNA-192 was also observed in previous miRNA profiling studies of the gut tissues of calves (Liang et al., 2014; Liang et al., 2016) and dairy cattle (Wang D. et al., 2016). The bta-miR-143, which had the highest abundance in all the intestinal regions, belongs to miR-143 family, and miR-143 has been reported to be highly expressed in the gut of humans (Gaulke et al., 2014) and mice (Singh et al., 2011). This likely reflects the important function in the gut that bta-miR-143 plays in maintaining smooth muscle cells, vascular homeostasis, epithelium regeneration, and epithelial tumor repression (Slaby et al., 2007; Elia et al., 2009; Chivukula et al., 2014). Functional enrichment for the most abundant miRNAs showed associations with development of the nervous system, vascular system, and digestive system, suggesting the critical role of abundantly expressed miRNAs in the bovine gut.

Although both environmental and host related factors could influence *E. coli* O157 shedding in cattle, since both NS and SS were raised in the same environment, we speculate that the identified DE miRNAs between SS and NS are host related mechanisms which regulate gut gene expression and alter gut environment in a manner that contributes to the shedding of *E. coli* O157. The distal jejunum and RAJ were the regions where most DE miRNAs were identified, and thus it is possible that certain miRNAs expressed in these regions may be involved in *E. coli* O157 super-shedding. For example, the miRNAs, bta-miR-2284j, bta-miR-378b, and bta-miR-2284d are simultaneously down-regulated in the distal jejunum and RAJ of SS. In addition, the intestinal mucosal immune surveillance components, Peyer's patches (PP) and isolated lymphoid follicles (ILFs) are known to be present in distal jejunum (Mutwiri et al., 1999) and RAJ (Naylor et al., 2003), respectively. Also, the attaching and effacing lesion caused by *E. coli* O157 was reported in the jejunum of challenged neonatal calves (Dean-Nystrom et al., 1997), and both innate and adaptive immune responses were detected in the RAJ of challenged cattle (Nart et al., 2008). Therefore, the miRNAs, bta-miR-2887 (down-regulated in RAJ of SS) and bta-miR-211 (up-regulated in

RAJ of SS), which potentially target mRNAs involved in host immune functions, may also play a role in *E. coli* O157 shedding in SS cattle.

Down-regulation of bta-miR-378b in all the intestinal regions of SS indicates its potentially important role in *E. coli* O157 super-shedding. According to miRbase (Release 21) (Griffiths-Jones et al., 2006), bta-miR-378b is encoded by miR-378 gene family, and miRNAs of this family were suggested to regulate lipogenesis in adipose tissues (Gerin et al., 2010). Also, miR-378 was detected in monocytes and T-cells (Oertli et al., 2011) and has been reported to regulate cytotoxicity of natural killer cells (Wang et al., 2012), indicating a potential role of miR-378 in mediating innate and adaptive immune functions. Indeed, in all the intestinal regions, the immune functions were enriched for the negatively correlated target transcripts of bta-miR-378b. The PRDM1 and CYLD were two predicted target transcripts of bta-miR-378b that were also negatively correlated with bta-miR-378b in RAJ, the known primary colonization site of *E. coli* O157 in cattle (Nart et al., 2008). The PRDM1 gene encodes a Blimp1 protein, which was known to positively regulate the differentiation of T-cells into long-lived plasma cells, and it is required for the release of antibodies by these cells (Martins and Calame, 2017). Lipopolysaccharide (LPS), a byproduct of Gram-negative bacteria, was reported to cause increase in expression of PRDM1 (Savitsky and Calame, 2006). CYLD was confirmed to be a NF-κB repressor to prevent inflammatory diseases and also, with the LPS being one of the factors that induces an increase in its expression (Courtois, 2008). The functions of these two transcripts in humoral and cell-mediated immune responses suggest their potential role in the host and gut microbial interactions. Down-regulation of bta-miR-378b suggested a trend of increased expression of both genes in RAJ of SS, which may be influenced by translocation of Gram-negative bacteria (as indicated above LPS may lead to altered expression of these genes), which is commonly observed when the host intestinal mucosal barrier is disrupted or immune defenses are deficient (Berg, 1999; Ryan et al., 2006). Indeed, our previous RNA-Seq based study of the rectal tissues of SS suggested possible immunodeficiency in RAJ of SS (Wang O. et al., 2016), and the capability of *E. coli* O157 to disrupt and invade cattle intestinal epithelium has also been demonstrated (Sheng et al., 2011). Future studies to measure LPS and to evaluate the abundance of Gram-negative bacteria in SS *vs* NS, as well as to examine the integrity of host intestinal mucosal barriers of SS are needed to validate the above speculation.

The bovine specific bta-miR-2284d was down-regulated in both the small and large intestine of SS, including in the RAJ. Although its expression level was low in all the tissues (average cpm = 2.2), it may still play a critical role in bovine physiology, as the number of its predicted targets is higher than that of other DE miRNAs except for bta-miR-211. Bta-miR-2284d has been reported in several bovine miRNA studies (Liu et al., 2014; Zhao et al., 2016); however, information about its function is limited. The correlation analysis suggests that this miRNA may play a regulatory function with regard to immunity in the bovine distal jejunum. Among the transcripts showing negative correlation with bta-miR-2284d in distal jejunum, the DE gene F3 (also called TF) was reported to be involved in recruiting leukocytes in the intestine of mice (Anthoni et al., 2007), and the PTGS2 gene (also called COX2) was suggested to promote humoral immune responses (Ryan et al., 2006). However, data of bacterial translocation, host mucosal integrity as well as the state of host immune defenses in the intestinal regions studied are not available, and the interaction between miR-378b/bta-miR-2284d and their target transcripts requires further validation. Nevertheless, these findings highlight the importance of further research on the role of miRNA regulation of immune functions including the intestinal mucosal barrier and immune defenses of SS cattle using histological and immunological methods, to identify miRNAs associated with host responses against *E. coli* O157. Also, to better understand the role of bta-miR-378b in super-shedding phenomena, it is worth further investigating whether it directly targets the genes associated with the immune system, or regulates immune functions through regulating lipid metabolism, as lipids are important components for cell signaling and cell proliferation involved in immune responses (Bensinger et al., 2008). In addition, to validate the interaction between bta-miR-2284d and potential targets, especially the targets involved in immune functions, will help understand the functions of this bovine specific miRNA, and how its regulation may influence *E. coli* O157 shedding in cattle.

A recent report suggested that fecal miRNAs were capable of maintaining normal gut microbiota (Liu et al., 2016) by entering bacteria cells including *Fusobacterium nucleatum* (*F. nucleatum*) and *E. coli*, to influence bacterial gene expression possibly through interaction with bacterial DNA/RNA (Liu et al., 2016). Although it is unclear whether the DE miRNAs reported in this study serve functions in the intestinal luminal spaces and their capability of entering bacterial cells including *E. coli* O157 are unknown; these miRNAs are worth further study as it is necessary to show the direct interaction with gut microbes and influence of *E. coli* O157 shedding in cattle.

One of the limitations of the current study is that the animals were not traced for shedding levels to determine whether they are transient shedders or persistent shedders. However, the miRNAs that may play a role in regulating the genes which influence the *E. coli* O157 shedding in cattle may serve as the candidates for future validation in larger population and for different ages of the animals.

In conclusion, this study investigated the miRNA expression in the whole intestine of *E. coli* O157 SS and NS beef steers. The miRNA profiling results indicated that the majority of expressed miRNAs were common within the regions of the intestinal tract that were investigated. Comparative expression analysis of miRNAs revealed that the distal jejunum, and RAJ may play an important role in host responses against *E. coli* O157, as most DE miRNAs between SS and NS were identified in these regions. Down-regulation of bta-miR-378b and bta-miR-2284d in multiple intestinal regions of SS suggested that these two miRNAs may differentially alter lipid metabolism and immune functions in the intestinal tract of in the SS vs NS, influencing host and *E. coli* O157 interactions and leading to its super-shedding in cattle. The identified miRNAs and their functions provide better understanding of the molecular mechanisms that

regulating colonization of this foodborne pathogen *in vivo*, which is vital for development of better strategies to reduce cattle super shedding of *E. coli* O157.

DATA AVAILABILITY STATEMENT

The miRNA-Seq data are available at NCBI Gene Expression Omnibus (GEO) database under accession number GSE96973.

ETHICS STATEMENT

The steers used in this experiment followed the Canadian Council of Animal Care Guidelines, and the protocol was reviewed and approved by the Animal Care Committee of Lethbridge Research Centre, Agriculture Agri-food Canada (Animal Care Committee protocol number: 1120; approved June 2011).

AUTHOR CONTRIBUTIONS

OW and LG conceived and designed the experiments. OW, MZ, YC, TM, and BS performed the experiments. OW, MZ, and LG analyzed the data. LG contributed reagents/materials/analysis tools. OW, MZ, TM, GP, KS, BS, and LG wrote and edited the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.634505/full#supplementary-material

Supplementary Figure 1 | Results of Euclidean distance based hierarchical clustering and PCA analysis based on miRNA expression profiles of each intestinal regions.

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Genetic Characterization of AmpC and Extended-Spectrum Beta-Lactamase Phenotypes in *Escherichia coli* and *Salmonella* From Alberta Broiler Chickens

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Horizontal gene transfer is an important mechanism which facilitates bacterial populations in overcoming antimicrobial treatment. In this study, a total of 120 *Escherichia coli* and 62 *Salmonella enterica* subsp. *enterica* isolates were isolated from broiler chicken farms in Alberta. Fourteen serovars were identified among *Salmonella* isolates. Thirty one percent of *E. coli* isolates (37/120) were multiclass drug resistant (resistant to ≥ 3 drug classes), while only about 16% of *Salmonella* isolates (10/62) were multiclass drug resistant. Among those, eight *E. coli* isolates had an AmpC-type phenotype, and one *Salmonella* isolate had an extended-spectrum beta-lactamase (ESBL)-type beta-lactamase phenotype. We identified both AmpC-type (bla_{CMY-2}) and ESBL-type (bla_{TEM}) genes in both *E. coli* and *Salmonella* isolates. Plasmids from eight of nine *E. coli* and *Salmonella* isolates were transferred to recipient strain *E. coli* J53 through conjugation. Transferable plasmids in the eight *E. coli* and *Salmonella* isolates were also transferred into a lab-made sodium azide-resistant *Salmonella* recipient through conjugation. The class 1 integrase gene, *int1*, was detected on plasmids from two *E. coli* isolates. Further investigation of class 1 integron cassette regions revealed the presence of an *aadA* gene encoding streptomycin 3''-adenylyltransferase, an *aadA1a/aadA2* gene encoding aminoglycoside 3''-O-adenyltransferase, and a putative adenylyltransferase gene. This study provides some insight into potential horizontal gene transfer events of antimicrobial resistance genes between *E. coli* and *Salmonella* in broiler chicken production.

Keywords: ***Escherichia coli*, *Salmonella*, bla_{CMY-2}, bla_{TEM}, antimicrobial resistance genes**

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INTRODUCTION

For decades, antimicrobial resistance (AMR) has been a global issue of grave concern. Understanding potential mechanisms and driving forces for dissemination of genes encoding antimicrobial resistance between bacteria will help reduce the prevalence of resistant bacteria and thereby reduce risks to human and animal health. Acquisition of new resistance genes occurs frequently and naturally among bacterial communities from humans, animals and environments as outlined in the model known as 'the epidemiology of AMR' (Prescott, 2006). However, the mechanism of dissemination of resistance genes is not yet fully understood.

Escherichia coli and *Salmonella* spp. are common bacterial causes of foodborne disease in humans as well as gastrointestinal disease in animals (Folster et al., 2011; Ghodousi et al., 2015). *E. coli* is a genetically diverse species which has both commensal and pathogenic strains (Leimbach et al., 2013). *Salmonella enterica* are enteric pathogens, and are closely related to commensal *E. coli*, sharing ~85% of their genomes in common at the nucleotide level (Mcclelland and Wilson, 1998; McClelland et al., 2000). Multi-drug resistant (MDR) *E. coli* and *Salmonella* could lead to the antibiotic choices of last resort for treating multidrug-resistant Gram-negative infections. Therefore, MDR *E. coli* and *Salmonella*, especially the ones that exhibit AmpC/ extended-spectrum beta-lactamase (ESBL) phenotypes, have become a major cause of clinical concern (Mohanty et al., 2010). However, these isolates are not always detected in routine susceptibility tests (Mohanty et al., 2010).

AmpC-type CMY beta-lactamase genes (bla_{CMY}) have been found on both the chromosome and plasmids of many gram negative bacteria such as *Klebsiella* spp., *Escherichia coli*, and *Salmonella* spp. CMY-2 is reported to be the most common plasmid-carried AmpC-type CMY in both *E. coli* and *Salmonella* isolates from various global regions including Asia, North America and Europe (Guo et al., 2014). Extended spectrum beta-lactamases (ESBLs) are beta-lactamases belonging mainly to Ambler class A, which includes TEM-, SHV-, CTX-M, GES, VEB enzyme families. ESBLs also include one enzyme family, the OXA- family, belonging to class D (Cantón et al., 2012). Isolates carrying plasmid-encoded AmpC can be easily misidentified as ESBLs due to their overlapping activity against beta-lactam antimicrobials. The inability to distinguish them could have significant treatment consequences (Hanson, 2003).

Mobile genetic elements, such as plasmids or DNA transposons, are the main mechanisms facilitating horizontal genetic transfer (HGT). Plasmid-mediated bla_{CMY-2} has been found to be the most predominant among other acquired *ampC* genes (Mata et al., 2012). The plasmids carrying bla_{CTX-M} or bla_{CMY} beta-lactamase genes have been associated with transferable replicon types IncA/C or IncI1 (Hopkins et al., 2006; Guo et al., 2014).

Antimicrobial use in the poultry industry improves animal health, welfare and production by preventing and treating animal disease resulting in lowered mortality, but may lead to the selection of AMR organisms (Diarra and Malouin, 2014). In Canada, the preventive use of ceftiofur in broiler chicken was voluntarily eliminated by the poultry industry in May 2014; the broiler chicken antimicrobial use (AMU)-AMR surveillance component of the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) detected a significant drop in ceftiofur use at the hatcheries between 2013 and 2014 (Agunos et al., 2017). The three most frequently reported antimicrobials by producers participating in the CIPARS farm surveillance between 2013 and 2015 were bacitracin, virginiamycin and avilamycin (Agunos et al., 2017). Avilamycin was approved around May 2013 and was first reported by producers in 2014.

The isolates used in this study were a subset of those described in the 2015 CIPARS annual report (Public Health Agency of Canada, 2015). The objectives of the study were threefold: 1. To further investigate AMR phenotypes in *E. coli* and *Salmonella* isolates from broiler chickens, 2. To identify genes responsible for ESBL/AmpC phenotypes in multi-drug resistant *E. coli* and *Salmonella* isolates and 3. To conduct conjugation assays to initially assess the potential for horizontal gene transfer between *E. coli* and *Salmonella*.

MATERIALS AND METHODS

Sampling, Bacterial Isolation, and Isolates Used in This Study

Fecal samples were taken from a single production unit on each of 30 registered premises/establishments (farms) participating in the CIPARS broiler chicken farm AMU/AMR surveillance in Alberta in 2015. Participating sentinel veterinarians were responsible for enrolling farms and collecting samples. Farms were chosen based on the veterinary practice profile and using specific inclusion and exclusion criteria. Samples were collected at pre-harvest, approximately one week prior to slaughter (broilers >30 days of age). Fecal samples consisted of 10 fecal droppings from each of the four quadrants of the chosen barn/ floor, pooled to represent the chosen production unit.

For the recovery of organisms, four pooled fresh fecal samples (equivalent to at least 10 individual droppings) were collected from four quadrants of the barn. Laboratory techniques are as follows (in brief): 1) *Salmonella:* fecal samples were weighed and Buffered Peptone Water (BPW) was added (1:10) and incubated at 35°C for 24 hours. A loopful was inoculated into a Modified Semisolid Rappaport Vassiliadis (MSRV) plate and incubated at 42°C for 24 to 72 hours. 2) *E. coli:* A drop of BPW aliquot prepared from above was inoculated on MacConkey agar and incubated at 35°C for 24 hours. It is important to note that the isolates recovered here were part of the CIPARS farm surveillance component and thus no selective media were used. The intent was to harmonize with the isolation/characterization procedures outlined in the CIPARS Report (Public Health Agency of Canada, 2015).

A total number of 120 *E. coli* and 62 *Salmonella* were isolated, banked, and shipped to the University of Calgary frozen on dry ice, by the Agri Food Laboratories Section of Alberta Agriculture and Forestry (Public Health Agency of Canada, 2015;

FoodNet Canada, 2017). *E. coli* J53 (KACC 16628), a recipient isolate for the conjugation experiment, was received from the Korean Agricultural Culture Collection (KACC), Agricultural Microbiology Division, National Academy of Agricultural Science. *E. coli* HB101carrying plasmid pRK600, used as a helper strain, was received from the Dong lab, University of Calgary.

Susceptibility Tests

Minimal Inhibitory Concentrations (MICs) of various antimicrobial agents were determined using Sensititre™ (TREK Diagnostic Systems, Inc.) Gram negative plates (CMV3AGNF) designed by the National Antimicrobial Resistance Monitoring System (Public Health Agency of Canada, 2015). The same panel of antimicrobial agents was used for both *E. coli* and *Salmonella* isolates (**Tables 2–4**). Antimicrobial resistance assays were conducted by the National Microbiology Laboratory (NML) St. Hyacinthine, and NML Guelph (Public Health Agency of Canada, 2015).

The disc diffusion method was used to compare antimicrobial resistance profiles of isolate donors and *E. coli* recipients (Tendencia, 2004). Antibiotic discs were purchased from either BD BBL™ or Oxoid companies. The diameters of the zones of inhibition were recorded and interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute, 2013). For the purposes of this study, isolates displaying intermediate resistance were categorized as sensitive.

Phenotype and Genotype Confirmation of ESBL/AmpC Genes

Two different ESBL/AmpC detection disc sets have been used to confirm ESBL/AmpC phenotypes. The first set is a combination of 4 individual discs of Cefotaxime/Cefotaxime + Clavulanic acid/Ceftazidime/Ceftazidime + Clavulanic acid, purchased from either BD BBL™ or Oxoid company. The second set is a combination of 4 individual discs of Cefpodoxime/Cefpodoxime + ESBL inhibitor/Cefpodoxime + AmpC inhibitor/Cefpodoxime + ESBL inhibitor + AmpC inhibitor, purchased from Mast Group company (D68C set).

In addition, AmpC and ESBL beta-lactamase genes were detected using PCR assays. A total of three AmpC (bla_{CMY-2}, bla_{FOX}, $bla_{ACT-1/MIR-1}$) and ten ESBL (bla_{TEM}, bla_{SHV} $bla_{CTX-M-1}$, $bla_{CTX-M-2}$, $bla_{CTX-M-8}$, $bla_{CTX-M-9}$, bla_{PER-1}, bla_{VEB}, bla_{IBC}/bla_{GES}, bla_{TLA}) beta-lactamase genes were screened in AmpC/ESBL positive isolates. Primers used in the PCR assays are listed in **Table 1**. PCR products at desired sizes were purified and sent for Sanger sequencing (http://www.ucalgary.ca/dnalab/sequencing) to confirm their sequences

Multi-drug resistant (MDR) *E. coli* and *Salmonella* isolates that were resistant to at least three drug classes and were confirmed to exhibit ESBL/AmpC phenotypes were selected for additional experiments. Seven drug classes were used in this study: beta-lactams (AMC = Amoxycillin + Clavulanic acid, AMP = Ampicillin, FOX = Cefoxitin, CRO = Ceftriaxone, TIO = Ceftiofur), aminoglycosides (GEN = Gentamicin, STR = Streptomycin), quinolones (NAL = Nalidixic acid, CIP = Ciprofloxacin), sulfonamides (SSS = Sulfisoxazole, SXT = Trimethoprim sulfamethoxazole), macrolides (AZM = Azithromycin), phenicols (CHL = Chloramphenicol), tetracyclines (TET =Tetracycline).

Plasmid Characterization/Replicon Typing

Plasmid miniprep was performed using an alkaline lysis method (Birnboim and Doly, 1984). The plasmid size was evaluated using agarose gel electrophoresis.

Replicon typing was performed using PCR assay as described previously, with primers listed in **Table 1** (Carattoli et al., 2005).

E. coli Phylogroups/*Salmonella* Serovars

MDR *E. coli* isolates exhibiting ESBL/AmpC phenotypes were assigned into one of four main phylogenetic groups by using a simplified two-step triplex polymerase reaction (Clermont et al., 2000). The results were confirmed using a quadruplex PCR assay which enabled us to classify isolates into a broader range of *E. coli* phylogroups as well as distinguish them from the cryptic clades II to V (Clermont et al., 2013).

An assay to classify *Salmonella* serovars was performed by the PHAC serotyping laboratory as described previously (Public Health Agency of Canada, 2015).

Detection of Integrons/Integrases

To further study other mobile genetic elements, *int1*, *int2* and *int3* genes encoding integrase of class 1, 2 and 3 integrons, respectively, were investigated by PCR assays using primers listed in **Table 1** as described previously (White et al., 2001). Primers for amplifying the class 1 and class 2 integron cassette regions were used to detect the presence of resistance gene cassettes (**Table 1**) (White et al., 2001). PCR products were purified and sent for Sanger sequencing (http://www.ucalgary.ca/dnalab/sequencing) to confirm their sequences.

Conjugation Experiment

Conjugation experiments were conducted using MDR isolates of interest as donors and *E. coli* J53 as the recipient with or without the presence of helper strain HB101/pRK600. *E. coli* J53 (F- *met pro* Azi^r Amp^s), an *E. coli* K-12 derivative strain, is resistant to sodium azide but sensitive to beta-lactams (Yi et al., 2012). This strain is also sensitive to other drugs (e.g. tetracycline, chloramphenicol, gentamicin, streptomycin) (Lei et al., 2019). Recipient and donor strains were inoculated into LB broth and cultured overnight at 37°C. The next day, cells were harvested, washed with saline, and mixed together in a ratio of 1:1, and spotted on to LB plates. They were also spotted individually on LB plates as controls. After overnight incubation at 37°C, mating spots were washed and resuspended in saline; and different dilutions were plated on LB media containing sodium azide (0.2 gL^{-1}) and ampicillin (100 μgml^{-1}) to select transconjugants. Control spots were transferred to the same selective media to make sure that no growth was observed. Conjugation frequency was calculated by taking the ratio of the number of colonies counted on selective plates (LB supplemented with sodium azide (0.2 gL^{-1}) + ampicillin (100 μgml^{-1})) for transconjugants over the number of colonies on selective plates (LB supplemented with

TABLE 1 | List of primers used in this study.

Primers	PCR product	Size (bp)	Reference
CIT-A 5'-ATGCAGGAGCAGGCTATTC-3' CIT-B 5'-TGGAGCGTTTTCTCCTGAAC-3'	bla_{CMY-2} FOd	689	(Mulvey et al., 2005)
fox-F 5'-TGTGGACGGCATTATCCAG-3' fox-R 5'-AAAGCGCGTAACCGGATTG-3'	bla_{FOX}	868	(Mulvey et al., 2005)
ent-F 5'-AGTAAAACCTTCACCTTCACCG-3' ent-R 5'-ATGCGCCTCTTCCGCTTTC-3'	$bla_{ACT-1/MIR-1}$	439	(Mulvey et al., 2005)
tem-F 5'-ATGAGTATTCAACATTTCCGT-3' tem-R 5'-TTACCAATGCTTAATCAGTGA-3'	bla_{TEM}	861	(Ryoo et al., 2005)
shv-F 5'-CCGGGTTATTCTTATTTGTCGCT-3' shv-R 5'-TAGCGTTGCCAGTGCTCG-3'	bla_{SHV}	831	(Ryoo et al., 2005)
C1-F 5'-GGACGTACAGCAAAAACTTGC-3' C1-R 5'-CGGTTCGCTTTCACTTTTCTT-3'	bla_{CTX-M} (CTX-M-1 group)	624	(Ryoo et al., 2005)
C2-F 5'-CGGTGCTTAAACAGAGCGAG-3' C2-R 5'-CCATGAATAAGCAGCTGATTGCCC-3'	bla_{CTX-M} (CTX-M-2 group)	891	(Ryoo et al., 2005)
C8-F 5'-ACGCTCAACACCGCGATC-3' C8-R 5'-CGTGGGTTCTCGGGGATAA-3'	bla_{CTX-M} (CTX-M-8 group)	490	(Ryoo et al., 2005)
C9-F 5'-GATTGACCGTATTGGGAGTTT-3' C9-R 5'-CGGCTGGGTAAAATAGGTCA-3'	bla_{CTX-M} (CTX-M-9 group)	947	(Ryoo et al., 2005)
PER-1-F 5'-GTTAATTTGGGCTTAGGGCAG-3 PER-1-R 5'-CAGCGCAATCCCCACTGT-3'	bla_{PER-1}	855	(Ryoo et al., 2005)
VEB-F 5'-ACCAGATAGGAGTACAGACATATGA -3' VEB-R 5'-TTCATCACCGCGATAAAGCAC-3'	bla_{VEB}	727	(Ryoo et al., 2005)
I/G-F 5'-GTTAGACGGGCGTACAAAGATAAT-3' I/G-R 5'-TGTCCGTGCTCAGGATGAGT-3'	bla_{IBC}/bla_{GES}	903	(Ryoo et al., 2005)
TLA-F 5'-CGCGAAAATTCTGAAATGAC-3' TLA-R 5'-AGGAAATTGTACCGAGACCCT-3'	bla_{TLA}	992	(Ryoo et al., 2005)
ChuA.1 5'-GACGAACCA ACGGTCAGGAT-3' ChuA.2 5'-TGCCGCCAGTACCAAAGACA-3'	*chuA*	279	(Clermont et al., 2000; Clermont et al., 2013)
YjaA.1 5'-TGAAGTGTCAGGAGACGCTG-3' YjaA.2 5'-ATGGAGAATGCGTTCCTCAAC-3'	*yjaA*	211	(Clermont et al., 2000; Clermont et al., 2013)
TspE4C2.1 5'-GAGTAATGTCGGGGCATTCA-3'	TspE4.C2	152	(Clermont et al., 2000; Clermont et al., 2013)

(Continued)

TABLE 1 | Continued

Primers	PCR product	Size (bp)	Reference
TspE4C2.2 5'-CGCGCCAACAAAGTATTACG-3' AceK.f 5′-AACGCTATTCGCCAGCTTGC-3′ ArpA1.r 5′-TCTCCCCATACCGTACGCTA-3'	*arpA*	400	(Clermont et al., 2013)
hep35 5'-TGCGGGTYAARGATBTKGATTT-3' hep36 5'-CARCACATGCGTRTARAT-3'	*int1, int2* and *int3*	491	(White et al., 2000; White et al., 2001)
hep58 TCATGGCTTGTTATGACTGT hep59 5'-GTAGGGCTTATTATGCACGC-3'	Class1 integron casset region	variable	(White et al., 2000; White et al., 2001)
hep74 5'-CGGGATCCCGGACGGCATGC ACGATTTGTA-3' hep51 5'-GATGCCATCGCAAGTACGAG-3'	Class2 integron cassette regions	variable	(White et al., 2001)
IncI1_F 5'-CGAAAGCCGGACGGCAGAA-3' IncI1_R 5'-TCGTCGTTCCGCCAAGTTCGT-3'	RNAI	139	(Carattoli et al., 2005)
IncA/C_F 5'-GAGAACCAAAGACAAAGACCTGGA-3' IncA/C_R 5'-ACGACAAACCTGAATTGCCTCCTT-3'	repA	465	(Carattoli et al., 2005)

sodium azide (0.2 gL^{-1})) for recipients. If there were no transconjugants obtained, a helper strain (HB101/pRK600) was added into the mating mix in the proportion of 1:1:0.5 (donor: recipient: helper strain) and spotted on LB plates as described. If there was no growth on plates selected for recipients, trypsin was added to the media to reverse the effect of colicin produced by the donors, and recover the recipients (Nomura and Nakamura, 1962; Dankert et al., 1980).

Salmonella isolate 112.2 was screened for spontaneously mutated colonies resistant to sodium azide (Azi^R) by plating on LB supplemented with sodium azide (0.2 gL-1). Then this Azi^R *Salmonella* was used as a recipient in conjugation with MDR isolates of interest as donors. Conjugation protocol was performed as described above.

Data Visualization Tools

Data visualization in this study was performed using following programs: Microsoft Excel 2013, R programming (R version 3.4.1).

RESULTS

Sampling, Isolation and Identification of Bacterial Strains

Four *E. coli* isolates were obtained from each farm, resulting in 120 *E. coli* isolates from 30 farms with a recovery rate of 100% (30/30). There were 23 of 30 farms *Salmonella* positive, with between one and four isolates identified per farm. Hence the recovery rate for *Salmonella* was about 77% (23/30). There were 14 different serovars identified among 62 *Salmonella* isolates (**Table 2**). The three most prevalent *Salmonella* serovars in our study were Enteritidis, Hadar, and Thompson.

Antimicrobial Susceptibility Testing

Antimicrobial resistance in *E. coli* and *Salmonella* isolates is described as follows. Isolates that were resistant to three or more antimicrobial classes were considered MDR. Thirty-one percentage of *E. coli* (37/120) were MDR and 16% of *Salmonella* (10/62) were MDR. About 4% of *E. coli* (5/120) were resistant to five antimicrobial classes, while none of *Salmonella* were resistant to five classes.

The majority of *Salmonella* isolates were resistant to streptomycin and tetracycline (**Table 2**). There were 8 *Salmonella* serotypes that were sensitive to all tested antimicrobials (Enteritidis, Typhimurium, Braenderup, Hartford, Infantis, Schwarzengrund, Senftenberg, Thompson). In addition to streptomycin and tetracycline, the majority of MDR *E. coli* showed resistance to sulfisoxazole (**Table 3**). Among *E. coli* that were phylo-typed, those belonging to groups D or E had diverse AMR patterns (**Table 4**). Overall, *E. coli* isolates showed more diversity in resistance phenotype between farms than did *Salmonella* (**Figure 1**).

Among MDR isolates, 19 *E. coli* and 10 *Salmonella* isolates with resistance to the beta-lactam class of antimicrobials and at least two other drug classes, were selected for further study. These isolates came from 19 farms.

ESBL/AmpC Phenotypes and Genotypes

Eight out of 19 MDR *E. coli* isolates were resistant to both penicillin and cephalosporin beta-lactam sub-classes and were confirmed as AmpC phenotype (**Table 5**). A unique *Salmonella*

TABLE 2 | AMR patterns in *Salmonella* isolates along with their serotypes.

Salmonella serotype	Resistance pattern													Ratio[a]
	beta-lactam					Macrolide	Phenicol	Quinolone		Aminoglycoside		Sulfonamide		Tetracycline	
	AMC	AMP	CRO	FOX	TIO	AZM	CHL	CIP	NAL	GEN	STR	SSS	SXT	TET	
Enteritidis															10/10
Braenderup															1/1
Hadar															
Pattern 1		R									R			R	6/10
Pattern 2		R												R	1/10
Pattern 3											R			R	3/10
Hartford															1/1
Heidelberg		R	R								R			R	1/1
Infantis															9/9
Kentucky											R			R	1/1
Mbandaka															
Pattern 1											R	R		R	1/4
Pattern 2														R	3/4
Schwarzengrund															2/2
Senftenberg															5/5
Thompson															10/10
Typhimurium															4/4
Worthington											R	R		R	2/2
I 6,7:k:-															2/2
Total															62/62

There were seven drug classes tested: 1. Beta-lactam (AMC, Amoxycillin + Clavulanic acid; AMP, Ampicillin; FOX, Cefoxitin; CRO, Ceftriaxone; TIO, Ceftiofur), 2. Aminoglycosides (GEN, Gentamicin; STR, Streptomycin), 3. Quinolones (NAL, Nalidixic acid; CIP, Ciprofloxacin), 4. Sulfonamides (SSS, Sulfisoxazole; SXT, Trimethoprim sulfamethoxazole), 5. Macrolides (AZM, Azithromycin), 6. Phenicols (CHL, Chloramphenicol), 7. Tetracyclines (TET, Tetracycline). Drugs were grouped in classes by clear/grey areas.
R, Resistant.
[a]*Ratio is defined as the number of isolates of the same resistance pattern over the total number of isolates.*
All the antimicrobials belonging to the same drug class were placed next to each other and separated from those in other drug classes by shading.

TABLE 3 | AMR patterns in all MDR *E.coli* isolates (≥3 drug classes).

Resistance to # classes	Resistance pattern													Ratio[a]
	beta-lactam					Macrolide	Phenicol	Quinolone		Aminoglycoside		Sulfonamide		Tetracycline	
	AMC	AMP	CRO	FOX	TIO	AZM	CHL	CIP	NAL	GEN	STR	SSS	SXT	TET	
5	R	R	R	R	R		R				R	R		R	3/5
	R	R	R	R	R		R			R	R	R		R	2/5
4	R	R	R	R	R					R	R	R		R	1/7
		R								R		R		R	4/7
		R									R	R		R	2/7
3	R	R	R	R	R						R			R	1/25
	R	R	R	R	R				R		R				1/25
	R	R									R			R	1/25
		R								R	R			R	1/25
		R								R	R	R	R		1/25
		R									R	R	R		1/25
		R									R			R	1/25
							R					R		R	1/25
										R	R	R		R	3/25
										R		R		R	7/25
											R	R	R	R	1/25
											R	R		R	6/25
Total															37/37

There were seven drug classes: 1. Beta-lactam (AMC, Amoxycillin + Clavulanic acid; AMP, Ampicillin; FOX, Cefoxitin; CRO, Ceftriaxone, TIO, Ceftiofur), 2. Aminoglycosides (GEN, Gentamicin; STR, Streptomycin), 3. Quinolones (NAL, Nalidixic acid; CIP, Ciprofloxacin), 4. Sulfonamides (SSS, Sulfisoxazole; SXT, Trimethoprim sulfamethoxazole), 5. Macrolides (AZM, Azithromycin), 6. Phenicols (CHL, Chloramphenicol), 7. Tetracyclines (TET, Tetracycline). Drugs were grouped in classes by clear/grey areas.
Antimicrobials belonging to the same drug class were placed next to each other and separated from those in other drug classes by shading.
[a]*Ratio is defined as the number of isolates of the same resistance pattern over the total number of isolates.*

TABLE 4 | AMR patterns in eight MDR *E. coli* strains carrying plasmids and their phylogroups.

E. coli phylogroup	Resistance pattern													Number of isolates	Ratio[a]
	beta-lactam					Macrolide	Phenicol	Quinolone		Aminoglycoside		Sulfonamide		Tetracycline	
	AMC	AMP	CRO	FOX	TIO	AZM	CHL	CIP	NAL	GEN	STR	SSS	SXT	TET	
A or C D or E	R	R	R	R	R		R				R	R		R	3	3/3
57.1	R	R	R	R	R						R			R	1	1/4
58.1	R	R	R	R	R		R			R	R	R		R	1	1/4
61.1	R	R	R	R	R					R	R	R		R	1	1/4
113.1	R	R	R	R	R				R		R				1	1/4
B1	R	R	R	R	R		R			R	R	R		R	1	1/1
Total															8	8/8

There were seven drug classes: 1. Beta-lactam (AMC, Amoxycillin + Clavulanic acid; AMP, Ampicillin; FOX, Cefoxitin, CRO=Ceftriaxone; TIO, Ceftiofur), 2. Aminoglycosides (GEN, Gentamicin; STR, Streptomycin), 3. Quinolones (NAL, Nalidixic acid; CIP, Ciprofloxacin), 4. Sulfonamides (SSS, Sulfisoxazole; SXT, Trimethoprim sulfamethoxazole), 5. Macrolides (AZM, Azithromycin), 6. Phenicols (CHL, Chloramphenicol), 7. Tetracyclines (TET, Tetracycline). Drugs were grouped in classes by clear/grey areas.

R, Resistant.

[a]*Ratio is defined as the number of isolates of the same resistance pattern over the total number of isolates.*

All the antimicrobials belonging to the same drug class were placed next to each other and separated from those in other drug classes by shading.

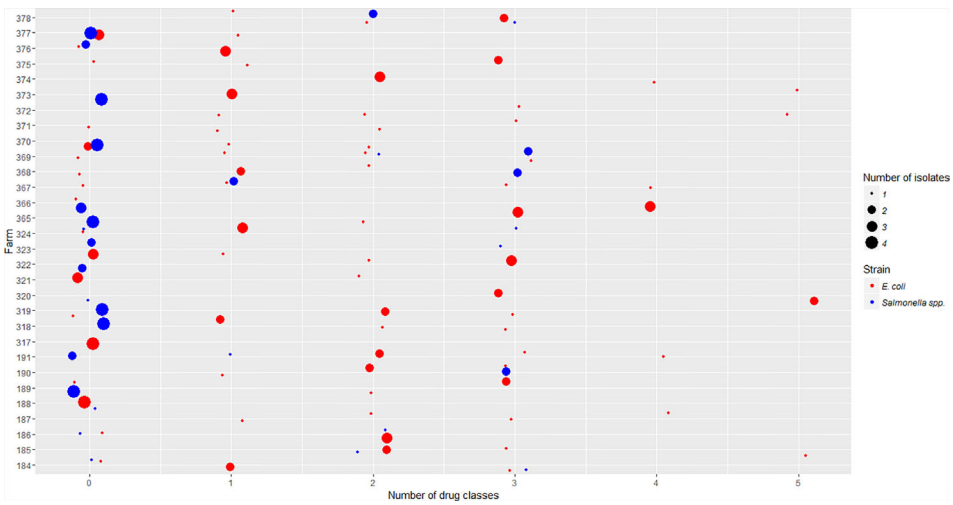

FIGURE 1 | *Salmonella* isolates resistant to differing number of drug classes were compared to *E. coli* isolates across participating farms. The x-axis is the number of drug classes to which isolates showed resistance. The y-axis is the code of individual farms participating in this project. The size of each dot represents the number of isolates obtained in each farm. The color represents whether the isolates are *E. coli* or *Salmonella* strains.

was resistant to both penicillin and cephalosporin sub-classes and was potentially an ESBL phenotype; in the presence of an ESBL inhibitor (clavulanic acid) the isolate showed sensitivity to penicillin (specifically amoxicillin). The ESBL phenotype was subsequently confirmed by disc diffusion method.

Plasmids extracted from nine isolates which either showed the AmpC or ESBL phenotype were used as templates in PCR reactions to detect a variety of AmpC and ESBL genes. The $bla_{\text{CMY-2}}$ gene, an AmpC-type gene, and the bla_{TEM} gene, an ESBL-type gene, were identified on plasmids from all nine *E. coli* and *Salmonella* isolates. The sequence of the bla_{TEM} gene identified in this study shared 100% identity with the sequence of the $bla_{\text{TEM-116}}$ gene found in *E. coli* strain MRC3 (accession no. KJ923009.1)

Plasmid Characterization

Nine isolates were found to carry IncI1 and IncA/C-type replicon plasmids (**Table 5**). The plasmids varied in size from approximately 7kb to larger than 20kb. The plasmid in one *Salmonella* isolate has a size larger than 20 kb with I1 and A/C-type replicon. Two *E. coli* isolates, 58.1 and 61.1, carried small plasmids (<10kb), while the rest carried larger ones (≥20 kb). All plasmids in *E. coli* isolates had IncI1 and IncA/C-type replicons.

E. coli Phylogroups/*Salmonella* Serovars of ESBL/AmpC-Positive Isolates

Both PCR methods confirmed that none of the *E. coli* isolates belonged to the group B2 (a group with high potential for

TABLE 5 | Characteristics of MDR *E. coli* and *Salmonella* isolates showing ESBL/AmpC phenotypes.

Strain	Isolate Number	Phylogroup (*E.coli*)/Serotype (*Salmonella*)	Plasmid type	ESBL/AmpC phenotype	bla_{CMY-2} gene	$bla_{TEM-116}$ gene	Integron/Integrase
E. coli	11.1	A[a]/A or C[b]	A/C, I1	AmpC	+	+	*aadA, aadA1a/A2*
	12.1	A[a]/A or C[b]	A/C, I1	AmpC	+	+	
	57.1	D[a]/D or E[b]	A/C, I1	AmpC	+	+	
	58.1	D[a]/D or E[b]	A/C, I1	AmpC	+	+	
	61.1	D[a]/D or E[b]	A/C, I1	AmpC	+	+	*int1*, *aadA*
	82.1	B1[a,b]	A/C, I1	AmpC	+	+	*int1, putative aadA1*
	89.1	A[a]/A or C[b]	A/C, I1	AmpC	+	+	
	113.1	D[a]/D or E[b]	A/C, I1	AmpC	+	+	
Salmonella	119.2	Heidelberg	A/C, I1	ESBL	+	+	

[a,b] *E. coli isolates were assigned into different phylogenetic group using both triplex and quadruplex PCR methods as described in previous studies, respectively (Clermont et al., 2000; 2013).*

pathogenicity) (**Tables 4** and **5**). Phylogroups of *E. coli* identified in this study were A, B_1 and D (triplex PCR) or A, B_1, C, D and E (quadruplex PCR). One MDR *Salmonella* isolate exhibiting the ESBL phenotype was identified as Heidelberg serovar.

Detection of Integrons/Integrases

Using DNA from plasmid extraction as a template in PCR reactions, the class 1 integrase gene *int1* was detected in two *E. coli* isolates that were from 2 different farms (**Table 5**). The class 1 integron cassette region was also detected in three isolates by PCR (**Table 5**). The PCR products were sequenced, and the results were confirmed by blasting the sequence against the NCBI database. The sequence matched the aminoglycoside resistance genes, *aadA* encoding streptomycin 3"-adenylyltransferase, *aadA1a*/*aadA2* encoding aminoglycoside 3"-O-adenyltransferase. The class 1 integron cassettes were also amplified from plasmid extraction from *E. coli* isolate 82.1. The results were confirmed by blasting the sequence against the NCBI database. The sequences matched the sequence of a putative adenylyltransferase found on a plasmid isolated from the *Salmonella* Heidelberg strain N418 (Accession no. CP009409).

Transfer of Plasmids Carrying Resistance Genes by Conjugation

Plasmids were mobilized from all, but one *E. coli* isolate 89.1, to an *E. coli* recipient J53 (**Table 6**). No growth was observed on selective plates of either the transconjugants or recipients when attempting to conjugate *E. coli* isolate 61.1. However, the conjugation experiment of this isolate was successful when the media were supplemented with trypsin to reverse the effect of colicin produced by the donors. Additionally, the *E. coli* isolate 113.1 required the presence of the helper *E. coli* strain HB101 carrying helper plasmid pRK600 to enable movement of the plasmid to recipients. All transconjugants were resistant to ampicillin which was used as a selective marker in conjugation experiments. Interestingly, their resistance phenotypes to other drugs, which were presented as "transferable AMR" in **Table 6**, were distinctly different, except for recipients that were conjugated with either donor 12.1 or 113.1. Although transconjugants obtained from conjugation assays with either donor 12.1 or 113.1 were resistant to the same drugs including amoxicillin, cefoxitin, ceftriaxone and ceftiofur, donor 12.1 produced *E. coli* transconjugants more efficiently (higher conjugation frequency) and did not require the presence of the helper strain (**Table 6**).

When using a lab-engineered sodium azide-resistant *Salmonella* as a recipient, we observed that plasmids from eight *E. coli* isolates with the exception of the isolate 89.1 mentioned above, were able to move to *Salmonella* with variable conjugation frequency (**Table 7**).

Farm Characteristics for Nine Isolates From Which Plasmids Were Mobilized

Seven 'conventional' (i.e., antimicrobials were used to some extent in all flocks) farms under the veterinary care of one

TABLE 6 | Conjugation frequency and AMR profile of transconjugants compared to donors (tested isolates) using *E. coli* J53 as a recipient.

Donor Strain	IsolateNumber	Conjugation frequency	Transferable AMR	Non-transferable AMR
E. coli	11.1	4×10^{-5}	AML, FOX, STR, TIO, CHL[a], CRO, TET	NAL
	12.1	13×10^{-3}	AML, FOX, TIO, CRO	NAL, CHL, TET
	57.1	14×10^{-5}	AML, STR, TET	FOX, TIO, CRO
	58.1	3×10^{-1}	AML, FOX, GEN, STR, TIO, CHL, CRO, TET	-
	61.1[b]	3×10^{-4}	AML, FOX	TIO, CRO, GEN, STR, TET, TIO
	82.1	5×10^{-2}	AML, FOX, GEN, STR, TIO, CHL[a], CRO	TET
	89.1	Non-transferable	N/A	AML, FOX, STR, TIO, CHL, CRO, TET
	113.1[c]	3×10^{-4}	AML, FOX, CRO, TIO	NAL, STR
Salmonella (Heidelberg)	119.2	9×10^{-2}	AML, TIO[a], CRO[a], TET[a]	STR

Antimicrobial abbreviation: AML, Amoxicillin; FOX, Cefoxitin; CRO, Ceftriaxone; GEN, Gentamicin; NAL, Nalidixic acid; CHL, Chloramphenicol; TET, Tetracycline; TIO, Ceftiofur; STR, Streptomycin.
[a] *Results were interpreted as intermediate (between resistant and sensitive) in recipient E. coli strains while donor strains (original isolates) were resistant to these antimicrobial agents.*
[b] *Donor strains produced colicin which is lethal to recipient strains. Trypsin was added into the media to rescue recipient strains.*
[c] *Helper strains were required to help transfer plasmid from donor strains to recipient strains.*

practice were represented by the nine ESBL/AmpC phenotyped isolates in this study. All of the farms, excluding the farm providing isolate 89.1, received their chicks from the same hatchery. All birds were Ross 308 strain. The most frequently used antimicrobials were bacitracin and salinomycin administered *via* feed (n = 5), followed by the combination of penicillin and streptomycin administered *via* water (n = 3). In addition, the feed were also reportedly medicated with the following antimicrobials avilamycin (n = 1), tylosin (n = 2), and coccidiostats such as decoquinate (n = 1), monensin (n = 1), the ionophore-chemical coccidiostat combination, narasin and nicarbazin (n = 2).

The number of chicks sampled per flock ranged from 14,790 to 55,000 within a single production unit. Age on the day of sampling ranged from 30 to 35 days old with an average weight ranging from 1.7 kg to 2.2 kg. The recorded floor space in the barns ranged from 8000 ft^2 to 30550 ft^2 and stocking density ranged from 0.54 to 0.67 ft^2 per bird. Reported percentage of mortality ranged from 2.47% to 7.19% of the birds placed within the barn.

Hydrogen peroxide was used on three of the farms for cleaning of water lines between flocks. Five of the farms also used chlorine for treatment of their water lines during the production cycle. Footbaths (n = 3), dedicated farm clothes (n = 4) and gloves (n =2) were methods of farm biosecurity utilized. Manure was stored onsite in the vicinity of the barn on three farms. The most frequently reported method of cleaning the barns after each production cycle was washing only (n = 6) and chlorine products were used for disinfection on four farms.

DISCUSSION

The three most prevalent *Salmonella* serovars in our study were Enteritidis, Hadar, and Thompson. Serovars Typhimurium and Heidelberg were also identified. According to the National Enteric Surveillance Program (NESP) 2013 Annual Report, the three most commonly reported serovars from human cases in Canada, which has remained unchanged since 2008, were Enteritidis, Heidelberg and Typhimurium (Government of Canada, 2015). Serovar Enteritidis is known as one of the most common *Salmonella* serovars found in broiler chickens, and the second most prevalent cause of *Salmonella* infection in humans after the serovar Typhimurium (Suzuki, 1994; Porwollik et al., 2005; Trampel et al., 2014). Serovars Hadar, Heidelberg, Mbandaka and Worthington were MDR. Of the 14 serovars in our study, the *Salmonella* serovar Heidelberg was the only MDR isolate with an ESBL phenotype. This is a of concern because in 2013-2014, a national outbreak of MDR *Salmonella* Heidelberg infections in the United States resulted in 200 hospitalized cases of 528 total cases (38%) (Gieraltowski et al., 2016). This outbreak was linked to chicken products from a single poultry company. In our study, six of the seven farms had chicks sourced from the same hatchery. There is potential for widespread dissemination of virulent bacteria over a wide geographical region if such strains are present among eggs or chicks at the hatchery level. However, there was no evidence of this in our study.

Urinary tract infections are one of the most common bacterial infections reported in primary care and the emergency department in Canada (Sanyal et al., 2019). Phylogroup B2 has been considered to be the most extra-intestinal virulent group (Clermont et al., 2000), and shown to have a strong association with the uropathogenic subpathotype (Hutton et al., 2018). To determine whether the MDR *E. coli* isolates that exhibited AmpC or ESBL phenotypes belonged to phylogroup B2, both triplex and quadruplex PCR assays were performed. Both assays confirmed that these isolates did not belong to group B2, therefore less likely caused urinary tract infections.

Both bla_{CMY-2} and bla_{TEM} genes may be present on plasmids isolated from AmpC/ESBL positive MDR *E. coli* and *Salmonella* isolates. The bla_{CMY-2} and bla_{TEM} genes had identical sequences in both *E. coli* and *Salmonella* isolates in this study. A previous study showed the evidence for the transfer of bla_{CMY-2}- carrying plasmids between *E. coli* and *Salmonella* isolates (Winokur et al., 2001). Interestingly, *E. coli* isolates had the AmpC beta-lactamase phenotype while *Salmonella* had the ESBL beta-lactamase phenotype. Even though both species carried both bla_{CMY-2} and bla_{TEM} genes, the phenotype differences suggest differential expression of these genes in these *E. coli* and *Salmonella* isolates.

The bla_{CMY-2} gene is the most common AmpC-type gene found in both *E. coli* and *Salmonella* from various sources: food, animals, and hospitals in multiple countries (Mulvey et al., 2005; Hiki et al., 2013; Cejas et al., 2014; Guo et al., 2014; Ghodousi et al., 2015). This gene has been hypothesized to have originated on the chromosome of *E. coli* and it could be induced with beta-lactams in some *Enterobacteriaceae* such as *Enterobacter cloacae, Citrobacter freundii, Serratia marcescens*, and *Pseudomonas aeruginosa* (Sanders, 1987; Philippon et al., 2002). Unlike these bacteria, *E. coli* and *Salmonella* lack systems to produce inducible AmpC enzymes. Mutations in the *ampC* promoter have increased the resistance to oxyimino-cephalosporins in *E. coli* (Caroff et al., 1999).

Plasmids are considered to be facilitators for disseminating beta-lactamase genes between various species such as *P. mirabilis*, *Achromobacter*, *Salmonella* and *E. coli* (Bobrowski et al., 1976; Levesque et al., 1982; Knothe et al., 1983; Bauernfeind et al., 1989). Molecular characterization of MDR plasmids is essential, yet complicated, because these plasmids are very diverse and promiscuous. The relatedness of plasmids can be analyzed using a PCR-based replicon typing method or whole genome sequence analysis (Carattoli et al., 2005). In previous

TABLE 7 | Conjugation frequency between donors (tested isolates) and a recipient strain (sodium azide-resistant *Salmonella*).

Strain	Isolate ID Number	Conjugation frequency	Isolate ID Number	Conjugation frequency
E. coli	11.1	4×10^{-9}	61.1	5×10^{-4}
	12.1	1×10^{-5}	82.1	2×10^{-2}
	57.1	3×10^{-7}	89.1	Non-transferable
	58.1	7×10^{-7}	113.1	2×10^{-4}
Salmonella (Heidelberg)	119.2	6×10^{-2}		

studies, bla_{CMY-2}-carrying plasmids found in either *E. coli* or *S. enterica* were most likely to belong to replicons I1 and A/C (Carattoli, 2009). Our results are also in accordance with these findings. bla_{TEM} genes have been reported to be located on plasmids of various replicon types such as A/C, I1, K, ColE, H12, etc. (Carattoli, 2009). In our study, bla_{TEM} gene was found on plasmids of replicons A/C or I1 in one *Salmonella* isolate and eight *E. coli* isolates. Previously, it was shown that plasmids encoding ESBL/AmpC genes in *E. coli* were highly promiscuous, resulting in the possibility of HGT between *E. coli* and related *Enterobacteriaceae* strains (Ewers et al., 2012).

In addition to plasmids, other mobile genetic elements (e.g. integrons containing gene cassettes and integrase) are also facilitating the spread of AMR genes (White et al., 2001). It was shown in multiple independent reports that there was an occurrence of integrons especially class 1 integrons and AMR genes (Leverstein-van Hall et al., 2002; Marashi et al., 2012; Di Cesare et al., 2016). More specifically, a significant association was found between integrons and resistance to certain antimicrobials including gentamicin, kanamycin, streptomycin, tobramycin, sulfafurazole, trimethoprim, ampicillin, chloramphenicol, and tetracycline (White et al., 2001). In our study, the integrase gene *int1* was detected in two out of nine AmpC/ESBL-producing isolates. Using specific primers to amplify class 1 integron cassette regions revealed the presence of the aminoglycoside resistance genes *aadA* encoding streptomycin 3''-adenylyltransferase, *aadA1a*/*aadA2* encoding aminoglycoside 3''-O-adenyltransferase, and a putative adenylyltransferase gene. *E. coli* isolate 11.1 carried a gene cassette in its variable region but did not carry the *int1* gene of the classical class 1 integron. This might be due to the disruption caused by IS*26* which was reported in a previous study (Dawes et al., 2010).

Conjugation *in vitro* showed that most of AmpC/ESBL positive MDR isolates carried transferable plasmids that can disseminate AMR phenotypes. The conjugation experiment on isolate 61.1 required the addition of trypsin into the media. Previous studies have shown that treatment of cells with trypsin reversed the inhibition activity caused by colicin (Nomura and Nakamura, 1962; Dankert et al., 1980). The isolate 61.1 likely harbored a colicin-producing plasmid which prevented conjugal transfer of the R-plasmid; hence, this would likely prevent conjugal transfer in the natural microbial community as well. It is interesting to note that in this study all ESBL/AmpC-producing MDR isolates, except for *E. coli* isolate 89.1, were from farms receiving their chicks from the same hatchery; and *Salmonella* serovar Heidelberg was one of them. In addition, we were able to transfer plasmids between strains: from *E. coli* to *E. coli*, from *E. coli* to *Salmonella*, from *Salmonella* to *E. coli*, and from *Salmonella* to *Salmonella*. Plasmids were transferable through conjugation from *E. coli* to *E. coli* or *Salmonella* to *E. coli* at higher frequencies compared to plasmids from *E. coli* to *Salmonella* or *Salmonella* to *Salmonella*.

In conclusion, this study investigated antimicrobial resistance phenotypes of *Escherichia coli* and *Salmonella* isolates from 30 broiler farms, which were obtained through the Canadian Integrated Program for Antimicrobial Resistance Surveillance. The study subsequently identified MDR isolates of *E. coli* and *Salmonella enterica* with ESBL/AmpC phenotypes and examined the sequences of the ESBL/AmpC genes in these isolates. In addition, plasmids from these MDR isolates were isolated and shown to carry the identical replicon type. We also performed conjugation assays between *E. coli* and *Salmonella* isolates to initially assess the potential for HGT. Overall, results suggested there are MDR bacteria in broiler chicken environments with characteristics that could potentially allow them to flourish in the broiler environment, and the possibility of natural HGT by conjugation between *E. coli* and *Salmonella* may readily occur in the broiler chicken house environment.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

SC, KL, SG, and CM conceptualized the research idea and obtained research funding from AAF (PI: SC). SG and AA developed the CIPARS AMU-AMR farm surveillance framework, farm surveillance tools (questionnaire) and protocols, and validated the recovery and AMR datasets. Bacterial isolation and initial antimicrobial susceptibility testing were performed by RC. TT and KL were responsible for experimental design. TT conducted research and laboratory analysis. NC conducted statistical analysis. TT and NC designed and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Prevalence and Antimicrobial Resistance of *Salmonella* Isolated From Dead-in-Shell Chicken Embryos in Shandong, China

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The present study was designed to explore the *Salmonella* prevalence and antimicrobial resistance characteristics in the context of chick mortality at hatching in China. Between December 2015 and August 2017, 1,288 dead-in-shell chicken embryos were collected from four breeder chicken hatcheries in Tai'an, Rizhao, Jining, and Heze, China. *Salmonella* isolates were successfully recovered from 6.7% of these embryos (86/1,288) and were evaluated for serotype, antimicrobial susceptibility, Class 1 integron prevalence, antimicrobial resistance gene expression, and multilocus sequence typing (MLST). *Salmonella* Thompson (37.2%), and *Salmonella* Infantis (32.6%) were the two most prevalent isolates in these chicken embryos, and 66.3% of isolates exhibited robust ampicillin resistance, while 55.8% of isolates exhibited multi-drug resistance (MDR). The majority of isolates harbored the bla_{TEM} gene (74.4%), with the *qnrS* gene also being highly prevalent (50.0%). In contrast, just 27.9% of these isolates carried Class 1 integrons. These 86 isolates were separated into four sequence types (STs), whereby ST26 (32.2%) was the most prevalent. Overall, these results suggested that *Salmonella* infections may be an important cause of chicken embryo mortality in China, and that efforts to support the appropriate use of antibiotics in managing poultry populations are essential.

Keywords: chicken embryos, class 1 integrons, antimicrobial resistance, MLST, *Salmonella*

INTRODUCTION

Salmonella is an important foodborne pathogen that can cause serious illness in humans and animals (1). Over 2,600 *Salmonella* serovars have been detected to date (2), and these bacteria cause illness in roughly 1 million patients per year in the USA alone, leading to approximately $365 million in medical costs (3). Salmonellosis is also highly prevalent in China and is particularly common in elderly or immunocompromised individuals (4, 5). Most *Salmonella* infections occur as a consequence of the consumption of contaminated pork, poultry, or other foods, with poultry in particular being commonly identified as an important *Salmonella* reservoir species. A range of *Salmonella* serovars can infect poultry, causing significant morbidity and mortality and enabling horizontal transmission of these

bacteria within flocks as well as vertical transmission of these bacteria to eggs, often resulting in embryonic mortality or death of newly hatched birds (6, 7). Prior work suggests that *Salmonella* infections are associated with 23.6% of dead breeder chicken embryos in Henan Province, China, with *Salmonella* Pullorum being the dominant serotype in this region (8). Similarly, *Salmonella* samples were isolated from 26.7% of dead-in-shell embryos in Jos, Central Nigeria, with *Salmonella* Hadar being dominant in this context (9). In order to control and prevent the spread of *Salmonella* through the food chain, it is vital that these sources of transmission and contamination be appropriately understood and managed.

Currently, *Salmonella* outbreaks are generally controlled *via* the application of antimicrobial agents. Widespread antibiotic application, however, has led to the emergence of antibiotic- and multidrug-resistant (MDR) *Salmonella* strains that can resist β-lactam and fluoroquinolone treatment, and that thus represent a major threat to global health (10–12). Such antimicrobial resistance can significantly increase treatment-related costs as well as rates of infection-related morbidity and mortality. The emergence of antibiotic-resistant *Salmonella* can occur in particular geographical regions and production sites, and may be confined to particular bacterial serotypes, emphasizing the importance of studying regional *Salmonella* epidemiology (13).

Bacterial genes associated with antibiotic resistance are commonly encoded by mobile genetic elements that can be transmitted between microbes, with DNA-based integrons being the primary mobile genetic elements responsible for the transmission of these genes *via* conjugation (14). The most common integrons in MDR *Salmonella* are class 1 integrons, which are also closely linked to resistance gene dissemination in a range of different pathogens (15).

Prior work has shown that the co-incubation of *Salmonella*-free and *Salmonella*-contaminated eggs can facilitate the horizontal transmission of these bacteria during hatching. After traversing the membrane, *Salmonella* can be extremely difficult to treat and generally further invades the egg whereupon it disrupts normal embryonic development (16, 17). Despite the critical importance of this pathogen, only a few studies have explored the prevalence of *Salmonella* in the context of chick mortality at hatching in Shandong, China (18). This study was therefore designed to assess *Salmonella* prevalence and antibiotic resistance characteristics in four breeder chicken hatcheries in this region in order to better understand the epidemiology of this foodborne pathogen.

MATERIALS AND METHODS

Sample Collection

Between December 2015 and August 2017, 1,288 dead-in-shell chicken embryos were collected from four breeder chicken hatcheries in Tai'an, Rizhao, Jining, and Heze. The lungs, heart, liver, and trachea were taken from each embryo and pooled, transported on ice, and analyzed within 6 h of collection in a laboratory. A bacterial culture was conducted as discussed previously (19). Briefly, 100 mL of buffered peptone water (BPW; Hopebiol, Qingdao, China) was combined with samples, followed by an 18-h incubation at 37°C. Next, 1 mL of pre-enrichment culture was combined with 10 mL of selenite cysteine (SC; Hopebiol, Qingdao, China) broth for 24 h at 42°C. A loop was then used to streak a sample of this SC broth culture on xylose lysine tergitol 4 (XLT4; Hopebiol, Qingdao, China) agar plates, followed by incubation for 24 h at 37°C. A bacterial genome extraction kit (QIAGEN, Mississayga, Ontario, Canada) was then used based on provided directions to isolate bacterial DNA, and polymerase chain reaction (PCR) amplification of the *invA* gene was used to confirm the identity of presumed *Salmonella* colonies (20).

Salmonella Serotyping

Salmonella isolate serotypes were established *via* the Kauffmann-White approach through slide agglutination using O and H antigen-specific sera (Tianrun Bio-Pharmaceutical, Ningbo, China) (21).

Antimicrobial Susceptibility Testing

A Kirby-Bauer disk diffusion approach was used to evaluate *Salmonella* sensitivity to treatment with 12 different common antibiotics, as per the protocols of the Clinical and Laboratory Standards Institute (22). Antibiotics used for these tests included ampicillin (AMP), cephalosporin/acid (CAC), cefazolin (CFZ), chloramphenicol (CHL), ciprofloxacin (CIP), nalidixic acid (NA), polymyxin B (PB), fosfomycin (FFN), gentamicin (GEN), tetracycline (TET), streptomycin (STR), and sulfamethoxazole (SXT). As a control, the ATCC 25922 and ATCC 35218 *Escherichia coli* strains were utilized and purchased from Beina Biotechnology Co., Ltd. All *Salmonella* isolates that were found to resist more than three antibiotic classes were defined as being MDR strains.

Class 1 Integrons and Antimicrobial Resistance Gene Detection

A bacterial genome extraction kit (QIAGEN) was used to isolate bacterial DNA, after which the *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, and *aac(6')Ib-cr* quinolone resistance genes were detected *via* PCR as detailed previously (23). Genes encoding β-lactamases, such as bla_{TEM}, bla_{PSE}, $bla_{\mathrm{CMY-2}}$, bla_{SHV}, $bla_{\mathrm{DHA-1}}$, bla_{OXA}, and $bla_{\mathrm{CTX-M}}$, were detected *via* PCR, as detailed previously (24, 25). Sequencing of all PCR products was then conducted. Class 1 integron gene cassettes were identified using primers and protocols discussed previously (26).

MLST

Seven housekeeping genes were used for multilocus sequence typing (MLST) profiling (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*), as defined by the University of College Cork (http://mlst.ucc.ie/). The *Salmonella enterica* MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Senterica) was used to assign STs to analyzed isolates.

Sequence-level relationships between *Salmonella* isolates were assessed by constructing an evolutionary phylogeny using MEGA6 *via* a maximum composite likelihood approach, with the topology of this phylogenetic tree being validated using 1,000

TABLE 1 | *Salmonella* prevalence in the context of chick mortality at hatching in Shandong.

Locations	No. of samples	No. of positive samples
Tai'an	313	78 (24.9%)
Rizhao	325	8 (2.5%)
Jining	325	0
Heze	325	0
Total	1,288	86 (6.7%)

bootstrap replicates (27). To analyze resistance phenotypes and the relatedness of resistance gene expression profiles within this phylogenetic tree, the EvolView software package (http://www.evolgenius.info/evolview/#login) was used.

RESULTS

Salmonella Prevalence

In total, we recovered *Salmonella* isolates from 6.7% of analyzed embryos (86/1,288), including 78 isolates from Tai'an (numbers 1–78) and 8 isolates from Rizhao (numbers 79–86). No isolate was recovered from Jining or Heze (**Table 1**).

Serotyping revealed four serotypes, including *S.* Thompson ($n = 32$), *S.* Infantis ($n = 28$), *S.* Enteritidis ($n = 25$), and *S.* Manhattan (*S.* Manhattan) ($n = 1$). *S.* Thompson (37.2%) and *S.* Infantis (32.6%) accounted for the majority of these isolates.

Antimicrobial Susceptibility Testing

These 86 *Salmonella* isolates were tested for resistance to 12 common antibiotics, revealing resistance rates as follows: ampicillin (66.3%), nalidixic acid (59.3%), tetracycline (47.7%), chloramphenicol (40.7%), sulfamethoxazole (38.4%), streptomycin (29.1%), and fosfomycin (2.3%). All *Salmonella* strains exhibited susceptibility or intermediate susceptibility to other tested antibiotics. Of these 86 isolates, 55.8% were classified as MDR isolates (**Figure 1**).

Class 1 Integron and Antimicrobial Resistance Gene Detection

A single quinolone resistance gene (*qnrS*) was detected in these 86 *Salmonella* isolates, with this gene being carried by 50.0% of analyzed strains. Two different β-lactamase genes were found to be expressed in these isolates, with bla_{TEM} being the more common of the two (74.4%), followed by bla_{PSE} (46.5%). No isolates harbored the $bla_{\mathrm{CTX-M}}$, $bla_{\mathrm{CMY-2}}$, bla_{SHV}, $bla_{\mathrm{OXA,}}$ or $bla_{\mathrm{DHA-1}}$ genes (**Figure 1**).

Integrons were detected in 24 of these 86 *Salmonella* isolates (27.9%), and all *Salmonella* isolates harboring integrons were classified as MDR isolates with the exception of one isolate that was only resistant to two antibiotics. All detected *Salmonella* integrons encoded resistance gene cassettes, including the *dfrA1-orfC* ($n = 13$), *dfrA1-catB3* ($n = 8$), *dfrA1-aadA1* ($n = 2$), and *dfrA16-aadA2* ($n = 1$) cassettes (**Figure 1**).

MLST

In an MLST analysis, these 86 *Salmonella* isolates were classified into four ST (Sequence Type) types, with ST26 being the most dominant (37.2%), followed by ST32 (32.6%), ST11 (29.1%), and ST292 (1.2%). These four STs were consistent with the observed serovars identified in this study, with ST26 corresponding to *S.* Thompson, ST32 to *S.* Infantis, ST11 to *S.* Enteritidis, and ST292 to *S.* Manhattan.

The majority of these ST strains were successfully grouped into a phylogenetic tree, which revealed no significant differences in drug-resistance gene expression patterns or drug-resistance spectra among strains within each ST (**Figure 1**).

DISCUSSION

In this study, we collected *Salmonella* isolates from 6.7% of analyzed chicken embryos, and the positive rate was similar to the eggs collected from poultry farms in Yangling (6.6%) (28) but lower than that from commercial chicken farms in China (25, 29). These differences in *Salmonella* isolation rates may be attributable to regional or seasonal differences, or to variations between studies with respect to the techniques used to collect samples. The relatively low isolation rate of *Salmonella* suggests that *Salmonella* is not the main cause of chicken embryo death, but it may be caused by other reasons, and further research is needed.

Serotyping is an effective approach to evaluating modes of transmission to develop strategies for preventing disease spread within poultry facilities (30). We found that *S.* Thompson, which is a member of *Salmonella* serogroup C1 commonly associated with disease in humans (31) and isolated in poultry and poultry eggs (8, 28, 32), was the most prevalent isolate in the present study. This is in contrast to studies in Shanghai and Sichuan that had identified *S.* Enteritidis as the most common serovar in commercial chicken farms (25, 33), while *S.* Indiana was found to be dominant in Shandong (34), and *S.* Weltevreden was dominant in Central Vietnam (35). Our study is the first to have reported the presence of *S.* Manhattan in Shandong, which is also found from broiler chickens in Kagoshima, Japan (36). Given that we observed clear overlap between the *Salmonella* serotypes isolated from chicken embryos and the strains known to cause human disease, this underscores the fact that *Salmonella* can be transmitted to humans through the consumption of contaminated food products (37).

Herein, we found that ampicillin and nalidixic acid were the most commonly resisted antibiotics, in line with findings from several other studies (38, 39), indicating that the use of these drugs may be widespread in laying hens. We also detected high rates of tetracycline resistance, consistent with the fact that this antibiotic is commonly used in the context of animal production (40). We found that the *Salmonella* isolates in the present study were largely sensitive to cephalosporin/acid, cefazolin, ciprofloxacin, polymyxin B, fosfomycin, and gentamicin, likely owing to the limited use of these antibiotics in the study area. We frequently detected MDR *Salmonella* isolates from dead-in-shell chicken embryos in the present study, consistent with findings

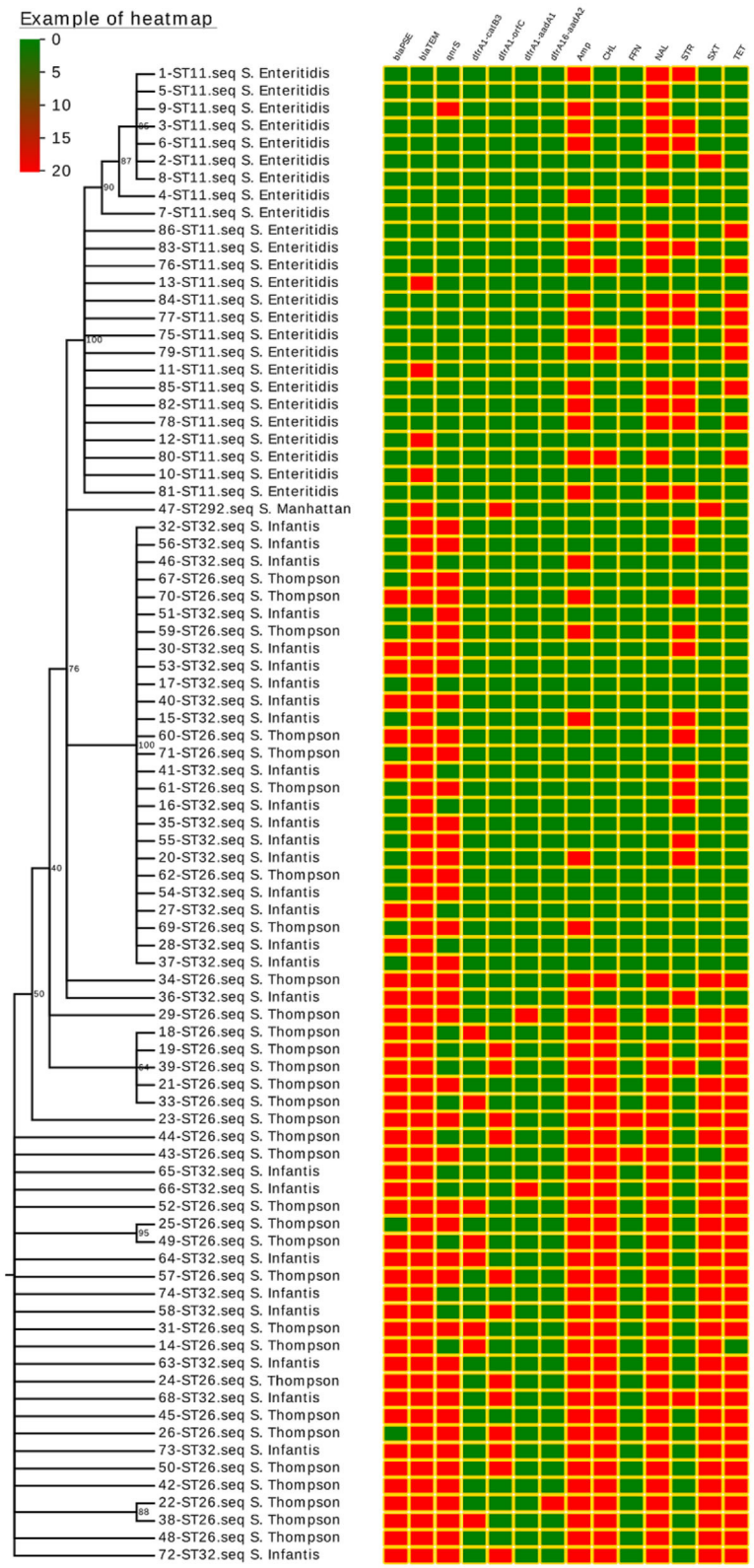

FIGURE 1 | *Salmonella* isolate phylogenetic relationships, drug-resistance gene expression (bla_{PSE}, bla_{TEM}, and *qnrS*), class 1 integron structure (*dfrA1-catB3*, *dfrA1-orfC*, *dfrA1-aadA1*, and *dfrA1-aadA2*), and antimicrobial resistance (AMP, CHL, FFN, NAL, STR, SXT, and TET). Isolated strain numbers are given with ST designations and serotypes. Red and green squares correspond to the presence or absence of a given gene, respectively, or to the resistance or lack of resistance to a particular antibiotic, respectively.

from commercial chicken farms in China (41). In addition, *S.* Thompson showed a high MDR rate (24/32, 75.0%) in this study, which was different from other study that most of *S.* Indiana showed MDR (34). These MDR *Salmonella* isolates are of particular concern because they represent a major threat to public health if transmitted to humans through the food chain (42).

We found that the *qnrS* gene was expressed by the majority of isolates in the present study, in contrast to a prior study of commercial chicken farms in Shandong where this gene was not detected (44). We found that 22 *Salmonella* isolates harboring the *qnrS* gene were resistant to nalidixic acid, indicating that these strains may exhibit a chromosomal quinolone resistance-determining region point mutation. The *aac(6')Ib-cr* gene is a key mediator of bacterial resistance to ciprofloxacin and norfloxacin treatment (43). However, no bacteria harboring this *aac(6')Ib-cr* gene were detected in the present study, in contrast to the findings of a prior analysis of commercial Chinese chicken farms, which found this gene to be present in >90% of *Salmonella* isolates (44). The high prevalence of PMQR genes in *Salmonella* isolates underscores the importance of prudently utilizing fluoroquinolones in order to minimize the development of high-level fluoroquinolone resistance.

We found that bla_{TEM} was the most common β-lactamase gene expressed among isolates in the present study, followed by bla_{PSE}, consistent with a similar report from commercial chicken farms in Shandong (34), although these results were inconsistent with those from a study of slaughterhouses and retail meat products in Sichuan, where the bla_{OXA} was the most common such gene, followed by bla_{TEM}, bla_{PSE}, and $bla_{\mathrm{CMY-2}}$ (25). We found that the majority of analyzed *Salmonella* isolates harboring bla_{TEM} and bla_{PSE} exhibited ampicillin resistance, indicating that β-lactamases may be the main mechanism in Gram-negative bacteria to overcome penicillin-derived antibiotics.

We found that 27.9% of our *Salmonella* isolates harbored integrons, consistent with a prior report from farm animals in Shandong (45), although this rate was higher than that reported for *Salmonella* isolates in the Netherlands (46). All but one of the *Salmonella* isolates harboring these integrons in the present study were classified as MDR isolates, consistent with a model wherein class 1 integrons are linked to the emergence of MDR in *Salmonella*. We additionally identified both *S.* Thompson and *S.* Infantis strains harboring these integrons.

An MLST approach led to the classification of these 86 *Salmonella* isolates into four STs, all of which have been previously linked to the incidence of human salmonellosis (31, 39). ST26 was the most prevalent ST in the present study, in contrast to the results of a prior study of breeder chicken flocks in nine Chinese provinces, which found ST92 to be the most prevalent in sample sites (18). We also observed a close relationship between STs and serovars. When a phylogenetic tree was used to assess relationships between ST genotypes and antibiotic resistance profiles, we observed marked similarities in drug-resistance characteristics for *Salmonella* isolates within each of these STs.

CONCLUSION

In summary, we explored the characteristics of *Salmonella* infections in the context of chicken mortality at hatching in Shandong, China. We found the clinically important *S.* Thompson and *S.* Infantis serovars to be dominant among isolates recovered in the present analysis, and the majority of other isolates were also related to salmonellosis in humans. Overall, our data emphasize the importance of conducting antibiotic susceptibility testing when choosing appropriate antibiotics to treat *Salmonella* infections in order to minimize the risk of further facilitating the spread of drug-resistant strains of these dangerous bacteria.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

XiaonZ, SS, and XiaomZ: data curation. SS and XiaomZ: formal analysis. SS: funding acquisition. XiaonZ, ZJ, and HT: investigation. ZJ, JY, GW, and FW: methodology. XiaonZ: writing. All authors contributed to the article and approved the submitted version.

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Abundance and Expression of Shiga Toxin Genes in *Escherichia coli* at the Recto-Anal Junction Relates to Host Immune Genes

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Shiga toxin (Stx) is the main virulence factor of Shiga toxin-producing *Escherichia coli* (STEC), and ruminants are the main reservoir of STEC. This study assessed the abundance and expression of *Stx* genes and the expression of host immune genes, aiming to determine factors affecting these measures and potential gene markers to differentiate *Stx* gene expression in the recto-anal junction of feedlot beef cattle. Rectal tissue and content samples were collected from 143 feedlot steers of three breeds (Angus, Charolais, and Kinsella Composite) over 2 consecutive years 2014 (n=71) and 2015 (n=72). The abundance and expression of *stx1* and *stx2* were quantified using qPCR and reverse-transcription-qPCR (RT-qPCR), respectively. Four immune genes (*MS4A1, CCL21, CD19*, and *LTB*), previously reported to be down-regulated in super-shedder cattle (i.e., > 10^4 CFU g^{-1}) were selected, and their expression was evaluated using RT-qPCR. The *stx1* gene abundance was only detected in tissue samples collected in year 2 and did not differ among breeds. The *stx2* gene was detected in STEC from all samples collected in both years and did not vary among breeds. The abundance of *stx1* and *stx2* differed ($P < 0.001$) in content samples collected across breeds (*stx1*:AN>CH>KC, *stx2*: AN=CH>KC) in year 1, but not in year 2. Expression of *stx2* was detected in 13 RAJ tissue samples (2014: n=6, 2015: n=7), while expression of *stx1* was not detected. Correlation analysis showed that the expression of *stx2* was negatively correlated with the expression of *MS4A1* (R=-0.56, P=0.05) and positively correlated with the expression of *LTB* (R=0.60, P=0.05). The random forest model and Boruta method revealed that expression of selected immune genes could be predictive indicators of *stx2* expression with prediction accuracy of *MS4A1 >LTB >CCL21 >CD19*. Our results indicate that the abundance of *Stx* could be affected by cattle breed and sampling year, suggesting that host genetics and environment may influence STEC colonization of the recto-anal junction of feedlot cattle. Additionally, the identified relationship between expressions of host immune genes and *stx2* suggests that the host animal may regulate *stx2* expression in colonizing STEC through immune functions.

Keywords: *Stx* gene, cattle breed, host immune genes, random forest model, Boruta algorithm

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) cause foodborne disease that can lead to hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC) (Karmali et al., 1983). Approximately, 2.8 million acute illnesses around the world are attributed to STEC (Majowicz et al., 2014), with 60,000 of these occurring in the US annually (Scallan et al., 2011). Many infections in humans are attributed to direct or indirect contact with food or water contaminated with cattle feces (Mir et al., 2016). Ruminants, especially cattle are the main reservoir who are asymptomatic carriers of O157 and non-O157 STEC strains with the recto-anal junction (RAJ) as the main colonization site (Wang et al., 2016). Most *E. coli* strains are commensals within the gut of cattle (Mir et al., 2016; Wang et al., 2016), and are shed into the environment through feces. Cattle that shed more than 10^4 CFU STEC per gram of feces are defined as "super-shedders" (SS), which are considered the primary source of STEC transmission on farms (Matthews et al., 2006). Although the incidence of *E. coli* O157:H7 causing disease in cattle is low, the prevalence of STEC including both *E. coli* O157:H7 and non-O157:H7 serotypes is not low in cattle ranging from 38.5%–75.0% (Cho et al., 2009). Both *E. coli* O157:H7 and non-O157:H7 serotypes can cause human disease and among non-O157 infections, up to 70% of human infections are attributed to six non-O157 STEC serogroups (O26, O45, O103, O111, O121, and O145) (Bosilevac and Koohmaraie, 2012).

Shiga toxins are the main virulence factors in STEC and other pathogenic bacterial species with the prototype toxins being designated as Shiga toxin 1a (Stx1a) and Shiga toxin 2a (Stx2a) (Melton-Celsa, 2014). These toxins differ in their virulence and host specificity (Fuller et al., 2011; Lee and Tesh, 2019; Petro et al., 2019) with Stx2 being most commonly associated with severe illness (HUS, hospitalization, and bloody diarrhea) in humans (Karmali et al., 1983; Panel et al., 2020). For example, 40% HUS, 41% hospitalization, and 43% bloody diarrhea cases reported in human were attributed to the detectable Stx2 (Panel et al., 2020). Therefore, identifying the abundance of *stx*1 and *stx*2 genes in cattle is important as they could harbor and shed STEC. However, information on the abundance and expression of *stx*1 and *stx*2 genes *in vivo* (e.g. in RAJ) of feedlot cattle is lacking. We hypothesize that the expression and abundance of *stx* genes at the RAJ is influenced by cattle breed and expression of host immune genes. Genetic variation in the host was found to be linked to the level of expression of immune genes in SS (Wang et al., 2018), which also affected the attachment and the colonization of the mucosa by STEC (Wang et al., 2018). The understanding of abundance and expression of *stx* genes in STEC from the main colonization site and its linkage with host immune gene expression will gain insights into the host-STEC interactions at the RAJ of feedlot cattle.

MATERIALS AND METHODS

Animal Populations and Sample Collection

The animal trial followed Canadian Council of Animal Care Guidelines and was approved by the Animal Care and Use Committee, University of Alberta (Animal Care Committee protocol number AUP00000882). In total, rectal tissue and contents were collected over 2 consecutive years (2014 and 2015) from 143 cattle representing Angus breed (AN, n=47), Charolais breed (CH, n=48), and a crossbreed named Kinsella Composite (KC, n=48) that were reared at the University of Alberta Roy Berg Kinsella Research Station. Sampling was performed when animals were slaughtered at a comparable age (Year 2014: 492 days ± 30 days; Year 2015: 496 days ± 22 days; P=0.11) in each year. Ten cm^2 rectal tissue was collected from RAJ and 10 ml rectum contents were squeezed from each steer within 30 min after slaughter at a federally approved abattoir. The samples were deep-frozen immediately in liquid nitrogen and stored at -80°C until use.

DNA and RNA Extraction

Tissue and content samples of RAJ were ground into fine powder in liquid nitrogen and mixed homogeneously before DNA and RNA extraction. DNA was isolated from 0.1 g powdered tissue using repeated bead beating and a column (RBBC) method (Yu and Morrison, 2004) and purified using the QIAamp DNA Stool Mini Kit (Qiagen, Germany). The quantity and quality of DNA were assessed based on absorbance at 260 and 280 nm using the ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). Trizol reagent (Invitrogen Corporation, Carlsbad, CA, USA) was used to isolate total RNA from 0.1 g powdered tissue following the manufacturer's protocol. RNA was purified using the RNeasy MinElute Cleanup kit (Qiagen, Valencia, CA, USA). Quality and quantity of RNA were assessed using Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA, USA) and Qubit 3.0 Fluorometer (Invitrogen, Carlsbad, CA, USA), respectively. DNA was extracted from 0.5 g of the RAJ contents from each steer using the same bead beating method described above. DNA was obtained from contents of 131 steers and were used for downstream analysis.

Assessment of Shiga Toxin Gene Abundance Using qPCR

The DNA extracted from contents and tissues was used to evaluate the abundance of *Stx* genes using quantitative PCR (qPCR) with primers for the detection of all subtypes of *stx1* and *stx2* (**Table 1**) and SYBR Green I reagent (Fast SYBR green master mix; Applied Biosystems, Foster City, CA, USA). The qPCR was conducted in triplicates for each sample on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the program of one cycle at 95°C for 20 s followed by 40 cycles of 3 s at 95°C, 30 s at 60°C. Melting curve analysis with a temperature gradient of 0.1°C/s from 60 to 95°C with fluorescence signal measurement at 0.1°C intervals was performed to make sure targeted products were amplified specifically. The standard curve method was used to quantify *stx1* and *stx2* copy numbers. The standard curve was constructed by genomic DNAs isolated from strain *E. coli* FUA 1403 and *E. coli* FUA 1400, which contain *stx1* and *stx2*, respectively. The formula to calculate the absolute copy

TABLE 1 | Primer sequences, amplicon sizes, and annealing temperature for qPCR assays.

Genes	Oligo sequence (5' to 3')	Amplicon size, bp	Reference	Annealing temperature (°C)
stx1	F: GTCACAGTAACAAACCGTAACA R: TCGTTGACTACTTCTTATCTGGA	95	Jothikumar & Griffiths, 2002	60
stx2	F: ACTCTGACACCATCCTCT R: CACTGTCTGAAACTGCTC	118	He et al, 2020	60
eae	F: TGCTGGCATTTGGTCAGGTC R: CGCTGA(AG)CCCGCACCTAAATTTGC	175	Delmas et al, 2009	60
CCL21	F: GCTATCCTGTTCTCGCCTCG R: ACTGGGCTATGGCCCTTTTG	222	Wang et al, 2016	60
LTB	F: TGGGAAGAGGAGGTCAGTCC R: TAGCTTGCCATAAGTCGGGC	215	Wang et al, 2016	62
CD19	F: CTCCCATACCTCCCTGGTCA R: GCCCATGACCCACATCTCTC	127	Wang et al, 2016	64
MS4A1	F: GCGGAGAAGAACTCCACACA R: GGGTTAGCTCGCTCACAGTT	206	Wang et al, 2016	64
β-actin	F: CTAGGCACCAGGGCGTAATG R: CCACACGGAGCTCGTTGTAG	177	Malmuthuge et al, 2012	60

number of standard curves is described as follows (Li et al., 2009):

$$Absolute\ copy\ number\left(\frac{\#}{gSample}\right) = \frac{Amount\left(\frac{gDNA}{gSample}\right) * 6.022 * 10^{23}\left(\frac{\#}{mol}\right)}{Length(bp) * 660\left(\frac{gDNA}{mol * bp}\right)}$$

where $6.022 * 10^{23}$ represents the Avogadro's constant (#/mol); Length (bp) is the length of template DNA; 660 represents the average mass of 1 bp double-strand DNA. The copy number of *stx1* or *stx2* was determined by relating threshold cycle (C_T) values to standard curves based on the following regression formula (Li et al., 2009): Y = -3.193 * log X + 35.003 (Y, C_T value; X, copy number of 16S rRNA gene) (r^2 = 0.996). The qPCR amplification efficiency was 88%–98%.

Detection of Expression of *Stx* and Host Immune Genes Using qRT-PCR

Total RNA (0.1 μg) was further subjected to reverse transcription to synthesize cDNA using a cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Single-stranded cDNA was amplified using $Oligo(dT)_{12\text{-}18}$ (Life Technologies, Carlsbad, CA, USA) and SuperScript™ II RT (Life Technologies, Carlsbad, CA, USA) was used to synthesize double-strand cDNA. Primers for the detection of *eae* expression are shown in **Table 1**. Quantitative RT-PCR of *stx1*, *stx2*, and *eae* was then performed using the double-strand cDNA and primers (**Table 2**) with the same thermal cycling program described above in triplicates for each sample. The expression of *stx1*, *stx2*, and *eae* was quantified by standard curve method described above.

In addition, four genes reported to be differentially expressed between SS and non-shedding (NS) cattle (Wang et al., 2016); chemokine (C-C motif) ligand 21 (*CCL21*), lymphotoxin beta (*LTB*), CD19 molecule (*CD19*), and 4-domains, subfamily A, member 1 (*MS4A1*) were selected to study their relationship with Stx gene abundance and expression. The same qPCR amplification conditions were used for the four genes with their respective primers (**Table 1**). Four commonly used housekeeping genes, including bovine *GAPDH*, 18S rRNA genes, *RPLP0*, and the β-actin gene, were also quantified by qPCR (Wang et al., 2016). As β-actin exhibited the most consistent Cq value it was used as the house-keeping gene for evaluating relative gene expression. The relative expression of each gene (*stx1*, *stx2*, and immune genes) was measured by ΔCq value, which was calculated as (Wang et al., 2016):

TABLE 2 | The prevalence analysis of *stx1* and *stx2* for samples collected from the rectal tissue and content in 2014 and 2015.

Sample type	Breed	Year 1 (2014)				Year 2 (2015)			
		No. (%) Stx1-positive	P value	No. (%) Stx2-positive	P value	No. (%) Stx1-positive	P value	No. (%) Stx2-positive	P value
Tissue	AN	0 (0)[a]	1	23 (100)	1	24 (100)	1	24 (100)	1
	CH	0 (0)		24 (100)		23 (100)		23 (100)	
	KC	0 (0)		24 (100)		24 (100)		24 (100)	
Content	AN	18 (78)	0.001***	22 (96)	<0.001***	1 (6)	0.069	17 (94)	0.272
	CH	7 (35)		20 (100)		0 (0)		24 (100)	
	KC	6 (27)		4 (18)		4 (17)		24 (100)	

[a]*Values presented here were numbers and percentages of Stx-positive samples. Fisher's exact test was used to examine the differential prevalence of stx1 and stx2 among three breeds within each sample type. For comparisons, P-values were included along with the level of statistical significance (P ≤ 0.001***).*

$$\Delta C_q = C_q target\ genes - C_q reference\ gene$$

with a higher ΔCq representing the lower expression while a lower ΔCq indicating higher expression. The qPCR amplification efficiency was 88%–98%.

Statistical Analysis

The PROC MIXED model in SAS (ver. 9.13; SAS Institute Inc., Cary, NC, United States) was used to analyze the *stx1* and *stx2* abundance as well as host gene expressions together with all potential 2- and 3-way interactions among breeds, years, and sample types. In this statistical model, breed, sample type, and year were analyzed as fixed effects with steers as the random effect. Interactions were removed from the model if they were not significant ($P > 0.05$). Least square means were compared using the Bonferroni mean separation method after the removal of insignificant interactions and the significance was considered at $P < 0.05$. The difference of prevalence of *stx1* and *stx2* was analyzed using Fisher's exact tests. Non-parametric Mann-Whitney U test in R (Mangiafico, 2020) was used to assess differences in host gene expression between Stx2+ (expressed) and Stx2- (not expressed) groups, with differences considered significant at $P<0.05$. Correlation analysis was performed based on Spearman's rank correlation coefficient (R) to identify relationships between expression of *stx2* and host genes using the "ggcorrplot" package in R with significance at $P<0.05$.

Isomap, a novel method for nonlinear dimensional reduction (Tenenbaum et al., 2000), was applied to determine the effect of breed, and sampling year on the expression of immune genes and *stx2* using the "RDRToolbox" package in R. In addition, Davis-Bouldin-Index (DBIndex) was used to compute Euclidean metrics to validate the clustering patterns of the expression of immune genes and *stx2*, with the value ≤ 1 indicating a well-separated cluster (Davies and Bouldin, 1979). Correspondence analysis (CA) was used to identify relationships among expression patterns using the "FactoMineR" package (Ringrose, 1992; Tekaia, 2016).

Identification of Potential Gene Markers for *Stx* Gene Expression Using Mathematic Models

The random forest model was used to identify predictive indicators for *stx2* expression with the "RandomForest" package in R. The host gene expression data were divided into two groups: *stx2*+ (expressed) and *stx2*- (not expressed). Two-thirds of each group was used as training data, and the rest (one-third) was used for validation. The accuracy rate (number of samples recognized correctly/total number of samples) was calculated to determine the model classification performance. The mean decrease in accuracy was used to assess the importance of host genes as predictive indicators of *stx2* expression. Variables with high mean decrease in accuracy indicate the higher contribution as compared to variables with low mean decrease accuracy (Han et al., 2016). The area under the ROC curve (AUC) was calculated to assess the robustness of the prediction model with the criteria being excellent (0.9–1.0), good (0.8–0.9), fair (0.7–0.8), weak (0.6–0.7), or fail (0.5–0.6) (Zhang et al., 2016). Moreover, the Boruta method, a random forest-based feature selection with the ability to remove less informative features, was used as a supportive approach to perform this prediction using the "Boruta" package in R (Kursa and Rudnicki, 2010).

RESULTS

Factors Affecting the Abundance and Prevalence of *stx1* and *stx2*

Sampling year significantly impacted the abundance and prevalence of *stx* genes identified in RAJ samples ($P<0.01$), therefore, the effect of breed on the prevalence and abundance of *stx1* and *stx*2 was analyzed separately for each year. The prevalence of *stx1* and *stx2* in tissue samples was not affected by breed in either year (**Table 2**). In year 1, the prevalence of *stx1* in contents was higher ($P = 0.001$, **Table 2**) in AN (n=18; 78%) compared to CH (n=7; 35%) and KC (n=6; 27%), and the

TABLE 3 | Abundance of *stx1* and *stx2* using q-PCR for samples collected from the rectal tissue and content in 2014 and 2015.

Year	Breed	AN		CH		KC		P-Value		
	Type	T	C	T	C	T	C	Breed	Type	Breed*Type
2014	*stx1*	N/D [a]	4.09 (5.20)	N/D	1.73 (5.79)	N/D	1.40 (5.47)	<0.0001***	<0.0001***	<0.0001***
	stx2	6.02 (0.08)	4.92 (1.01)	5.31 (0.05)	5.91 (0.22)	5.70 (0.05)	1.00 (4.65)	<0.0001***	<0.0001***	<0.0001***
2015	*stx1*	6.78 (0.02)	0.25 (1.11)	6.82 (0.03)	N/D	6.76 (0.03)	N/D	0.31	<0.0001***	0.28
	stx2	5.70 (0.02)	4.58 (1.58)	5.73 (0.03)	4.91 (0.20)	5.67 (0.03)	5.06 (0.31)	0.17	<0.0001***	0.12

[a] *The value was presented as Mean (SE) after log_{10} transformation (gene copy numbers/g sample). T represents tissue samples, C represents contents. For content and tissue samples, the lowest abundance that can be detected corresponds to 200 (2.3 after log_{10} transformation) gene copies/g and 40 (1.5 after log_{10} transformation) gene copies/g, respectively. Therefore, stx gene abundance that lower than 2.3 log_{10}(gene copies/g) and 1.5 log_{10}(gene copies/g) for content and tissue samples was defined as "underdetermined" ("N/D") which is assumed to be "0" in our analysis, respectively. For comparisons among different factors and among interaction effects, P-values were included along with the level of statistical significance ($P \leq 0.001$***).*

prevalence of *stx2* was higher (P < 0.001, **Table 2**) in AN (n=22; 96%) and CH (n=20; 100%) than in KC (n=4; 18%). However, the prevalence of *stx1* and *stx2* in content samples collected in year 2 was not affected by breed (P_{stx1} = 0.069, P_{stx2} = 0.272, **Table 2**) with a tendency for breed to affect the prevalence of *stx1*.

The abundance of *stx1* and *stx2* was affected (P < 0.001) by sample type (tissue *vs*. contents) for both years (**Table 3**). An interaction effect between breed and sample type for the abundance of *stx1* and *stx2* was detected in year 1 (P_{stx1}<0.001, P_{stx2}<0.001, **Table 3**), but not in year 2 (P_{stx1} = 0.28, P_{stx2} = 0.12, **Table 3**). In year 1, the abundance of *stx1* in contents was affected by breed with its abundance higher in AN> CH> KC (P<0.001, **Table 3**), while its abundance in rectal tissue was under the detection limit (**Table 3**). For *stx2*, it was detected in both tissue and content samples in year 1 with no difference in the abundance of *stx2* in tissue samples (**Table 3**), but with the higher abundance in rectal contents of AN and CH as compared to KC steers (P<0.0001, **Table 3**). For year 2, the abundance of *stx1* or *stx2* did not differ among breeds for either tissue or contents (**Table 3**), with the abundance of *stx1* and *stx2* in tissue being higher compared to that in contents (P_{stx1}<0.001, P_{stx2}<0.001, **Table 3**), respectively.

Expression of *stx1* and *stx2* Associated With the Rectal Tissue of Beef Steers

Expression of bacterial *stx1* was not detected, and bacterial *stx2* (defined as *stx2*+) was only detected in mucosal tissue from 13 cattle (2014: n=6, 2015: n=7, **Table S1**). The expression of *stx2* was more common in KC (n=9; 70%) than in AN (n=2; 15%) and CH (n=2; 15%). The non-parametric Kruskal-Wallis test showed that *stx2* expression did not differ among breeds (ΔCq_{AN}=5.04; ΔCq_{CH}=5.11; ΔCq_{KC}=5.04; $P_{=}$ 0.31), but there was a trend for difference between sampling years ($\Delta Cq_{Year\ 2014}$ = 4.94; $\Delta Cq_{Year\ 2015}$ = 5.15; P = 0.06).

Expression of Selected Immune Genes in RAJ Tissue From Beef Steers

In year 1, the expression of four selected immune genes was not affected by breed. In year 2, only expression of *CD19* and *CCL21* differed among breeds (P_{CD19} = 0.02, P_{CCL21} = 0.0035, **Table 4**).

TABLE 4 | Quantification for relative expressions of four host gene among breeds and differed RFI using qRT-PCR for rectal tissue samples collected in 2014 and 2015.

Year	Immune genes	AN	CH	KC	P-Value
2014	*MS4A1*	2.80 (0.36)[a]	3.42 (0.29)	3.76 (0.44)	0.13
	CD19	-0.14 (0.38)	-0.32 (0.52)	-0.06 (0.35)	0.91
	CCL21	3.88 (0.45)	4.64 (0.35)	4.82 (0.46)	0.26
	LTB	-0.96 (0.48)	-0.97 (0.60)	-1.30 (0.44)	0.86
2015	*MS4A1*	3.76 (0.27)	3.65 (0.25)	4.26 (0.30)	0.26
	CD19	3.61 (0.28)	3.50 (0.29)	4.51 (0.24)	0.02*
	CCL21	5.94 (0.25)	4.90 (0.21)	5.87 (0.23)	0.0035***
	LTB	4.31 (0.44)	4.36 (0.40)	5.47 (0.36)	0.07

[a]*The value was presented as Mean (SE) of ΔCq value that was calculated from each tissue sample under different year, breed, and feed efficiency. For comparisons among different factors and interaction effects, P-values were included with the level of statistical significance (P < 0.05*, P ≤ 0.001***).*

TABLE 5 | Expression differences for four host genes between Stx2+ and Stx2- samples using non-parametric Mann-Whitney U test.

Immune genes	Mean		Z-score	P-Value
	Stx2-	Stx2+		
MS4A1	3.65	3.44	0.92	0.36
CD19	1.90	1.54	0.49	0.62
CCL21	5.02	5.04	0.08	0.94
LTB	1.90	1.30	0.61	0.54

For comparisons between Stx2+ and Stx2- group, P > 0.05 indicates no significant difference.

There was no difference (P_{MS4A1} = 0.36, P_{CD19} = 0.62, P_{CCL21} = 0.94, P_{LTB}=0.54, **Table 5**) in the expression of the four genes between *stx2*+ and *stx2*- steers. Visually, host gene expression patterns from tissue samples were affected by year among all samples ($Value_{Year}$ = 0.81, **Figure 1A**) as well as among *stx2*+ samples ($Value_{Year}$ =0.75, **Figure 1B**). However, host gene expression patterns did not differ among breeds based on DBIndex clustering value among all samples ($Value_{breed}$=9.30, **Figure S1A**) or among *stx2*+ samples ($Value_{breed}$=1.64, **Figure S1B**).

Association Between Expressions of *stx2* and Host Immune Genes

Expression of *stx2* was negatively correlated with the expression of *MS4A1* (R=-0.56, P=0.05, **Table 6**) and positively correlated with the expression of *LTB* (R=0.60, P=0.05, **Table 6**). Neither *CD19* nor *LTB* clustered with *Stx2*+ samples but *CD19* and *LTB* were positively correlated (R=0.98, P=0.00, **Table 6**). Correspondence analysis revealed that most of the samples (12 out of 13, outlier: KC14.105) grouped together in the CA plot with *MS4A1* and *CCL21* (**Figure 2**). In the correspondence analysis (CA), Dimension 1 (Dim1) represented up to 94% of the importance with *CD19* and *LTB* contributing the most to Dim1, with Dim2 only representing 4.14% of the variation (**Figure 2**).

Prediction Model to Discover Potential Gene Markers for *stx2* mRNA Abundance

Further analysis using a random forest model classifier based on expressions of four host immune genes *MS4A1, LTB, CCL21, CD19* revealed the accuracy for predicting *stx2* mRNA abundance was 96.5% for the training data and 93.6% for the validation data. The AUC value of 0.908 for the ROC curve also revealed a high accuracy and a robust prediction (**Figure 3A**). As an indicator of *stx2* expression, the prediction accuracy of *MS4A1, LTB, CCL21, CD19* was 47.55%, 45.35%, 41.44%, 36.80%, respectively. Further Boruta analysis also revealed that all four immune genes were attributes for *stx2* expression, with the ranking *MS4A1* > *LTB* = *CD19* > *CCL21* (**Figure 3B**).

DISCUSSION

This study characterized the abundance, prevalence, and expression of the *stx1* and *stx2* at the recto-anal junction in feedlot steers of three breeds over 2 consecutive years. Several

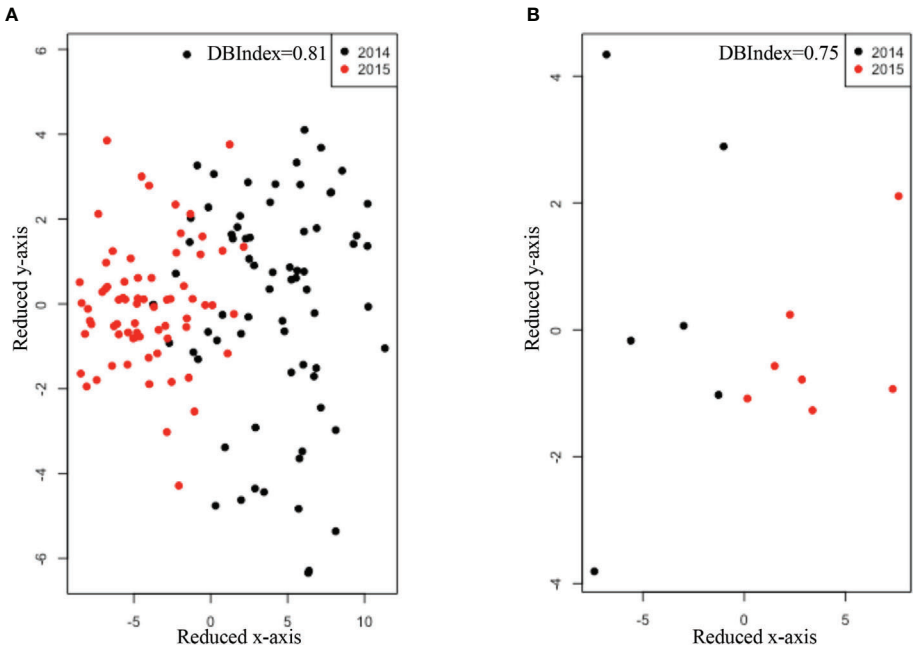

FIGURE 1 | Comparisons of host gene expression patterns using non-parametric method Isomap and DBIndex value for sampling year effect **(A)** among all samples **(B)** as well as among Stx2+ samples. Black dots and red dots refer to samples collected in 2014 and 2015, respectively. DBIndex value was shown on the right corner of each figure. The lower DBIndex value, the well-separated cluster pattern.

TABLE 6 | Correlation analysis among relative expressions of host genes and *stx2* expression among Stx2+ samples.

		Stx2RNA	*MS4A1*	*CD19*	*CCL21*	*LTB*
Stx2RNA	R-Value	1.00	-0.56	0.51	-0.44	0.60
	P-Value	0.00	0.05*	0.08	0.13	0.03*
MS4A1	R-Value		1.00	-0.55	0.39	-0.56
	P-Value		0.00	0.05*	0.19	0.05*
CD19	R-Value			1.00	0.19	0.98
	P-Value			0.00	0.53	0.00***
CCL21	R-Value				1.00	0.09
	P-Value				0.00	0.78
LTB	R-Value					1.00
	P-Value					0.00

R-value was defined as the correlation coefficient ranged from -1 to 1. For correlations with different genes, P-values were included along with the level of statistical significance (P ≤ 0.05, P ≤ 0.001***).*

studies have quantified the copy number of *stx1* and *stx2* in cattle feces using qPCR, with estimates ranged from 0 to 5.6 $\log_{10}$(gene copies/g) (Imamovic and Muniesa, 2011; Verstraete et al., 2014). Our estimates of the copy number of *stx1* and *stx2* in contents are within these ranges, with 1.24 to 4.13 $\log_{10}$(gene copies/g) (year 1, *stx1*), 0 to 0.45 $\log_{10}$(gene copies/g) (year 2, *stx1*), 0.86 to 5.38 $\log_{10}$(gene copies/g) (year 1, *stx2*), and 4.51 to 5.09 $\log_{10}$(gene copies/g) (year 2, *stx2*). However, there was a markable difference in the copy number of *stx* in tissue samples when compared to RAJ contents. Stx genes associated with RAJ tissue samples ranged from 5.62 to 6.07 $\log_{10}$(gene copies/g) (year 1, *stx2*), 6.71 to 6.85 $\log_{10}$(gene copies/g) (year 2, *stx1*), and 5.61 to 5.76 $\log_{10}$(gene copies/g) (year 2, *stx2*). We speculate that the high *stx* copy numbers detected from tissues likely represents the higher possibility of the STEC colonization on RAJ mucosa. Indeed, a previous study has reported that the abundance of *E. coli* O157 strain was inconsistent between RAJ tissues and content samples (Keen et al., 2010), suggesting that Stx carrying bacteria were associated with the epithelium of RAJ in the steers in addition to their presence in digesta. Based on our results, digesta samples only present a proportion of the actual STEC that inhabit in the RAJ of cattle, with the higher population directly colonizing epithelial tissue. These suggest that it should include fecal samples together with rectal mucosa swabs or biopsies to have more accurate estimation of *stx* gene abundance in cattle.

Our study further revealed that the abundance and prevalence of the *stx* genes was affected by breed and sampling year, and such effects were *stx* type dependent. However, a previous study found no relationship between cattle breed and the presence of *stx* at the RAJ (Mir et al., 2016). The inconsistency between our and previous findings may be due to differences in breed, age [calf (Mir et al., 2016) *vs.* steer], and diets of the cattle. In this study, Angus, Charolais, and Kinsella Composite breeds were used to examine the abundance and prevalence of *stx* genes, while previous studies collected samples from hybrid Angus-Brahman beef calves (Mir et al., 2016). Steers in our study were fed a high gain diet and slaughtered at similar body weight, but still differed in *stx1* and *stx2* prevalence across breeds, suggesting the highly individualized response to STEC colonization. Therefore, host genetics may alter the gut environment through influences on immunity and the microbiome (Wang et al., 2018), which may influence the prevalence of STEC and the prevalence

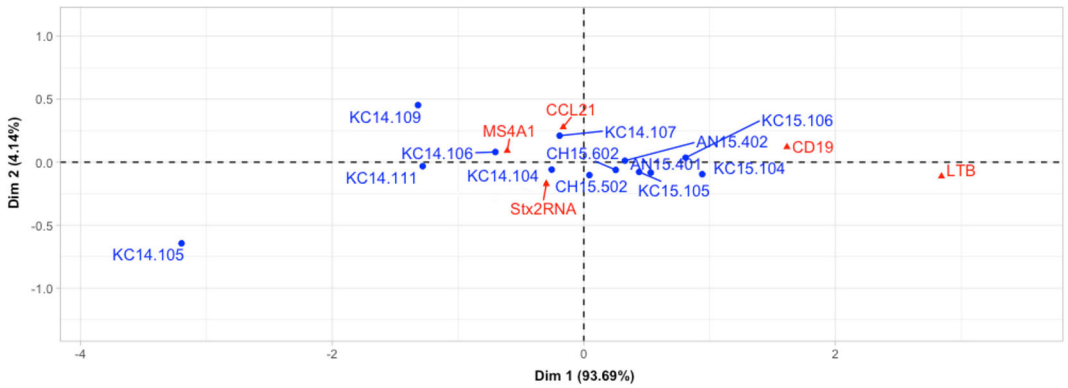

FIGURE 2 | Assessment of associations between host immune gene expressions and Stx2+ samples using correspondence analysis. Red triangles and blue dots refer to host genes and Stx2+ samples, respectively. For example, "AN14.105" means the number of this sample is 105, breed is Angus, and was collected in 2014.

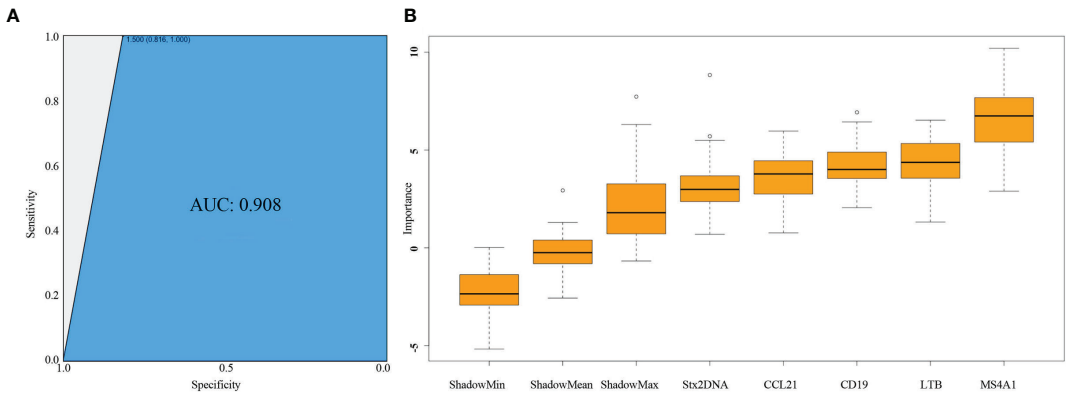

FIGURE 3 | Assessment of Random Forest model using ROC curve and Boruta method. **(A)** Assessment of classification performance of random forest model using area under ROC (AUC). Sensitivity (y-axis) represents the fraction of samples with positive *Stx2* expression that the test correctly identifies as positive. Specificity (x-axis) represents the fraction of samples without *Stx2* expression that the test correctly identifies as negative. **(B)** Rank of host immune genes as markers for *Stx2* expression prediction using Boruta method. ○ represents the outliers in each Z-score.

and abundance of *stx* genes in the samples. The observed differences between sampling years suggest that environmental factors together with host genetics impact the prevalence of the *stx* genes in the RAJ of steers. Higher ambient temperatures have been shown to be associated with increased prevalence of both *stx1* and *stx2* in the rectal mucosa of both dairy and beef cattle (Fernandez et al., 2009; Tahamtan et al., 2010). For our study, the average ambient temperatures were similar between the two years (3.25°C for 2014 *vs.* 5.63°C for 2015) and as a result it is unlikely to account for the difference in detection of *stx1* and *stx2* between years. Other ecological factors such as seasonality, water and soil sources, and factors associated with farm management may also contribute to varied STEC colonization. Future long term monitoring studies are needed to determine to what extent these environmental factors contribute to the prevalence of both *stx1* and *stx2* in the RAJ of cattle.

Although the presence of both *stx1* and *stx2* genes were detected, only expression of *stx2* was found in the RAJ tissue of beef steers. Severe STEC infections that result in HUS are mostly associated with *stx2* as its product is 400 times more toxic (as quantified by LD_{50} in mice) than the product of *stx1* (Riley et al., 1983). *Stx2*-producing *E. coli* strains were reported to be in 71% (34 out of 48) of children with HUS, while only 40% (four out of 10) of patients were associated with *stx1*-producing *E. coli* strains (Ludwig et al., 2001). It is noticeable that the prevalence of *stx2* gene expression in steers (8.5% for year 1, 9.7% for year 2) is similar to the reported super shedder rate [~10% (Matthews et al., 2006)], suggesting the expression of *stx2* might be highly correlated with super shedding (SS) and cattle with *stx2* expression might potentially be SS. Interestingly, all *stx2*+ samples were from KC steers in 2014,suggesting KC might be more prominent carriers of STEC and further highlighting the role of breed.

We further speculate that the *stx2*+ cattle may have higher colonization of STEC. As the adherence factor intimin encoded by *eae* gene enables STEC colonization (Farfan and Torres, 2012)

and the presence of *eae* is correlated with the formation of attaching and effacing (A/E) lesions (Wieler et al., 1996) and *E. coli* O157:H7 colonization in bovine RAJ (Sheng et al., 2006), the expression of *eae* was also assessed in this study. The expression of *eae* was detected in nine out of 131 RAJ tissue samples (Data not shown). Of these, only two samples were *stx2* positive. A previous study isolated 326 *E. coli* strains from 304 fecal samples of clinically healthy wild boars, and found that 10 samples were *eae* positive belonging to different *E. coli* strains (Alonso et al., 2017). Besides, only one *stx2*+ *eae*+ *E. coli* strain (*E. coli* O145:H28) was characterized to date and was reported to be associated with HUS in human (Alonso et al., 2017). Although the occurrence of *eae*, alone or in combination with *stx2* is sporadic, diverse *E. coli* serotypes exist in beef cattle and among them certain serotypes could be potential human pathogens. Compared to previous studies only reported expressions of *eae* and *stx* from fecal samples, our study is the first to report expressions of these two genes on RAJ mucosa. The detection of *stx*+, *eae*+, and *stx2*+*eae*+ cattle suggests the importance to include all serotypes instead of only *E. coli* O157:H7 for future SS research in practice to the prevention of SS transmission and the mitigation of potential human infections. Future study is needed to isolate *E. coli* serotypes who carry *stx*+, *eae*+, and *stx2*+*eae*+ genes and evaluate their abundances in RAJ and feces of beef steers to verify whether they are SS. Although the abundance of O157 strains were not quantified in this study, our study highlights the importance to use marker genes to assess all STEC populations as opposed to only *E. coli* O157:H7. In addition to *eae* genes, Enterohemorrhagic *E. coli* autotransporters (Eha) A and B autotransporters that can colonize on bovine epithelia are vital adhesin factors in STEC and are higher prevalent among STEC strains (97% and 93%, respectively) (Wells et al., 2009; Easton et al., 2011). Particularly, *EhaA* promoted adhesion to primary epithelial cells of bovine RAJ and should be explored to identify relationships between *EhaA* and host immunity for fundamental understanding of host-STEC interactions and STEC colonization. Other adhesin factors that play a role in STEC colonization on bovine epithelia such as hemorrhagic coli pili (HCP), EspP rope-like fibers (Farfan and Torres, 2012) should also be explored to identify relationships between STEC adhesin factors and host immune gene expressions.

Previous studies have identified differences in the expression of *MS4A1, CD19, CCL21, LTB* genes at the RAJ of super-shedder *vs.* non-shedders (Corbishley et al., 2014; Wang et al., 2016). These genes are involved in B cell proliferation (Uchida et al., 2004), B cell receptor signaling pathway (Karnell et al., 2014), and the migration of B cells from bone narrow to lymphoid tissues (Bowman et al., 2000), as well as the induction of the inflammatory response system (Browning et al., 1995). The observed higher relative expression of *CD19* (a membrane co-receptor found on all B cells) in KC steers and the higher relative expression of *CCL21* in AN and KC than CH in 2015, suggests that expression of this gene in cattle is influenced by breed. Breed-driven gene expression against infections and biological processes have been explored in bovine tissues and cells. Examples include, the reduced expression of the *ALDOA* (Fructose-bisphosphate aldolase A) gene in the longissimus muscle of Wagyu- as compared to Piedmontese-sired offsprings (Lehnert et al., 2007), and the up-regulation of *CD9* (CD9 antigen) and *BoLA-DQB* (BoLa Class II histocompatibility antigen, DQB*101 beta chain) in the macrophage of Sahiwal compared to Holstein cattle in response to *Theileria annulate* infection (Glass and Jensen, 2007). In our previous study, the variation in expression of immune genes between SS and NS, could be due to the genetic variation (Wang et al., 2016), suggesting future genome wide association studies (GWAS) are needed to identify the genotypes and/or SNPs responsible for expression of immune genes that could directly or indirectly affect STEC colonization and expression of their virulence genes.

Lymphotoxin beta (LTB) induces the immune response and is crucial for the initiation of Lymphoid follicle (ILF) development (McDonald et al., 2005). Lymphoid follicles (ILFs) in the bovine rectum are regarded as the reservoir of secretory antibodies in the gut, serving as a frontline defensive system in the gastrointestinal (GI) tract (Tsuji et al., 2008). The positive correlation between *stx2* expression and relative expression of *LTB* suggests that cattle with higher *stx2* expression have lower *LTB* expression, which may lead to decreased production of lymphotoxin, reduced ILF development in the RAJ. Impaired ILF has been associated with a 10 to 100-fold increase in the colonization of *Enterobacteriaceae* in ileum of mice (Bouskra et al., 2008), and 100-fold increase in anaerobic bacteria in the small intestine of mice (Fagarasan et al., 2002). Also, a previous study indicated that super-shedders harbor a distinct fecal microbiota compared to non-shedder (Xu et al., 2014). These suggest that changes in *LTB* expression could lead to impaired ILF function and altered microbiota, which could promote STEC colonization in cattle. Expression of *MS4A1* was negatively correlated with *stx2* expression and *MS4A1* was in the dominant position of *stx2*+ samples from the correspondence analysis, suggesting the vital role of *MS4A1* in regulating *stx2* expression and partially reflecting a strengthened adaptive immunity in *stx2*+ cattle. *MS4A1* encodes CD20 which is expressed from late pro-B cells through memory cells with its function to enable optimal B cell immune response and against T-independent antigens (Kuijpers et al., 2010). Hence, these indicate that *MS4A1* is the key gene in connecting *stx2* expression to host adaptive immunity, and their negative correlation suggest the establishment of host recognition mechanisms for *stx2* expression.

To our knowledge, this study is the first to explore whether host gene markers were related to *stx* expression and potential STEC colonization using artificial intelligence-based approaches (Random Forest model and Boruta method). Based on results of mean decrease accuracy in the Random Forest Model and Boruta method and the biological functions of these four immune genes, our results highlight the relationship between host immune genes and *stx2* expression. Of the genes studied, *MS4A1* was the best predictor of *stx2* expression and it was in the *stx2*+ sample cluster in the CA map. We used the non-parametric dimensionality reduction method, Isomap, to assess the

relationship between the expression of host genes and *stx2*, and results supported the *stx2* expression is closely associated with host gene expression patterns. Isomap was initially developed for computational visual perception (Tenenbaum et al., 2000) and then used to investigate ecosystem crosstalk (Mahecha et al., 2007), human disease phenotypes, and gene expression (Dawson et al., 2005). Compared to principal component analysis (PCA), this approach is less restricted since it does not require any specific distribution (*i.e.* normal distribution) of data (Shlens, 2014). The clustering patterns generated by PCA were similar to Isomap results, which could be due to the limited number of genes analyzed. But the Isomap approach is suitable for mammalian studies since interactions among genetics, environment, and microbes are in nature nonlinear (Nicholson et al., 2004). Regardless, our previous studies have reported 57 differential expressed genes between SS and NS (Wang et al., 2018) and many genes are interplay in cattle to affect their immunity and microbiota, the complexity of gene-gene interactions should be taken into account for future studies. Further explorations to investigate more DE genes and their interactions either at the individual or whole transcriptome level could identify and verify the predictiveness of host genes as markers of *stx2* expression. In addition to the genetic background that alters the predictiveness of random forest model, mucosa attached microbes (bacteria and viruses) can also impact on host immune gene expressions which should also be considered for the future construction of the prediction model. Our previous study (Wang et al., 2018) identified relationships between RAJ mucosa-associated bacteria and expression of 19 out of 57 DE immune genes identified from SS compared to NS. Although four immune genes were not part of these 19 DE genes, future studies to include the expression of these genes are needed for the better understanding of STEC colonization and its relationship with host immune genes and model construction.

CONCLUSION

Taken together, our results revealed that cattle genetic background (breed) and sampling year could affect the abundance and prevalence of STEC *stx1* and *stx2* genes in the RAJ of feedlot cattle. We identified the relationships between *stx2* expression and the expression of host immune genes, and found that *stx2* expression could be driven by expression of particular host immune genes (e.g., *MS4A1*). Our study established a model to correlate host gene expression to *stx2* expression, suggesting that its expression can be driven by the host. Although *Stx* detection from feces is a more direct method, the findings from this study revealed that it may not represent the true population of STEC colonized in RAJ which can be influenced by the tissue immune genes. Future studies are needed to elucidate mechanisms behind host-STEC interactions by applying methods including genome wide association studies (GWAS) that determines potential genetic variations related to host-STEC interactions and also explore digesta and mucosal attached microbiota variations to develop methods for the potential precise identification of STEC in cattle.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee, University of Alberta.

AUTHOR CONTRIBUTIONS

ZP and YC performed experiments. ZP, MG, and LG were involved in experimental design and methodology development. ZP, TM, MG, and LG were involved in data analysis. ZP wrote the draft manuscript. TM, MG, GP, and LG contributed to manuscript revisions. GP, TM, and LG were involved in securing the funding for the project. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.633573/full#supplementary-material

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Characteristics of *Salmonella* From Chinese Native Chicken Breeds Fed on Conventional or Antibiotic-Free Diets

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Salmonella is a common food-borne Gram-negative pathogen with multiple serotypes. Pullorum disease, caused by *Salmonella* Pullorum, seriously threatens the poultry industry. Many previous studies were focused on the epidemiological characteristics of *Salmonella* infections in conventional antibiotic use poultry. However, little is known about *Salmonella* infections in chicken flocks fed on antibiotic-free diets. Herein, we investigated and compared *Salmonella* infections in three Chinese native breeders fed on antibiotic-free diets, including the Luhua, Langya, and Qingjiaoma chickens, and one conventional breeder, the Bairi chicken, via analyzing 360 dead embryos in 2019. The results showed that the main *Salmonella* serotypes detected in a total of 155 isolates were *S.* Pullorum (82.6%) and *S.* Enteritidis (17.4%). Coinfection with two serotypes of *Salmonella* was specifically found in Bairi chicken. The sequence type (ST) in *S.* Pullorum was ST92 ($n = 96$) and ST2151 ($n = 32$), whereas only ST11 ($n = 27$) was found in *S.* Enteritidis. The *Salmonella* isolates from three breeder flocks fed on antibiotic-free diets exhibited phenotypic heterogeneity with a great variety of drug resistance spectrum. Most of the isolates among three chicken breeds Luhua (64.9%, 50/77), Langya (60%, 12/20) and Qingjiaoma (58.3%, 7/12) fed on antibiotic-free diets were resistant to only one antibiotic (erythromycin), whereas the rate of resistance to one antibiotic in conventional Bairi chicken isolates was only 4.3% (2/46). The multidrug-resistance rate in *Salmonella* isolates from layer flocks fed on antibiotic-free diets (20.2%, 22/109) was significantly ($P < 0.0001$) lower than that from chickens fed on conventional diets (93.5%, 43/46). However, high rate of resistance to erythromycin (97.4%∼100%) and streptomycin (26%∼41.7%) were also found among three breeder flocks fed on antibiotic-free diets, indicating resistance to these antibiotics likely spread before antibiotic-free feeding in poultry farms. The findings of this study supplement the epidemiological data of salmonellosis and provide an example of the characteristics of *Salmonella* in the chicken flocks without direct antibiotic selective pressure.

Keywords: multilocus sequence typing, serotype, antibiotic resistance, chicken, *Salmonella*, antibiotic-free

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INTRODUCTION

Salmonella is a clinically common food-borne gram-negative pathogen with over 2,600 serotypes (1). It is demonstrated that *Salmonella* is predominantly found in poultry, eggs and dairy products (2). *Salmonella* species are considered as intracellular pathogens and carry a number of virulence factors for entry and survival in the intracellular environment, including *Salmonella* pathogenicity islands (SPIs) and *Salmonella* virulence-plasmids (3). *Salmonella* can spread not only horizontally but also vertically through eggs (chicken embryos) (4). When *Salmonella* colonizes the fallopian tubes, it can settle in the reproductive tract of poultry and contaminate fresh eggs, and contaminated chicken embryos may die due to the pathogenicity of *Salmonella* (5). The non-dead chicken embryos will still carry *Salmonella* after hatching, which will cause healthy chicks to be infected with *Salmonella* disease. For example, *Salmonella enterica* serovar Gallinarum biovar Pullorum (*S.* Pullorum), the causative agent of pullorum disease (PD) in chickens, results in a high mortality rate among embryos and chicks, as well as weakness and white diarrhea (6). Therefore, improper treatment of *Salmonella* infection may greatly increase cost on the disease management and flock breeding (7, 8).

However, strains of *Salmonella* spp. with antibiotic resistance are now widespread in both developed and developing countries (9). The emergence of *Salmonella* with antimicrobial resistance is mainly promoted by the use of antibiotics in animal feed to promote the growth of food animals, and in veterinary medicine to treat bacterial infections in those animals (2). This poses a high risk of zoonotic disease caused by the transmission of multidrug-resistant *Salmonella* strains from animals to humans via the ingestion of contaminated food or water (10, 11). To limit the negative impacts, the European Union Commission, U.S., China and many other countries banned antibiotics use for enhancing growth in livestock in 2006, 2017, and 2020, respectively (12–14). Recent studies have shown that antibiotic resistance patterns from agricultural settings can be indistinguishable, and a better understanding of the background data is required for effective agricultural management (15). A few studies (16, 17) investigated the characteristics and antibiotic resistance profile of *Salmonella* from antibiotic-free poultry or chicken meat. However, little is known about the characteristics of *Salmonella* in Chinese native chicken flocks reared on an antibiotic-free diet.

There are a variety of indigenous layer breeds in China, including the Luhua chicken, Langya chicken, Qingjiaoma chicken and Bairi chicken. The Luhua chicken has a unique black and white feather color and produces high-nutrition eggs. The Langya chicken has a small body size and high egg production. The Qingjiaoma chicken has cyan feet and black spots in body and feather. The Bairi chicken has a small body size and a *U*-shaped back. No antibiotics were used during the entire feeding process for the Luhua, Langya and Qingjiaoma chickens for at least 4 years. Earlier research in our previous study found that the detection rate of *Salmonella* in dead embryos could evaluate the *Salmonella* infection rate in chicken flocks (18, 19). In the current study, we mainly investigated the serotypes and antibiotic resistance profiles of *Salmonella* from dead embryos of Chinese native breeders fed on antibiotic-free or conventional diets in 2019. This study will help to supplement the epidemiological data of *Salmonella* infection in Chinese chicken flocks fed on antibiotic-free diets.

MATERIALS AND METHODS

Samples and *Salmonella* Isolation

A total of 360 dead chicken embryos (18 days of incubation) were used to isolate *Salmonella* from three Chinese native layer breeders fed on antibiotic-free diets and one conventional native breeder with 90 dead embryos in each farm in 2019. In 2020, dead embryos, cloacal swabs, feed samples and waterline drip samples (nipple drinkers) were collected for *Salmonella* isolation. The Luhua breeder has been not fed with antibiotic for 6 years and its flock size is 200,000. The Langya and Qingjiaoma chickens were fed on antibiotic-free diets for 4 years in the same breeder farm with flock sizes 10,000 and 50,000, respectively. The three chicken flocks fed on antibiotic-free diets had ever used antibiotics to treat bacterial diseases before antibiotic-free feeding and they were 1-day or about 18 weeks old when the antibiotic-free diet was started. The conventional Bairi breeder farm used antibiotics to promote growth intermittently prior to this study with flock size 50,000. These native breeder flocks were generally maintained for 1.0∼1.5 years, and therefore the chicken embryos from these flocks fed on antibiotic-free diets were probably in the 4th−6th generation. All of these chicken farms are located in eastern China. The Bairi chicken farm is 42 km away from Luhua layer farm and 271 km away from Langya and Qingjiaoma chicken farm (**Figure 1A**).

The liver, spleen and large intestine were taken from the dead chicken embryos with sterile forceps and placed in sterile microcentrifuge tubes (20). Discolored embryos, engorged blood vessels or liver necrosis were usually observed in these dead embryo samples. *Salmonella* strains were isolated from these samples using the Chinese National Standard method (GB 4789.4-2010) with some modifications. Briefly, each embryo sample was added into 4.5 mL of buffered peptone water (BPW, Land Bridge Technology, Beijing, China) and the BPW mixture was incubated at 37°C for 14 h for pre-enrichment. Approximately 0.5 mL of pre-enriched cultures were inoculated into 4.5 mL tetrathionate broth base (TTB, Qingdao Hope Bio-technology Co., Ltd.). After 20 h of incubation at 37°C for selective enrichment, one loopful of each TTB broth culture was streaked onto Xylose-Lysine-Tergitol 4 (XLT4) agar (Qingdao Hope Bio-technology Co., China) plates and incubated at 37°C for 48 h (21). About 3∼5 suspected *Salmonella* colonies were identified by polymerase chain reaction (PCR) assays with primers designed for *Salmonella invA* (product of 331 bp) and *S.* Pullorum *iPAJ* (740 bp) (19). Only one colony with the same morphology per sample was picked and confirmed by MALDI Biotyper SMART (Beckman Coulter Inc, US). Bacterial DNA was extracted using TIANamp Bacterial DNA Kit (TIANGEN, Beijing, China) according to the manufacturer's instructions. PCR was performed in a 25.0 μL mixture containing 12.5 μL of 2 × Taq Master Mix (Vazyme Nanjing, China), 9.5 μL ddH_2O, 1.0 μL of sample DNA, and 1.0 μL of each primer.

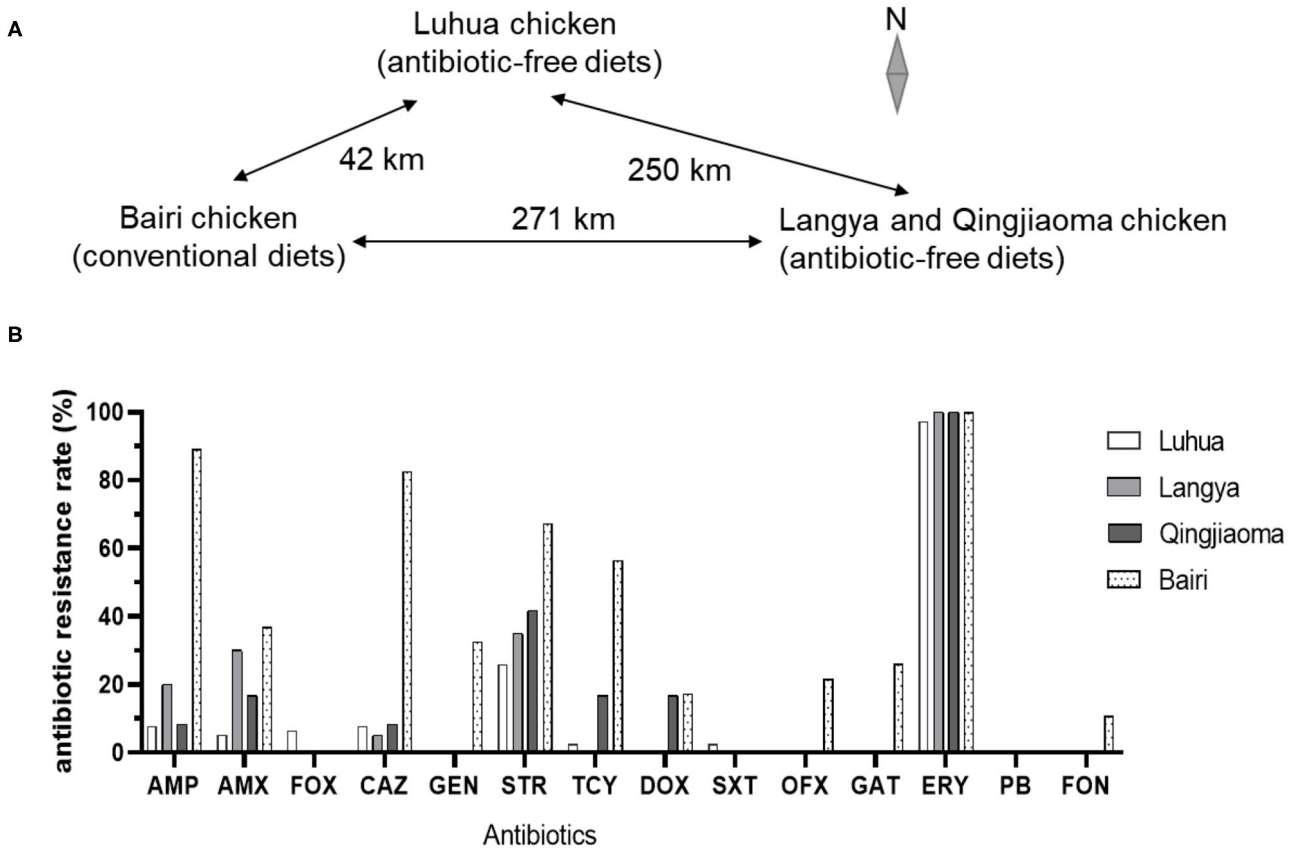

FIGURE 1 | (A) Geographical distribution of the Chinese native layer farms in this study. **(B)** Resistance rates of *Salmonella* isolates from four Chinese native breeder flocks to different antibiotics. Antibiotic susceptibility was tested by the Kirby-Bauer disc diffusion test method and the results of antibiotic resistance rates were calculated from three repeats.

PCR reactions were conducted using annealing at 55°C for *invA* and 58°C for *iPAJ*. The standard strain of *S.* Enteritidis (CVCC3377) and *S.* Pullorum (CVCC535) purchased from the China Veterinary Culture Collection Center (Beijing, China) were used as control strains.

Salmonella Serotyping

According to the manufacturer's instructions from the *Salmonella* serotyping kit (Tianrun Bio-Pharmaceutical, Ningbo, China), all isolates used in this study were serotyped by slide agglutination using a commercial *Salmonella* antisera kit (Tianrun Bio-Pharmaceutical, Ningbo, China). The kit contained Vi antiserum and monovalent and polyvalent H and O antisera with a total of 60 factors. A single colony of *Salmonella* on the nutrient agar plate was mixed with polyvalent O antisera first, then with the specific monovalent antisera testing agglutination within 60 sec. Once the O and H antigens are identified, the serotype can be determined according to the Kauffmann-White scheme (22, 23).

Multilocus Sequence Typing

Seven housekeeping genes (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*) were used to characterize Multilocus sequence typing (MLST) of *Salmonella* isolates according to the instructions from the University of Warwick (http://mlst.warwick.ac.uk/mlst/). The primer pairs for the PCR amplification of internal fragments of these genes were used according to the protocols on the EnteroBase website (https://enterobase.readthedocs.io/en/latest/mlst/mlst-legacy-info-senterica.html). All PCR reactions were conducted by using an annealing temperature of 55°C. Gene products were sequenced (Sangon Biotech, Shanghai, China) and the allele number of the corresponding sequence for each of the seven housekeeping genes was obtained by sequence alignment with BioEdit software based on the "*Salmonella enterica* MLST Database." The sequence type (ST) was assigned according to the Achtman seven Gene MLST scheme as described online (http://mlst.warwick.ac.uk/mlst/dbs/Senterica) (20, 24).

Antimicrobial Susceptibility Testing

According to the Kirby Bauer method recommended by the World Health Organization and the manual of clinical and Laboratory Standards Institute (CLSI, 2017), antimicrobial susceptibility testing of the *Salmonella* isolates obtained in this study was performed with a total of 14 antibiotics (Hangzhou Binhe Microorganism Reagant Co., Ltd., China), including ampicillin (AMP; 10 μg), cefoxitin (FOX; 30 μg), ceftazidime

TABLE 1 | The serotypes, MLST and number of *Salmonella* isolates from Chinese native breeder flocks fed on antibiotic-free or conventional diets.

Breeder	Antibiotic use	No. of isolates			
		Salmonella	*S.* Pullorum		*S.* Enteritidis
			ST92	ST2151	ST11
Luhua	No	77	71	0	6
Langya	No	20	0	20	0
Qingjiaoma	No	12	5	7	0
Bairi	Yes	46	20	5	21

(CAZ; 30 μg), erythromycin (ERY; 15 μg), gentamicin (GEN; 10 μg), streptomycin (STR; 10 μg), tetracycline (TCY; 30 μg), sulfamethoxazole (SXT; 25 μg), ofloxacin (OFX; 5 μg), gatifloxacin (GAT; 5 μg), amoxicillin (AMX; 20 μg), doxycycline (DOX; 30μg), florfenicol (FON; 30 μg) and polymyxin B (PB; 300 IU) (25). *E. coli* (ATCC 25922) and (ATCC 35218) were used as quality control strains according to the CLSI M100-S27 guideline. Multiple drug resistance (MDR) was defined as bacteria isolates with resistance to one or more antibiotics in three or more antibiotic classes. The MDR rates of *Salmonella* isolates in these chicken breeds were calculated by the number of MDR isolates divided by the number of screening isolates. The total *S.* Pullorum and *S.* Enteritidis isolates from three antibiotic-free chicken breeds to different kinds of antibiotics were aggregated, respectively, and the relative antibiotic resistance rate was presented as a percentage and compared with that from the conventional Bairi chicken breed.

Data Analysis

The Chi-squared test or Fisher's exact test were used for analyzing the data (26).

RESULTS

Serotypes of *Salmonella*

In this study, a total of 155 *Salmonella* isolates were recovered from 360 dead chicken embryos from three Chinese native breeder flocks fed on antibiotic-free diets and one conventional layer breeder Bairi chicken in 2019 (**Table 1** and **Supplementary Table 1** in the **Supplemental Material**). The number of dead embryos positive for *Salmonella* was 143 (39.72%), and coinfection with two serotypes of *Salmonella* was found in 12 dead embryos (13.3%) of Bairi chicken. The 155 *Salmonella* isolates were divided into *S. Gallinarum* biovar Pullorum (*S.* Pullorum, $n = 128$) and *S.* Enteritidis ($n = 27$). The positive detection rate of *Salmonella* in the Luhua chicken (85.6%) was the highest, followed by 51.1% in the Bairi chicken, 22.2% in the Langya chicken and 13.3% in the Qingjiaoma chicken. The average isolation rate of *Salmonella* in three breeder flocks fed on antibiotic-free diets was 40.4%, lower than 51.1% in conventional Bairi chicken. *S.* Pullorum was found in all four chicken flocks, whereas *S.* Enteritidis was detected specifically in Luhua and Bairi breeder flocks. The results indicated the prevalence of *S.* Pullorum in Chinese native chicken flocks fed on both antibiotic-free and conventional diets.

In order to evaluate the *Salmonella* infection in breeder flocks after implementing the *Salmonella* eradication project and strengthening feeding management, various samples were collected from Luhua and Langya breeder flocks in 2020. Compared with the high isolation rate of *Salmonella* (85.6%) in Luhua breeder flocks in 2019, the infection rate of *Salmonella* in Luhua chicken was remarkably reduced to 2.08% (5/240) in 2020 by examining 140 dead embryos and 100 cloacal swabs ($P < 0.0001$) (**Table 2**). However, for Langya chicken flocks without *Salmonella* eradication project implementation, the isolation rate of *Salmonella* spp. (13.91%, 32/230) was significantly lower than that in Luhua chicken flocks ($P < 0.0001$). The 32 *Salmonella*-positive samples included 29 of 100 dead embryos (29%), 2 of 15 feed (13.33%), 1 of 100 cloacal swabs (1%) and 0 of 15 waterline drip samples (nipple drinkers) (**Table 2**).

MLST

Only three STs were identified in the 155 *Salmonella* isolates, including ST92 (61.9%, $n = 96$), ST2151 (20.6%, $n = 32$) and ST11 (17.4%, $n = 27$) (**Table 1**). ST92 and ST2151 were identified in *S.* Pullorum, whereas only ST11 was found in *S.* Enteritidis. For the Luhua and Bairi chickens, ST92 *S.* Pullorum was the prevalent isolate, but for the Langya and Qingjiaoma chickens, the most numerous isolate was ST2151 *S.* Pullorum.

Antimicrobial Susceptibility Testing

All of the 155 *Salmonella* isolates from chicken embryos were tested for resistance against 14 commonly used antibiotics (**Figure 1B**). Approximately equally high proportions of *Salmonella* isolates from three breeder flocks fed on antibiotic-free diets (98.2%, 107/109; 97.4% for Luhua, 75 of 77; 100% for Lanya, 20 of 20; 100% for Qingjiaoma, 12 of 12) and conventional Bairi chicken (100%, 46/46) were resistant to ERY ($P = 0.36$) (**Figure 1B** and **Figure 2**). A high resistance rate of *Salmonella* to STR was also found among the three breeder flocks fed on antibiotic-free diets, ranging from 26 to 41.7% (**Figure 1B**). But the rate of resistance to STR was significantly lower ($P < 0.0001$) in antibiotic-free-fed chicken (29.4%, 32/109) than in conventional chicken isolates (67.4%, 31/46). Moreover, the rates of resistance to AMP and AMX differed significantly ($P < 0.0005$) between chicken breeds fed on antibiotic-free diets (10.1%, 11/109; 11.0%, 12/109) and conventional Bairi chicken breed (89.1%, 41/46; 37.0%, 17/46) isolates (**Figure 2**), respectively. The resistant rates of *Salmonella* isolates from Bairi chicken to GEN, OFX, GAT, and FON were 10.9%∼32.6%, but no isolate showed resistance to the above four antibiotics among the other three chicken breeds fed on antibiotic-free diets (**Figure 1B**). Among the *Salmonella* isolates from chicken breeds fed on antibiotic-free and conventional diets, the significantly different resistance rates to other antibiotics were as follows, respectively: to CAZ, 7.3% and 82.6% ($P < 0.0001$); to GEN, 0 and 32.6%; to TCY, 3.7% and 56.5% ($P < 0.0001$); to DOX, 1.8% and 17.4%; to OFX, 0 and 21.7% and to GAT, 0 and 26.1% (**Figure 2**). All *Salmonella* isolates used in this study were sensitive to PB, which was a drug of last resort for the treatment

TABLE 2 | Comparison of the infection rates of *Salmonella* spp. from various samples in Luhua and Langya breeds in 2020.

Breeder	Improvements	Total isolates/% (positive/total)	No. of isolates from various samples (positive/total)			
			Dead embryos	Cloacal swabs	Feed sample	Waterline drip
Luhua	*Salmonella* eradication, improved feeding management	2.08% (5/240)	5/140	0/100	NA	NA
Langya	No	13.91% (32/230)	29/100	1/100	2/15	0/15

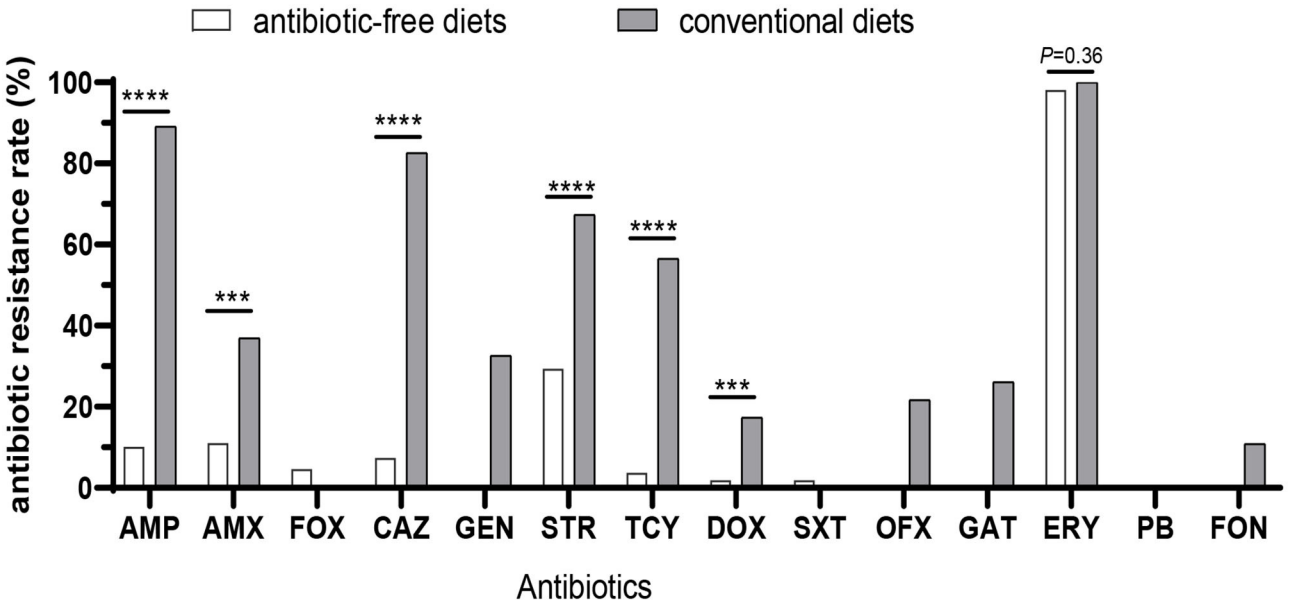

FIGURE 2 | Comparison of the resistance rates of *Salmonella* isolates between three Chinese native breeder flocks fed on antibiotic-free diets and one breeder flock fed on conventional diets. The total *Salmonella* isolates from three Chinese breeder flocks fed on antibiotic-free diets were aggregated and presented as a percentage. The difference was analyzed by chi-squared test. ***$P < 0.0005$; ****$P < 0.0001$.

of MDR *Enterobacteriaceae* infection. Moreover, the rates of resistance in antibiotic-free-fed chicken and conventional chicken isolates to FOX (4.6% and 0, respectively) and SXT (1.8%, 0, respectively) were low (**Figure 2**).

Most of the isolates among three chicken breeds Luhua (64.9%, 50/77), Langya (60%, 12/20) and Qingjiaoma (58.3%, 7/12) fed on antibiotic-free diets were resistant to only one antibiotic (ERY), whereas the rate of resistance to one antibiotic in conventional Bairi chicken isolates was only 4.3% (2/46) (**Figure 3**). The largest proportion in conventional Bairi chicken was occupied by MDR isolates, up to 93.5% (43/46). However, the MDR rate in Luhua, Langya and Qingjiaoma chicken isolates was 15.6% (12/77), 30% (6/20) and 33.3% (4/12), respectively (**Figure 3**). The total MDR rate in isolates from chickens fed on antibiotic-free diets (20.2%, 22/109) was significantly ($P < 0.0001$) lower than that from chickens fed on conventional diets (93.5%, 43/46) (**Figure 4A**). One isolate (1/77) from the Luhua chicken was shown to be susceptible to all antibiotics tested in this study (**Figure 3**). Moreover, the MDR profile of both *S.* Pullorum and *S.* Enteritidis isolated from chickens fed on antibiotic-free diets exhibited a diverse drug resistance spectrum (**Supplementary Table 1**). By comparing the *S.* Pullorum isolates from chicken flocks fed on conventional and antibiotic-free diets, we showed that the MDR rate of conventional breeder chicken isolates (100%, 25/25) was much higher than that from three breeder flocks fed on antibiotic-free diets (21.4%, 22/103) ($P < 0.0001$) (**Figure 4B**). Approximately 62.1% of *S.* Pullorum isolates from three breeder flocks fed on antibiotic-free diets were resistant to only one antibiotic ERY, followed by 15.5% of *S.* Pullorum isolates that were resistant to two antibiotics tested in this study (**Figure 4B**). For *S.* Enteritidis, the MDR rate from the conventional Bairi chicken was up to 85.7% (18/21), whereas no isolate with MDR was found among a total of 6 isolates from three chicken breeds fed on antibiotic-free diets (**Figure 4B**).

DISCUSSION

Among three chicken breeds (Luhua, Langya and Qingjiaoma) fed on antibiotic-free diets and one conventional Bairi chicken, different serotypes and ST types of *Salmonella* were identified.

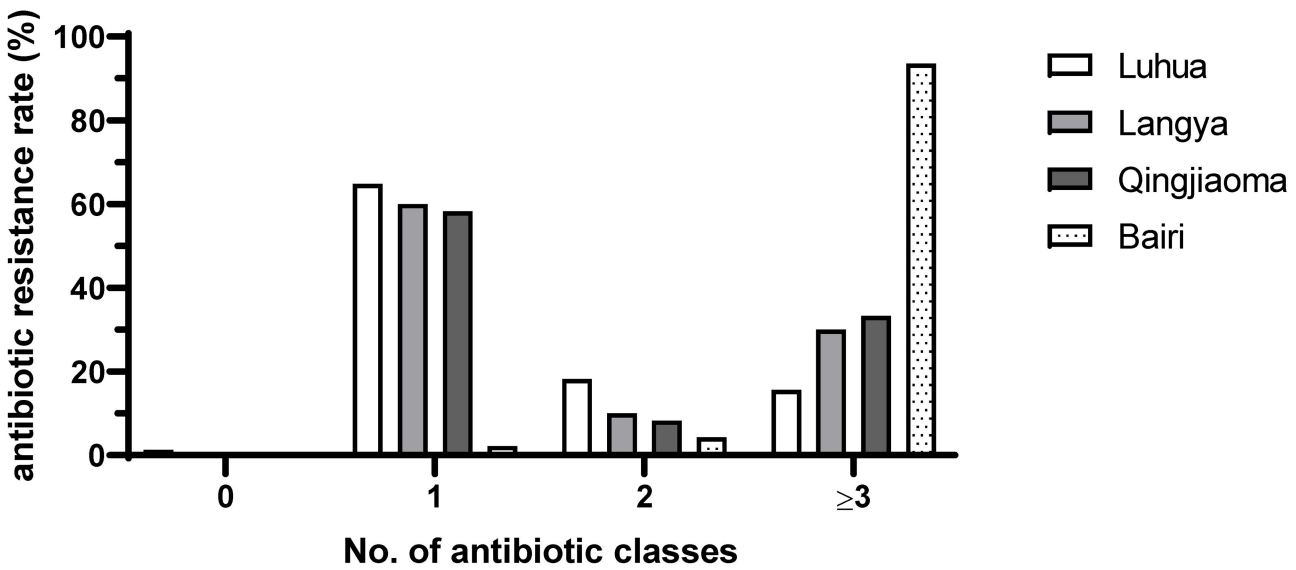

FIGURE 3 | Summary of resistance rates of *Salmonella* isolates to one, two and three or more antimicrobial classes from four Chinese native breeder flocks in this study. The percentages of resistance to one, two and three or more antimicrobial classes were analyzed by the number of isolates divided by the number of all screening isolates from the chicken flocks.

However, the dominating serotype among these breeder flocks in this study was *S.* Pullorum, which was significantly different from the prevalent isolates (100% *S.* Enteritidis) from fecal swabs and chicken embryos of large-scale breeder farms in China (19). Zhao et al. (27) investigated the prevalence and characteristics of *Salmonella* in free-range chickens in China and showed that a total of 38 *Salmonella* isolates (38/300, 12.7%) were recovered and the most common serotype was *S.* Enteritidis (81.6%). Certainly, some *Salmonella* species may be missed in these flocks due to the choice of methods and media used for isolation of *Salmonella*. We were not able to differentiate the susceptibility of these chicken breeds to *Salmonella* infection due to lack of data in the study. However, the *Salmonella* infection rates in embryos were higher than that in samples from cloacal swabs and farm environments. These farms were located in different regions of China east, and different native breeder flocks had different diets with or without antibiotics added, so it seemed that the infection rate of *Salmonella* may be significantly associated with geographical distribution and feeding management level in China (18). The prevalence of *Salmonella* associated with chick mortality at hatching was investigated in three hatcheries in Jos, central Nigeria. The results showed that 45(9%) of the 500 samples were positive for *Salmonella* and the prevalent serotypes were *S.* Kentucky (75.6%) and *S.* Hadar (24.4%) (28). Bailey et al. (29) tracked the serotype of *Salmonella* through integrated broiler chicken operations in the US. The results showed that the rate of *Salmonella*-positive samples from the hatchery in 1999–2000 was the highest and the predominant serotype found in hatchery samples was *S.* Senftenberg. An association between the serotypes found in the hatchery and those found on the final processed carcasses was observed.

PD caused by *S.* Pullorum is strongly associated with vertical transmission directly from contamination of the egg in the genital tract or indirectly from chick-to-chick contact in the hatchery (30). It was demonstrated that *S.* Pullorum colonized both the ovary and the oviduct of hens and led to 6% of laid eggs being infected by *S.* Pullorum via more than one mechanism of egg infection (31). *S.* Pullorum is not excreted extensively in the feces, unlike many other *Salmonella* serotypes that are more frequently associated with human food poisoning (32). In the current study, *S.* Pullorum isolates from dead embryos in these chicken flocks exhibited limited genetic diversity in ST and only ST92 and ST2151 were determined among the total 128 isolates. Hu conducted the whole-genome sequencing of a panel of 97 *S.* Pullorum isolates between 1962 and 2014 from four countries across three continents, and Hu also found most of the strains belonged to ST92 (33). However, the *S.* Pullorum isolates in these chicken flocks fed on antibiotic-free diets exhibit phenotypic heterogeneity with relatively low antibiotic resistance rates, providing an example of *Salmonella* characteristics for the chicken production system without direct selective pressure. The most effective means of controlling pullorum disease is a combination of stringent management procedures and eradication by a serological test (34). In the United States, PD was brought under control after the implementation of the National Poultry Improvement Plan and the vaccination of flocks (35). The European Union also established a regulation focused on preventing, monitoring or eradicating *Salmonella* in poultry, and the incidence of salmonellosis had decreased since 2003 (36). However, related control programs for *S.* Pullorum eradication and available vaccines were still absent in China. The positive rate of *Salmonella* isolates from Luhua chicken in 2019 was the highest, however, *Salmonella* serological tests had been regularly

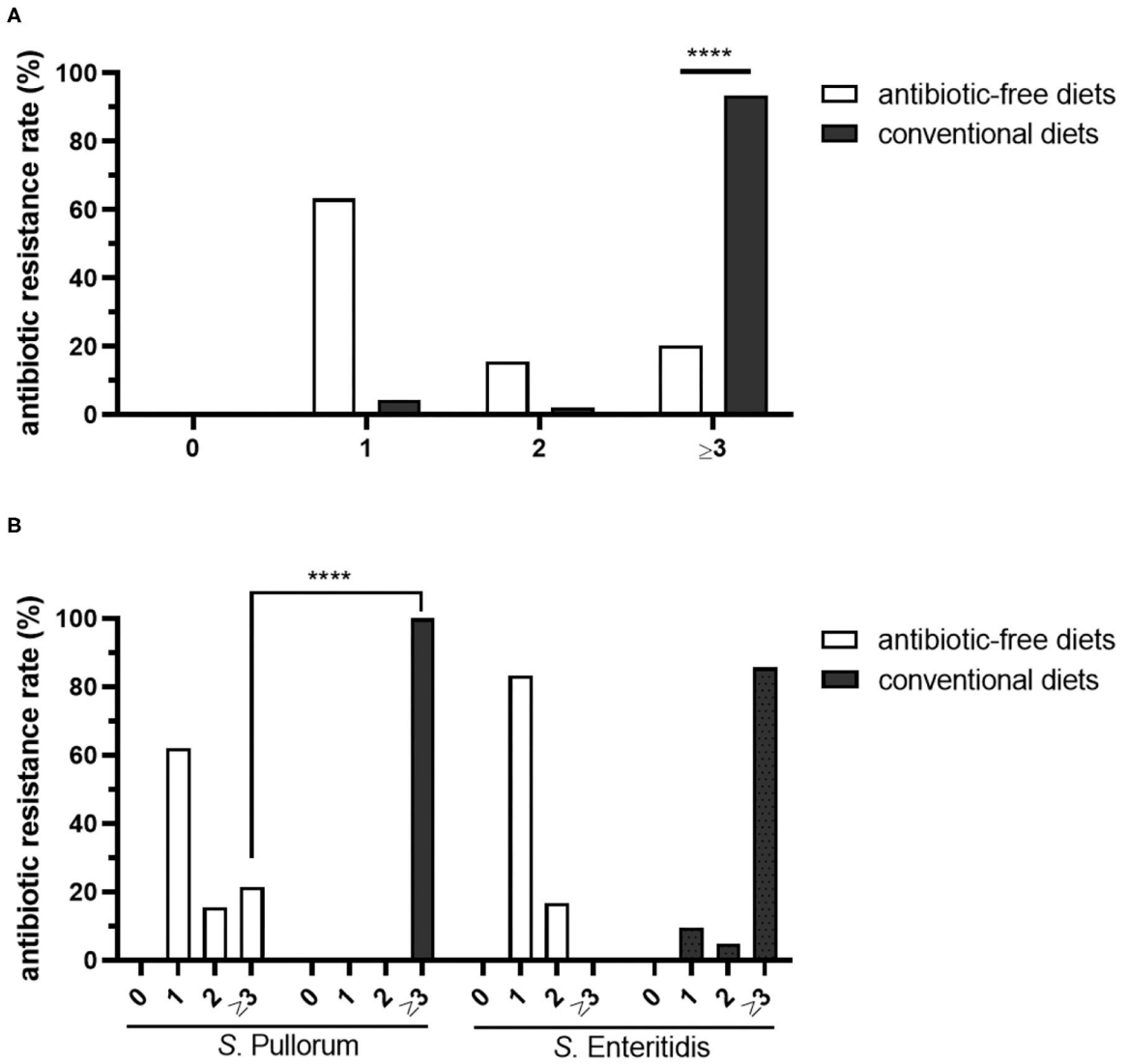

FIGURE 4 | Statistics of antibiotic resistance rates of *Salmonella* isolates from Chinese native breeder flocks fed on antibiotic-free diets or conventional diets. **(A)** Comparison of the antibiotic resistance rates of *Salmonella* isolates. **(B)** Antibiotic resistance rates of different *Salmonella* serotypes. The total *Salmonella* spp., *S.* Pullorum and *S.* Enteritidis isolates from three antibiotic-free-fed breeder flocks to different kinds of antibiotics were aggregated together respectively, and the difference of resistant rates to antibiotics in isolates from breeder flocks fed on antibiotic-free or conventional diets was analyzed with chi-squared test. ****$P < 0.0001$.

done to eliminate positively infected chickens promptly since 2019. And with daily feeding management strengthening, the infection rate of *Salmonella* in Luhua chicken was dramatically reduced to 2.08% in the survey of 2020, lower than 13.91% in Langya chicken.

S. Enteritidis is the serovar most frequently associated with egg infection due to its unique long term ability to colonize the ovary and the oviduct of laying hens and its spread and persistence in the parental breeder flock population (37). The frequency of egg contamination by *S.* Enteritidis depends on the level of contamination of the flock, and eggs are more likely to become internally contaminated around the onset of lay (38, 39). The isolation rate of *S.* Enteritidis in the present study was 17.4% (27/155) and all were resistant to erythromycin. This was quite different from a survey with rectal swabs collected from three Chinese large-scale conventional chicken farms, which showed 80.8% (63/78) of MDR isolates with the most common serovar being *S.* Enteritidis (88.5%) (21). Unlike *S.* paratyphoid serovars that only infect humans by causing enteric fever, *S.* Enteritidis is a zoonotic pathogen of substantial concern to global human and animal health (40). Many studies using whole genome sequencing linking epidemiology, phylogeny and virulotyping are performed with *Salmonella* isolates from the harmonized monitoring of poultry and from human disease to facilitate attribution studies and identify trends associated with virulence and stress-response genes (41, 42).

Among the 14 antibiotics used in this study, the resistance rates of all *Salmonella* isolates to 11 antibiotics in conventional chicken were higher than those from chickens fed on antibiotic-free diets. The average MDR rate (20.2%, 22/109) of *Salmonella* isolates from chickens fed on antibiotic-free diets was significantly lower than the rates of 100% among 63 isolates examined by Yang et al. (19) from conventional farms in a similar geographical region, and also lower than that from other poultry farms in China (43). These data indicated that the use of antibiotics may promote the development of MDR *Salmonella* (44). Liu et al. (45) found high abundances of aminoglycoside, sulfonamide and tetracycline resistance genes in one antibiotic-free layer farm without direct antibiotic selective pressure. Similarly, the high resistance rate to streptomycin belonging to aminoglycoside antibiotic was also found in *Salmonella* isolates from antibiotic-free-fed layer farms in this study. Besides, high resistance rate to erythromycin was seen amongst three antibiotic-free-fed layer flocks in this study. The mechanisms of erythromycin resistance in *Salmonella* contain modification of the ribosomal target of macrolides and hydrolysis of the macrolide lacton ring catalyzed by erythromycin esterases (such as *ereA* and *ereB*) (46). Modification of the ribosomal target of macrolides is a common mechanism, and confers broad cross-resistance to macrolide-lincosamide-streptogramin antibiotics. This modification can occur by mutation and methylase encoded by *erm* (erythromycin ribosome methylase) genes (47). Antibiotics were used to treat bacteria diseases in the three flocks of layer breeder in this study 4–6 years ago, but those resistance antibacterial and/or genes may still circulate within the living environment of flocks. Management practices and contaminated eggs, and feces or wastewater have been attributed to the spread and persistence of antibiotic resistant *Salmonella* in the environment (2, 44). Together, these data provided an example of the *Salmonella* antibiotic resistance profiles in the chicken flocks fed on antibiotic-free diets. To the best of our knowledge, there have been no previous studies that investigated the characteristics of *Salmonella* infection in such native layer flocks fed on antibiotic-free diets in China.

In summary, the current study showed that majority of *Salmonella* isolates from three Chinese native breeder flocks fed on antibiotic-free diets were ST92 and ST2151 *S.* Pullorum and ST11 *S.* Enteritidis. The antibiotic resistance rates and MDR rates in three chicken breeds fed on antibiotic-free diets were significantly lower than that from a conventional Bairi chicken farm. Moreover, the *Salmonella* isolates in these chicken flocks fed on antibiotic-free diets exhibit phenotypic heterogeneity with a diverse drug resistance spectrum, providing an example for the occurrence of antibiotic resistance in the chicken production system without direct selective pressure.

DATA AVAILABILITY STATEMENT

The original contributions generated for this study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use of Shandong Agricultural University (SDAUA-2018-027). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

LC and QL performed the experiments and analyzed the data. LC and GH worked on the manuscript writing. SS designed the experiments and worked on the manuscript. ZJ, YS, and SY helped do the experiments. JQ provided support for experiments. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.607491/full#supplementary-material

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First Isolation and Molecular Characterization of *bla*$_{CTX-M-121}$-Producing *Escherichia coli* O157:H7 From Cattle in Xinjiang, China

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Su Z, Tong P, Zhang L, Zhang M, Wang D, Ma K, Zhang Y, Liu Y, Xia L and Xie J (2021) First Isolation and Molecular Characterization of bla$_{CTX-M-121}$-Producing Escherichia coli O157:H7 From Cattle in Xinjiang, China. Front. Vet. Sci. 8:574801. doi: 10.3389/fvets.2021.574801

The bovine *Escherichia coli* O157:H7 is a major foodborne pathogen causing severe bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome in humans. Cattle are recognized major reservoir and source of *E. coli* O157:H7. We investigated the antibiotic resistance, molecular profiles, and intrinsic relationship between 21 isolates of *E. coli* O157:H7 from cattle farms and slaughtering houses in Xinjiang. Using pulsed-field gel electrophoresis (PFGE) molecular typing, two types of PFGE were revealed through cluster analysis, including clusters I and II, with 66 and 100% similarity of PFGE spectra between 21 isolates. We also detected that 18 isolates (86%) carried at least one virulence gene, 16 isolates (76%) carried the *eae* gene, and 7 (33%) carried the *stx1* + *stx2* + *eae* + *hly* + *tccp* genes. Eighteen isolates were susceptible to antibiotics. Three isolates were resistant to antibiotics, and two were multidrug resistant. One of the two multidrug-resistant isolates detectably carried the *bla*$_{CTX-M-121}$ gene. This is the first finding of the *bla*$_{CTX-M-121}$ gene detected in *E. coli* O157:H7 isolated from cattle in Xinjiang. The *bla*$_{CTX-M-121}$ gene is transferable between the bacterial strains via plasmid transmission. The results indicated that *E. coli* O157:H7 may have undergone clonal propagation in cattle population and cross-regional transmission in Xinjiang, China.

Keywords: *E. coli* O157:H7, virulence genes, antibiotic resistance, PFGE, bovine

INTRODUCTION

Escherichia coli O157:H7 is a major foodborne pathogen that causes severe bloody diarrhea, hemorrhagic colitis (HC), and hemolytic uremic syndrome (HUS) in humans (1). *E. coli* O157:H7 was first recognized as a pathogen contributing to an outbreak of HC associated with hamburger consumption in 1982 (2). Since then, *E. coli* O157:H7 outbreaks have been reported in the United States, Canada, Japan, and China (3–6). *E. coli* O157:H7 has been reportedly detected in healthy cattle worldwide (7). The infected, asymptomatic cattle irregularly excrete *E. coli* O157:H7, resulting in contaminating food and water in the environment, as well as infecting humans and other animals (8). Cattle are recognized major reservoir and source of *E. coli* O157:H7.

Pathogenic virulence of *E. coli* O157:H7 is attributable to genes coding for Shiga toxin (Stx), the intestinal cell shedding site [locus of entericyte effacement (LEE)] virulence island, and the large plasmid pO157 (9). Stx, comprising Stx1 and Stx2, is able to induce cell necrosis and tissue lesions, and Stx2 is more potent than Stx1 (10, 11). The LEE region encodes a type III secretion system, and the secreted proteins *E. coli* secreted proteins (Esp) and translocated intimin receptor (Tir). Both Esp and Tir are required for intimate attachment and A/E lesion formation (12). The LEE region also encodes intimin, an outer membrane protein adhesin (Eae) that mediates the intimate attachment of bacteria to the host epithelial cell surface (13). In addition, Tir cytoskeleton-coupling protein (TccP) stimulates actin polymerization during the formation of A/E lesion (14). The large plasmid pO157 carries genes coding for type II secretion systems, such as hemolysin (Hly) and ToxB. All these virulence factors of *E. coli* O157:H7 reportedly regulate the adhesion of pathogenic bacteria to intestinal epithelial cells, causing the shedding of intestinal cells. These virulence genes have been used to identify bacterial strains isolated from various sources in epidemiological studies (6, 15, 16).

Antimicrobials have been the mainstay for the prevention and treatment of bacterial diseases in animals. However, their use is getting limited due to rising antibiotic resistance, which has become a serious problem worldwide, especially in developing countries where the quality, distribution, and use of antibiotics in human and veterinary medicine is not strictly regulated (15, 17). Extended-spectrum cephalosporins (ESCs), especially the third- and fourth-generation cephalosporins, are classified by the World Health Organization (WHO) to treat infections of multidrug-resistant Gram-negative bacteria (18). However, acquisition of genes encoding extended spectrum β-lactamases (ESBLs), especially CTX-M enzymes, by *E. coli* plays an important role in the resistance to ESCs (19). The genes encoding these enzymes, i.e., bla_{CTX-M} genes, are usually located on transferable plasmids, which also carry resistance genes for other types of antimicrobials (i.e., fluoroquinolones, aminoglycosides). These plasmids mediate the spread of drug resistance between bacteria *via* conjugation (20). *E. coli* O157:H7 isolates collected from humans and animals have shown resistance to a variety of antibiotics; therefore, the emergence of multidrug resistant (MDR) *E. coli* O157:H7 has become a public health issue (21, 22).

The sustainability of cattle industry and food safety depend upon the effective prevention and control of bovine pathogenic microorganisms. Xinjiang is one of largest cattle-raising regions in China. To further assess the potential public health impact of *E. coli* O157:H7 in Xinjiang, we investigated the pathogenicity and antibiotic resistance of isolates collected from farms and slaughterhouses. We examined the intrinsic relationship among different isolates and assessed the potential dissemination of MDR profiles *in vitro*.

MATERIALS AND METHODS

Sample Collection

Total samples (n = 2,439) included 1,155 fresh feces, 1,236 rectal swabs, and 48 carcass swabs that were collected from 18 beef cattle and dairy farms (industrial, semi-industrial, and traditional farms, with a herd size of 200–8,000 cattle) and one slaughterhouse in the region of Akesu, Bole, Changji, Tacheng, Urumqi, Wujiaqu, and Yili in Xinjiang, China between October 2012 and March 2017. Samples were collected from Xinjiang brown cattle, Holstein cattle, Simmental cattle, and Angus cattle (1–7 years old, 400–800 kg body weight). Approximately 25 g of fecal samples were collected from each animal by rectal palpation or during defecation using disposable sleeve gloves and then placed in sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI, USA). Rectal swabs were collected when rectal palpation is not applicable or no bowel movement is observed. Sterile cotton swabs (length, 150 mm; Copan Italia SpA) were used to collect mucus samples from the rectal anal junction. Sterile cotton swabs were also used to swab ∼10 cm^2 of carcass surface. All samples were transported in icebox to the laboratory and stored at 4°C until processed within 2 h.

Escherichia coli O157:H7 Isolation

Selective enrichment was carried out according to the method reported by Mersha et al. (23) with minor modifications. One gram of each feces was aseptically added to 9 ml of modified tryptone soya broth containing 20 mg/L novobiocin (mTSB + n) (Hopebio, Qingdao, China) and incubated at 37°C for 16 h. To all the swab samples, 90 ml of mTSB + n was added and homogenized using a vortex mixer. After incubation for 16 h, all the samples were processed for immunomagnetic separation (IMS) using anti-*E. coli* O157 Dynabeads (Dynal, Invitrogen, USA) as follows. One microliters of the enriched broth culture was put in a sterile screw cupped Eppendorf tubes to which 20 μl of anti-O157:H7 immunomagnetic beads was added, followed by shaking at ambient temperature for 30 min. The tubes were then kept inside the manual magnetic particle concentrator. The beads were washed thrice using 300 μl phosphate-buffered saline (PBS) buffer for each wash. Finally, 100 μl of PBS was added in each tube and mixed gently (24). Fifty microliters of the mixture was streaked onto Sorbitol MacConkey agar containing 0.05 mg/L cefixime and 2.5 mg/L potassium tellurite (CT-SMAC) (Hopebio, Qingdao, China) and incubated at 37°C for 20–24 h to develop colonies. Pale-colored colonies were purified by repeated streak plating until a uniform colony morphology was obtained (25). One or more of the colonies were individually selected as presumptive *E. coli* O157 per sample. *E. coli* CICC 21530 (O157:H7, *stx1* + *stx2* + *eae* + *hly* + *tccp*) (26, 27) and ATCC 25922 strains were used as positive and negative controls, respectively. Two genes (*rfbEO157* and *fliCH7*) were used to identify *E. coli* O157:H7 (28). Pink colonies (suspected the general *E. coli*) were purified by restreaking on McConkey agar and confirmed by PCR method as described by Teichmann et al. (29) (**Table 1**). The PCR amplicons (10 μl) were subjected to electrophoresis on a 1.2% agarose gel in 1× Tris–acetate–EDTA (TAE) buffer at 115 V for 30 min and stained with SYBR Green (Fermentas, Germany). The positive isolates were each inoculated in separate TSB and incubated overnight at 37°C, from which glycerol stocks were made and then stored at −80°C for further analysis.

TABLE 1 | Primers used in PCR to detect targeted genes.

Gene	Primer oligonucleotide sequences (5′-3′) (forward/reverse)	Amplicon size (bp)	Annealing temperature (°C)	Reference
bla$_{CTX-M-U}$	ATGTGCAGYACCAGTAARGT/TGGGTRAARTARGTSACCAGA	593	50	(35)
bla$_{CTX-M-1G}$	GTTACAATGTGTGAGAAGCAG/CCGTTTCCGCTATTACAAAC	1,018	50	(35)
bla$_{CTX-M-2G}$	ATGATGACTCAGAGCATTCG/TGGGTTACGATTTTCGCCGC	865	55	(36)
bla$_{CTX-M-9G}$	ATGGTGACAAAGAGAGTGCA/CCCTTCGGCGATGATTCTC	870	60	(37)
bla$_{TEM}$	ATGAGTATTCAACATTTCCGT/TTACCAATGCTTAATCAGTGA	861	48	(38)
bla$_{SHV}$	CCGGGTTATTCTTATTTGTCGCT/TAGCGTTGCCAGTGCTCG	1,081	48	(39)
cmlA1	CCGCCACGGTGTTGTTGTTATC/CACCTTGCCTGCCCATCATTAG	698	59	(39)
eae	CATTATGGAACGGCAGAGGT/ACGGATATCGAAGCCATTTG	375	52	(31)
fliCH7	TACCATCGCAAAAGCAACTCC/GTCGGCAACGTTAGTGATACC	247	58	(28)
hly	CACACGGAGCTTATATTCTGTCA/AATGTTATCCCATTGACATCATTTGACT	319	45	(32)
rfbEO157	CTACAGGTGAAGGTGGAATGG/ATTCCTCTCTTTCCTCTGCGG	327	58	(28)
*stx*1	GAAGAGTCCGTGGGATTACG/AGCGATGCAGCTATTAATAA	130	54	(30)
*stx*2	TTAACCACACCCACGGCAGT/GCTCTGGATGCATCTCTGGT	346	54	(30)
tccP	CGCCATATGATTAACAATGTTTCTTCAC/CTCGAGTCACGAGCGCTTAGATGTATT	700∼1,000	58	(14)
sul1	CGGCGTGGGCTACCTGAACG/GCCGATCGCGTGAAGTTCCG	433	65	(40)
tetA	GCTACATCCTGCTTGCCTTC/CATAGATCGCCGTGAAGAGG	210	55	(41)
tetE	AAACCACATCCTCCATACGC/AAATAGGCCACAACCGTCAG	278	55	(41)
tetG	GCTCGGTGGTATCTCTGCTC/AGCAACAGAATCGGGAACAC	468	55	(41)

Analysis of Virulence Genes

Genomic DNA contents were extracted from 21 *E. coli* O157:H7 isolates as confirmed by PCR serotyping. In brief, 3–5 colonies were individually suspended in 200 μl of sterile distilled water. Bacterial suspensions were then heated at 95°C for 10 min centrifugation at 13,400 × g for 10 min to obtain the supernatant containing the template DNA and were transferred into 1.5-ml Eppendorf tubes without nuclease and stored at −20°C.

A multiplex PCR procedure was used to detect the *stx1* and *stx2* genes (30), and a single PCR procedure was used to detect the *eae* (31), *hly* (32), and *tccp* (14) genes. The primers, conditions, and references cited are listed in **Table 1**. *E. coli* CICC 21530 was used as a positive control for all the five virulence genes, while ATCC 25922 was used as a negative control. Amplification of the targeted gene was carried out using EX Taq (TaKaRa, Dalian, China) with the following PCR program: 94°C for 4 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 10 min. The annealing temperature was adjusted according to the primer Tm value (**Table 1**).

Antimicrobial Susceptibility Tests

Antibiotic susceptibility was tested using the Kirby–Bauer disk diffusion technique. Antibiotic disks of 6 mm in diameter obtained from OXOID, UK, containing ampicillin (AMP, 10 μg/disk), piperacillin (PIP, 100 μg/disk), cefotaxime (CTX, 30 μg/disk), ceftazidime (CAZ, 30 μg/disk), cefepime (FEP, 30 μg/disk), aztreonam (ATM, 30 μg/disk), ampicillin-sulbactam (SAM, 10/10 μg/disk), piperacillin-tazobactam (TZP, 100/10 μg/disk), amoxicillin-clavulanic acid (AMC, 20/10 μg/disk), gentamicin (GEN, 10 μg/disk), amikacin (AMI, 30 μg/disk), streptomycin (STR, 10 μg/disk), cotrimoxazole (SXT, 25 μg/disk), chloramphenicol (CHL, 30 μg/disk), levofloxacin (LEV, 5 μg/disk), ciprofloxacin (CIP, 5 μg/disk), tetracycline (TET, 30 μg/disk), and polymyxin B (PB, 300 U/disk) (20). *E. coli* ATCC25922, purchased from China Center of Industrial Culture Collection (CICC), was used as a quality control strain in the susceptibility tests. The ESBL-producing isolates were determined by double-disk synergy tests according to CLSI (33). Isolates shown to be resistant to at least three different classes of antimicrobial agents were determined to be multidrug resistant (MDR) (34).

Detection of Antibiotic Resistance Genes

The following resistance determinants were investigated by PCR: *bla*$_{CTX-M}$ [the CTX-M-type genes were detected using universal primers *bla*$_{CTX-M-U}$ (35), and the entire CTX-M-type genes were amplified using the primers *bla*$_{CTX-M-1G}$ (35), *bla*$_{CTX-M-2G}$ (36), or *bla*$_{CTX-M-9G}$ (37)], *bla*$_{TEM}$ (38), and *bla*$_{SHV}$ (39), which encode β-lactamases, chloramphenicol efflux pumps [*cmlA1*(39)], sulfonamide resistance gene [*sul1* (40)], and the *tetA* (41), *tetE* (41), and *tetG* (41) tetracycline efflux pumps. *bla*$_{TEM}$ and *bla*$_{SHV}$ genes were amplified by double PCR; *tetA*, *tetE*, and *tetG* genes were amplified by triplex PCR, while other resistant genes were amplified by single PCR. Primers used for the different genes are listed in (**Table 1**). The PCR products were sent to Sangon Biotech Co., Ltd. (Shanghai, China) for sequence determination. The DNA sequences and deduced amino acid sequences were compared with sequences reported in GenBank to confirm the subtypes of the β-lactamase gene.

Conjugation Experiments and Plasmid Analysis

Sodium azide-resistant *E. coli* J53 was used as a recipient and conjugated to a *bla*$_{CTX-M}$-producing isolate by filtration. Transconjugants were selected on Mac Conkey agar containing cefotaxime or ceftazidime (4 μg/ml) and sodium azide (200 μg/ml). ESBL and antibiotic susceptibility was also tested in selected transconjugants, and the presence of *bla* genes was determined using PCR as described above. The resistance plasmids carried by transconjugants were typed using PCR-based replicon typing (42).

Epidemiological Typing

All the 21 *E. coli* O157:H7 isolates were characterized by pulsed field gel electrophoresis (PFGE) using the CHEF-MAP-PER System (Bio-Rad Laboratories, Hercules, CA, USA) as described by Gautom (43). Briefly, chromosomal DNA of *E. coli* O157:H7 isolates was isolated, and the inserts were digested with *Xba*I (TaKaRa Dalian, China) for 16 h at 37°C. The electrophoresis was performed at 6.0 V/cm for 18.5 h with an angle of 120 at 14°C. The pulse time was increased from 0.5 to 60 s. The *Salmonella* serotype Braenderup H9812 (ATCC BAA-664) was chosen as the molecular weight marker. Gels were then stained in ethidium bromide (1.0 mg/L). Isolates were considered to belong to the same PFGE cluster when the similarity index was >80% (44).

RESULTS

Isolation and Presence of Virulence Genes

To investigate the virulence and antibiotic resistance of *E. coli* O157:H7, we collected 2,439 samples from farms and slaughterhouses in Xinjiang regions (**Table 2**). We successfully isolated *E. coli* clones from all the feces (100%), rectal swabs (100%), and carcass swabs (100%). Studying these *E. coli* isolates, we detected that 21 isolates were the *E. coli* O157:H7 strain (19 isolates collected from cattle farms and 2 isolates obtained from one slaughterhouse). As shown in **Table 2**, the isolation rates of *E. coli* O157:H7 in feces, rectal swabs, and carcass swabs were 0.7% (8/1155), 1% (11/1236), and 4% (2/48), respectively.

Of the 21 *E. coli* O157:H7 isolates, 18 (86%) carried at least one virulence gene and 3 (14%) did not carry any (**Table 3**). Using PCR technique, we detected that seven (33%) possessed only *stx2*, seven (33%) isolates were positive for *stx1 and stx2*, and only one (5%) isolate had just *stx1* gene. The *eae* gene and *hly* gene were detected in 16 (76%) and 14 (67%) *E. coli* O157:H7 isolates, respectively. *Tccp* in combination with *hly* and *eae* was found in 12 (57%) isolates. In total, six diverse virulence profiles were determined, including *stx1*/*stx2*/*eae*/*hly*/*tccp* (seven isolates), *stx2*/*eae*/*hly*/*tccp* (five isolates), *stx2* (two isolates), *eae* (two isolates), *stx1*/*eae*/*hly* (one isolate), and *eae*/*hly* (one isolate) (**Table 3**).

Antibiotic Resistance Spectrum and Distribution of Antibiotic Resistance Genes

Studying the resistance of isolated *E. coli* O157:H7 to antibiotics, we detected that one isolate (Y4-A103) was resistant to tetracycline and carried the *tetA* gene, which encodes a tetracycline efflux pump. Y4-A109 and Y4-C21-1 were MDR isolates with the resistant patterns: AMP/CAZ/CHL/CIP/CTX/LEV/PIP/SXT/TET (Y4-A109) and AMP/CHL/CIP/CTX/LEV/PIP/SXT/TET (Y4-C21-1). In particular, the Y4-A109 was an ESBL-producing isolate carrying the *bla*$_{CTX-M-121}$ gene (**Table 3**). Although both Y4-A109 and Y4-C21-1 isolates were resistant to chloramphenicol and sulfonamides, the *cmlAI* and *sulI* genes were not detectable in these isolates, indicating other genes involved in the resistance to chloramphenicol and sulfonamides. In addition, those two MDR isolates (Y4-A109 and Y4-C21-1) simultaneously harbored five virulence genes (*stx1*/*stx2*/*eae*/*hly*/*tccp*).

Transferability of *bla*$_{CTX-M}$ Genes and Plasmid Replicon Typing

Studying transferability, we detected that the *bla*$_{CTX-M-121}$ gene of the *E. coli* O157:H7 Y4-A109 isolate was transferable to the recipient strain azide-resistant *E. coli* J53 by conjugation at a frequency of approximately 10^{-6} per donor cell after coincubation of bacteria. We also determined that the resistance of Y4-A109 to ampicillin, cefotaxime, ceftazidime, cotrimoxazole, and tetracycline was also transferable to the recipient. However, plasmid replicon carrying these resistance genes in Y4-A109 remained to be determined.

Epidemiological Typing

Overall, the genetic relatedness ranged from 66 to 100% among the 21 isolates (**Figure 1**). Furthermore, the studied isolates shared ≤80% genetic similarity to the reference strain 21530. Seventeen of the 21 isolates were grouped into two clusters using >80% similarity of the Dice coefficient. Isolates Y4-A20-1 and Y4-A41-2 (cluster II) were simultaneously isolated from different cattle at Yili in 2015 but shared identical pattern of PFGE, virulence genes, and antibiotic susceptibility (**Figure 1**). This suggests that potential pathogen transmission might occur from animals to animals within the farm. In addition, three drug-resistant isolates were all identified from Yili. However, they were genetically distantly related (<71% similarity of the Dice coefficient). Noticeably, the PFGE profiles of two isolates from the slaughterhouse were identical to those from the farms. However, Y4-C21-2 carried *stx1* gene, which was absent from its identical farm isolate, whereas Y4-C21-1 appeared to be resistant to eight drugs tested, which were not observed in its identical counterparts (**Figure 1**).

DISCUSSION

In this communication, we reported, for the first time, that the *bla*$_{CTX-M-121}$ gene was detected in *E. coli* O157:H7 isolated from cattle in Xinjiang. The *bla*$_{CTX-M-121}$ gene belongs to the *bla*$_{CTX-M-9}$ group. Rao et al. (45) reported the *bla*$_{CTX-M-121}$ gene detected in two *E. coli* isolates collected from farm ducks in China. Zhou et al. (46) identified the *bla*$_{CTX-M-121}$ gene in one *E. coli* isolated from healthy people in Guangdong Province. Jin (47) reported the *bla*$_{CTX-M-121}$ gene in chicken *E. coli* isolated from Guangdong Province. The cephalosporins are used to treat infectious disease such as bovine respiratory infection and

TABLE 2 | Sample collection and isolation of *E. coli*.

Location	Source	Farm type	Sample size and types	Numbers and rates (%) in isolation of *E. coli*	Numbers and rates (%) in isolation of *E. coli* O157:H7
Akesu	Farms	IST	354 feces	354 (100%)	4 (1%)
Bole	Farms	I	82 rectal swabs	82 (100%)	0
			43 feces	43 (100%)	0
Changji	Farms	IT	211 rectal swabs	211 (100%)	0
			46 feces	46 (100%)	0
Tacheng	Farms	IT	134 feces	134 (100%)	0
Urumqi	Farms	IST	467 rectal swabs	467 (100%)	3 (0.6%)
			90 feces	90 (100%)	0
Wujiaqu	Farms	S	79 rectal swabs	79 (100%)	0
			8 feces	8 (100%)	0
Yili	Farms	IST	480 feces	480 (100%)	4 (0.8%)
			397 rectal swabs	397 (100%)	8 (2%)
	Slaughterhouse		48 carcass swabs	48 (100%)	2 (4%)

I, industrial farm; S, semi-industrial farm; T, traditional farm.

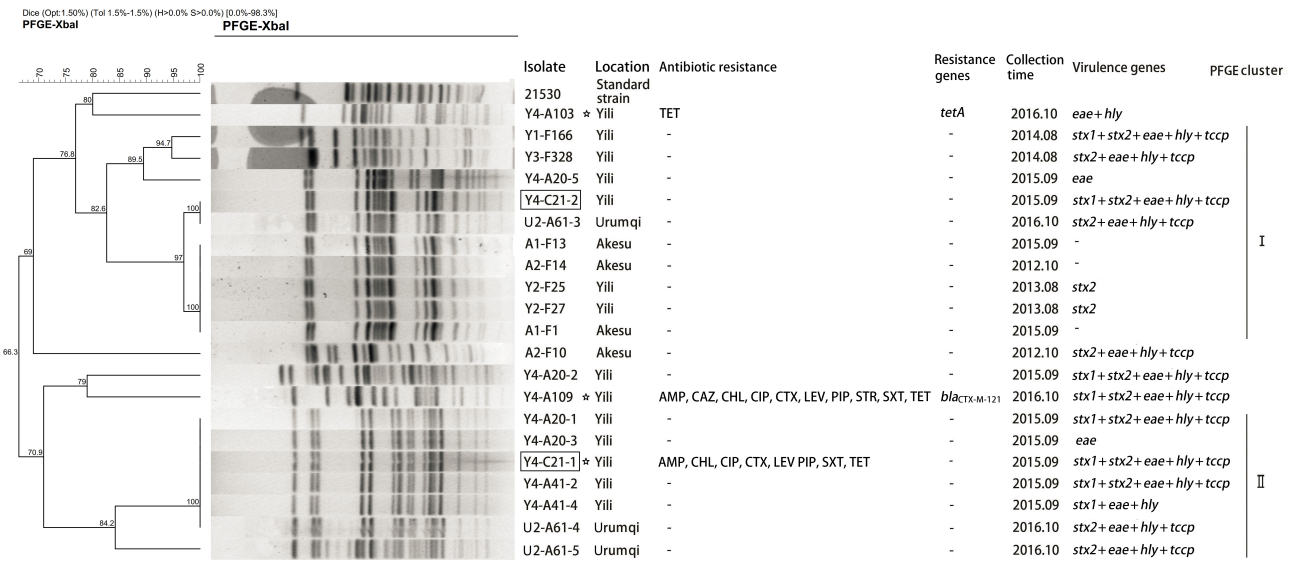
TABLE 3 | Typing antibiotic resistance and virulence genes in *E. coli* O157:H7 isolates.

Locations	Isolates	Antibiotic resistance	Resistance genes	Virulence genes
Akesu	A1-F1	–	–	–
	A1-F13		–	–
	A2-F10	–	–	*stx2*, *eae*, *hly*, *tccp*
	A2-F14	–	–	–
Urumqi	U2-A61-3		–	*stx2*, *eae*, *hly*, *tccp*
	U2-A61-4	–	–	*stx2*, *eae*, *hly*, *tccp*
	U2-A61-5	–	–	*stx2*, *eae*, *hly*, *tccp*
Yili	Y1-F166	–	–	*stx1, stx2*, *eae*, *hly*, *tccp*
	Y2-F25	–	–	*stx2*
	Y2-F27	–	–	*stx2*
	Y3-F328	–	–	*stx2*, *eae*, *hly*, *tccp*
	Y4-A20-1	–	–	*stx1, stx2*, *eae*, *hly*, *tccp*
	Y4-A20-2	–	–	*stx1, stx2*, *eae*, *hly*, *tccp*
	Y4-A20-3		–	*eae*
	Y4-A20-5	–	–	*eae*
	Y4-A41-2	–	–	*stx1, stx2*, *eae*, *hly*, *tccp*
	Y4-A41-4	–	–	*stx1, eae*, *hly*
	Y4-A103	TET	*tetA*	*eae*, *hly*
	Y4-A109	AMP, CAZ, CHL, CIP, CTX, LEV, PIP, STR, SXT, TET	$bla_{CTX-M-121}$	*stx1, stx2*, *eae*, *hly*, *tccp*
	Y4-C21-1	AMP, CHL, CIP, CTX, LEV PIP, SXT, TET	–	*stx1*, *stx2*, *eae*, *hly*, *tccp*
	Y4-C21-2	–	–	*stx1, stx2*, *eae*, *hly*, *tccp*

Resistance to Ampicillin (AMP), Ceftazidime (CAZ), Chloramphenicol (CHL), Ciprofloxacin (CIP), Cefotaxime (CTX), Levofloxacin (LVE), Piperacillin (PIP), Streptomycin (STR), Trimethoprim-sulfamethoxazole (SXT), Tetracycline (TET). –, undetectable.

mastitis, which may promote production and dissemination of β-lactamase genes (48).

Besides the transferable $bla_{CTX-M-121}$ gene between the bacterial strains via plasmid transmission, we also detected a wide spectrum of virulence genes, including the *stx1*, *stx2*, *eae*, *hly*, and *tccp* genes in *E. coli* O157:H7 isolates, which were consistent with the virulence gene types of *E. coli* O157:H7 from bovine in Jiangsu Province (49). We also detected three isolates of *E. coli* O157:H7 lacking any of these virulence genes, which was similar to the bovine *E. coli* O157:H7 isolates reported by Akomoneh et al. (50). *E. coli* O157:H7 isolates possessing only *eae* or *stx2* gene were similar to the isolates obtained from cattle in USA and milk in Nigeria, respectively (51, 52). The attendance of the *eae* gene in O157:H7 STEC



Isolate	Location	Antibiotic resistance	Resistance genes	Collection time	Virulence genes	PFGE cluster
21530	Standard strain					
Y4-A103 ☆	Yili	TET	*tetA*	2016.10	*eae+hly*	
Y1-F166	Yili	-	-	2014.08	*stx1+stx2+eae+hly+tccp*	
Y3-F328	Yili	-	-	2014.08	*stx2+eae+hly+tccp*	
Y4-A20-5	Yili	-	-	2015.09	*eae*	
Y4-C21-2	Yili	-	-	2015.09	*stx1+stx2+eae+hly+tccp*	
U2-A61-3	Urumqi	-	-	2016.10	*stx2+eae+hly+tccp*	
A1-F13	Akesu	-	-	2015.09	-	I
A2-F14	Akesu	-	-	2012.10	-	
Y2-F25	Yili	-	-	2013.08	*stx2*	
Y2-F27	Yili	-	-	2013.08	*stx2*	
A1-F1	Akesu	-	-	2015.09	-	
A2-F10	Akesu	-	-	2012.10	*stx2+eae+hly+tccp*	
Y4-A20-2	Yili	-	-	2015.09	*stx1+stx2+eae+hly+tccp*	
Y4-A109 ☆	Yili	AMP, CAZ, CHL, CIP, CTX, LEV, PIP, STR, SXT, TET	*bla*$_{CTX-M-121}$	2016.10	*stx1+stx2+eae+hly+tccp*	
Y4-A20-1	Yili	-	-	2015.09	*stx1+stx2+eae+hly+tccp*	
Y4-A20-3	Yili	-	-	2015.09	*eae*	
Y4-C21-1 ☆	Yili	AMP, CHL, CIP, CTX, LEV PIP, SXT, TET	-	2015.09	*stx1+stx2+eae+hly+tccp*	
Y4-A41-2	Yili	-	-	2015.09	*stx1+stx2+eae+hly+tccp*	II
Y4-A41-4	Yili	-	-	2015.09	*stx1+eae+hly*	
U2-A61-4	Urumqi	-	-	2016.10	*stx2+eae+hly+tccp*	
U2-A61-5	Urumqi	-	-	2016.10	*stx2+eae+hly+tccp*	

FIGURE 1 | Dendrogram of *Xba*I pulsed-field gel electrophoresis profiles of *E. coli* O157:H7 isolates. The box indicates that the strains isolated from slaughterhouse. The asterisk represents the resistant strains.

(Shiga toxin-producing *E. coli*) isolates resulted in the formation of a highly virulent subpathotype, Enterohemorrhage *E. coli* (EHEC) (53), which was observed in two MDR isolates in the present survey.

In addition, the *tet* resistance gene has been increasingly detected in bovine O157 and non-O157 STEC isolates worldwide (54–56). Our studies also revealed, for the first time, the presence of the *tetA* gene detected in bovine *E. coli* O157:H7 in Xinjiang. Horizontal gene transfer plays a key role in bacterial evolution and transmission of antibiotic resistance genes (57). Resistance traits located in genetic mobile elements, such as plasmids, transposons, and integrons, can be transferred to different strains or bacterial species (58, 59). It is conceivable that virulence gene and drug-resistance gene are carried by the same genetic element; cotransfer may occur under the selection of antibiotics to result in stable virulence clones, thereby leading to production of drug-resistant pathogenic bacteria and persistent bacterial infection in humans and food animals.

We found that the isolates obtained in the same geographical location at the same time had similar PFGE patterns and vice versa, indicating that clonal propagation in cattle population and cross-regional transmission. *E. coli* O157:H7 with identical PFGE pattern (100% similarity) carry different virulence genes and different drug resistance phenotypes, suggesting that the virulence and drug resistance carried by *E. coli* O157:H7 may be acquired or lost during the evolution and transfer of the same cluster of strains. The β-lactam-resistant *E. coli* O157:H7 may give β-lactam resistance to other pathogenic enterobacteria via plasmid-mediated conjugation, thereby posing potential challenges in the management of their associated infectious disease in cattle (60).

E. coli O157:H7 was prevalent in 2–15% population of cattle and other animals in China (47). Our results revealed that the overall isolation rate at ∼0.9% (21 of 2,439 samples) of *E. coli* O157:H7 and the *bla*$_{CTX-M}$ gene detected in 1 of 21 isolates indicated that the transmission of the *bla*$_{CTX-M}$ gene in *E. coli* O157:H7 population was at an early stage in Xinjiang. Thus, it is important to not only continuously monitor but also identify methods to intervene in the transmission of *bla*$_{CTX-M}$ genotypes to *E. coli* and other bacterial strains, thereby minimizing potential dissemination of β-lactam resistance from the cattle production to their surrounding environment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee of Xinjiang Agricultural University. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

ZS and PT conceived and designed the experiments. LZ, MZ, DW, and KM performed the experiments. YZ and YL analyzed the date. PT, LX, and JX contributed to the writing of the manuscript. All authors read and approved the article.

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Chromosomal Integration of Huge and Complex *bla*$_{NDM}$-Carrying Genetic Elements in Enterobacteriaceae

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In this study, a detailed genetic dissection of the huge and complex *bla*$_{NDM}$-carrying genetic elements and their related mobile genetic elements was performed in Enterobacteriaceae. An extensive comparison was applied to 12 chromosomal genetic elements, including six sequenced in this study and the other six from GenBank. These 12 genetic elements were divided into five groups: a novel IME Tn*6588*; two related IMEs Tn*6523* (SGI1) and Tn*6589*; four related ICEs Tn*6512* (R391), Tn*6575* (ICE*Pvu*ChnBC22), Tn*6576*, and Tn*6577*; Tn*7* and its derivatives Tn*6726* and 40.7-kb Tn*7*-related element; and two related IMEs Tn*6591* (GI*sul2*) and Tn*6590*. At least 51 resistance genes, involved in resistance to 18 different categories of antibiotics and heavy metals, were found in these 12 genetic elements. Notably, Tn*6576* carried another ICE Tn*6582*. In particular, the six *bla*$_{NDM}$-carrying genetic elements Tn*6588*, Tn*6589*, Tn*6575*, Tn*6576*, Tn*6726*, and 40.7-kb Tn*7*-related element contained large accessory multidrug resistance (MDR) regions, each of which had a very complex mosaic structure that comprised intact or residual mobile genetic elements including insertion sequences, unit or composite transposons, integrons, and putative resistance units. Core *bla*$_{NDM}$ genetic environments manifested as four different Tn*125* derivatives and, notably, two or more copies of relevant Tn*125* derivatives were found in each of Tn*6576*, Tn*6588*, Tn*6589*, and 40.7-kb Tn*7*-related element. The huge and complex *bla*$_{NDM}$-carrying genetic elements were assembled from complex transposition and homolog recombination. Firstly identified were eight novel mobile elements, including three ICEs Tn*6576*, Tn*6577*, and Tn*6582*, two IMEs, Tn*6588* and Tn*6589*, two composite transposons Tn*6580a* and Tn*6580b*, and one integron In1718.

Keywords: Enterobacteriaceae, chromosomal integration, *bla*$_{NDM}$, multidrug resistance, mobile genetic elements

INTRODUCTION

New Delhi metallo-β-lactamase (NDM) is able to hydrolyze nearly all β-lactams except aztreonam and thus mediates resistance to penicillins, cephalosporins, and carbapenems (Yong et al., 2009). It is hypothesized that the bla_{NDM} gene is originally integrated into the *Acinetobacter* chromosome from an unknown environmental species and then captured by two copies of IS*Aba125*, giving rise to IS*Aba125*-composite transposon Tn*125* (Poirel et al., 2012). With the transposition of Tn*125*, bla_{NDM} is disseminated among *Acinetobacter*, Enterobacteriaceae and *Pseudomonas* species; Tn*125* and its bla_{NDM}-carrying derivatives, with various truncations and deletions, can be found in the accessory resistance regions of bacterial plasmids or chromosomes (Wu et al., 2019). There are reports of chromosomal location of bla_{NDM} in Enterobacteriaceae species including *Escherichia coli* (Pfeifer et al., 2011; Poirel et al., 2011; Shen et al., 2017; Reynolds et al., 2019), *Providencia stuartii* (Poirel et al., 2011), *Proteus mirabilis* (Girlich et al., 2015), *Klebsiella pneumoniae* (Sakamoto et al., 2018), and *Proteus vulgaris* (Kong et al., 2020), but few of them are subjective to detailed genetic dissection of bla_{NDM}-carrying accessory resistance regions (Girlich et al., 2015; Sakamoto et al., 2018; Reynolds et al., 2019; Kong et al., 2020).

Integrative and conjugative elements (ICEs) and integrative and mobilizable elements (IMEs) (Bellanger et al., 2014; Delavat et al., 2017; Botelho and Schulenburg, 2021) are two different types of mobile genetic elements which are frequently integrated into bacterial chromosome, contributing to dissemination of resistance genes. ICEs have the ability to transfer between cells because of their self-encoded conjugation function. It is typically composed of *attL* (attachment site at the left end), *int* (integrase), *xis* (excisionase), *rlx* (relaxase), *oriT* (origin of conjugative replication), *cpl* (coupling protein), a P (TivB)- or F (TivF)-type T4SS machinery (mating pair formation), and *attR* (attachment site at the right end). IMEs are not self-transmissible, and they achieve the intercellular mobility with the help of other conjugative elements that encode proteins involved in complete conjugation function. IMEs typically have *attL*, *int*, *rlx*, *oriT*, and *attR*, but contained no conjugal transfer genes. Tn*7* is a unit transposon with the ability to integrate into bacterial chromosomes and plasmids, and it encodes five core transposition determinants TnsA and TnsB (transposases), TnsC (regulator), and TnsD and TnsE (DNA-binding proteins), as well as three TnsB-binding sites and four TnsB-binding sites at its left and right ends, respectively (Peters, 2014).

In this work, whole-genome sequencing identified four $bla_{NDM-1/-3}$-carrying genetic elements plus two additional genetic elements harboring other resistance genes in the chromosomes of four isolates of *Providencia rettgeri*, *Proteus mirabilis*, and *K. pneumoniae*. An extension sequence comparison was then applied to a collection of 12 chromosomal genetic elements (including the above six ones sequenced in this work) that could be grouped into ICEs, IMEs, and Tn*7* unit transposon and its derivatives. Data presented here gave a detailed genetic dissection of the huge and complex bla_{NDM}-carrying genetic elements and their related mobile genetic elements in multiple Enterobacteriaceae species.

MATERIALS AND METHODS

Bacterial Strains

The four chromosomal bla_{NDM}-carrying isolates (**Table S1**) were screened from more than two hundred bla_{NDM}-carrying Enterobacteriaceae isolates routinely collected from China hospitals and livestock farms. *Providencia rettgeri* 1701091 and *Proteus mirabilis* 1701092 (**Table S1**) were recovered in 2017 from the chicken intestinal contents in two different China livestock farms. *K. pneumoniae* QD23 and *Providencia rettgeri* 51003 were recovered from the urine specimens of two different patients with nosocomial infections in two Chinese public hospitals in 2015 and 2017, respectively. Bacterial species identification was performed using genome sequence-based average nucleotide identity analysis (http://www.ezbiocloud.net/tools/ani) (Richter and Rosselló-Móra, 2009).

Sequencing and Sequence Assembly

Bacterial genomic DNA was isolated using the UltraClean Microbial Kit (Qiagen, NW, Germany) and sequenced from a sheared DNA library with average size of 15 kb (ranged from 10 to 20 kb) on a PacBio RSII sequencer (Pacific Biosciences, CA, USA), as well as a paired-end library with an average insert size of 350 bp (ranged from 150 to 600 kb) on a HiSeq sequencer (Illumina, CA, USA). The paired-end short Illumina reads were used to correct the long PacBio reads utilizing *proovread* (Hackl et al., 2014), and then the corrected PacBio reads were assembled *de novo* utilizing *SMARTdenovo* (https://github.com/ruanjue/smartdenovo).

Sequence Annotation and Comparison

Open reading frames (ORFs) and pseudogenes were predicted using RAST 2.0 (https://rast.nmpdr.org/) (Brettin et al., 2015) combined with BLASTP/BLASTN searches (Boratyn et al., 2013) against the UniProtKB/Swiss-Prot database (https://web.expasy.org/docs/swiss-prot_guideline.html) (Boutet et al., 2016) and the RefSeq database (https://www.ncbi.nlm.nih.gov/refseq/) (O'Leary et al., 2016). Annotation of resistance genes, mobile elements, and other features were carried out using the online databases including CARD (https://card.mcmaster.ca/browse) (Jia et al., 2017), ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) (Zankari et al., 2012), ISfinder (https://www-is.biotoul.fr/) (Siguier et al., 2006), INTEGRALL (http://integrall.bio.ua.pt/)? (Moura et al., 2009) and Tn Number Registry (https://www.ucl.ac.uk/eastman/tn-number-registry) (Roberts et al., 2008). Multiple and pairwise sequence comparisons were performed using MUSCLE 3.8.31 (Edgar, 2004) and BLASTN, respectively. Gene organization diagrams were drawn in Inkscape 1.0 (https://inkscape.org/en/). Heatmaps were plotted with MeV 4.9.0 (Saeed et al., 2003).

Conjugal Transfer

Conjugal transfer experiments were carried out with rifampin-resistant *Escherichia coli* EC600 or sodium azide-resistant *E. coli* J53 being used as a recipient, and the 1701092 or QD23 isolate as a donor. Three milliliters of overnight cultures of each of donor and recipient bacteria were mixed together, harvested and resuspended in 80 µl of Brain Heart Infusion (BHI) broth (BD Biosciences). The mixture was spotted on a 1 cm^2 hydrophilic nylon membrane filter with a 0.45 µm pore size (Millipore) that was placed on BHI agar (BD Biosciences) plate and then incubated for mating at 37°C for 12 to 18 h. Bacteria were washed from filter membrane and spotted on Muller–Hinton (MH) agar (BD Biosciences) plates, for selecting an *E. coli* transconjugant carrying *bla*$_{NDM}$ or carrying *tetA*(C). Then 200 mg/L sodium azide (for J53) or 1,000 mg/L rifampin (for EC600), together with 4 mg/L imipenem (for *bla*$_{NDM}$) or 8 mg/L tetracycline [for *tetA*(C)] was used for transconjugant selection.

PCR Identification

All the wild-type and transconjugant strains was subjected to PCR amplification followed by amplicon sequencing, for determining the sequences of bacterial 16S rRNA genes (Frank et al., 2008), the presence of key markers such as *bla*$_{NDM}$, *tetA* (C), *int*, and *parM*, and also the location/boundary of mobile genetic elements such as Tn*6588*, Tn*6589*, Tn*6576*, Tn*6577*, and Tn*6590* (data not shown).

Phenotypic Assays

Activity of Ambler class A/B/D carbapenemases in bacterial cell extracts was determined by a modified CarbaNP test (Feng et al., 2016). Bacterial antimicrobial susceptibility was tested by BioMérieux VITEK 2 and interpreted as per the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2020).

Nucleotide Sequence Accession Numbers

The complete chromosome sequences of the 1701091, 1701092, QD23, and 51003 isolates were submitted to GenBank under accession numbers CP042860, CP042857, CP042858, and CP042861 respectively.

RESULTS

Genome Sequencing for Dissection of Chromosomal *bla*$_{NDM}$-Carrying Genetic Elements

The complete genome sequences of four *bla*$_{NDM}$-carrying isolates *Providencia rettgeri* 1701091, *Proteus mirabilis* 1701092, *K. pneumoniae* QD23, and *Providencia rettgeri* 51003 were determined in this work through high-throughput genome sequencing. A total of six chromosome-borne accessory resistance regions were identified: *bla*$_{NDM-1/-3}$-carrying Tn*6588*, Tn*6589*, Tn*6576*, and 40.7-kb Tn*7*-related element from strains 1701091, 1701092, QD23, and 51003, respectively; *tetA*(C)- and *bla*$_{CTX-M-14}$-carrying Tn*6577* were from strain 1701092; and *strAB*-carrying Tn*6590* was from strain 51003.

TABLE 1 | Major features of genetic elements characterized in this work.

Group	Genetic element	Accession number	Presence (+) or absence (–) of *bla*$_{NDM}$	Chromosomal nucleotide position	Length (bp)	Host bacterium	Reference
Novel IME	Tn*6588*	CP042860	+	4048158.4148181	100,024	*Providencia rettgeri* 1701091	This study
Tn*6523*-related IMEs	Tn*6523*	AF261825	–	Not applicable	42,451	*Salmonella enterica* Typhimurium DT104	(Boyd et al., 2000)
Tn*6512*-related ICEs	Tn*6589*	CP042857	+	4033353.4127100	93,748	*Proteus mirabilis* 1701092	This study
	Tn*6512*	AY090559	–	Not applicable	88,549	*Providencia rettgeri* 107	(Boltner et al., 2002)
	Tn*6575*	MH160822	+	Not applicable	146,895	*Proteus vulgaris* BC22	(Kong et al., 2020)
	Tn*6576*	CP042858	+	4485620.5019721	534,102	*K. pneumoniae* D23	This study
	Tn*6577*	CP042857	–	3239622.3377168	137,547	*Proteus mirabilis* 1701092	This study
Tn*7*-related elements	Tn*7*	KX117211	–	Not applicable	14,067	*E. coli* 3.5-R3	(Peters and Craig, 2001)
	Tn*6726*	AP018750	+	5052592.5228430	175,839	*K. pneumoniae* KP64	(Sakamoto et al., 2018)
	40.7-kb Tn*7*-related element	CP042861	+	31628.72403	40,776	*Providencia rettgeri* 51003	This study
Tn*6591*-related IMEs	Tn*6591*	AE014073	–	2598547.2614010	15,464	*Shigella flexneri* 2457T	(Boyd et al., 2000)
	Tn*6590*	CP042861	–	2252695.2268315	15,621	*Providencia rettgeri* 51003	This study

For each group, all the fully sequenced and non-redundant bla$_{NDM}$-carrying genetic elements available in GenBank (last accessed 15 December 2019) are included.

A detailed sequence comparison was applied to a collection of 12 chromosomal genetic elements, which included the above mentioned six genetic elements sequenced in this study, together with six additional ones from GenBank (four reference/prototype ones Tn*6523*, Tn*6512*, Tn*7*, and Tn*6591*, and two bla_{NDM}-carrying ones Tn*6575* and Tn*6726*). These 12 genetic elements could be further divided into five distinct groups: a novel IME Tn*6588*; two related IMEs Tn*6523* and Tn*6589*; four related ICEs Tn*6512*, Tn*6575*, Tn*6576* and Tn*6577*; Tn*7* and its two derivatives Tn*6726* and 40.7-kb Tn*7*-related element; and two related IMEs Tn*6591* and Tn*6590* (**Table 1**). Six (Tn*6588*, Tn*6589*, Tn*6575*, Tn*6576*, Tn*6726*, and 40.7-kb Tn*7*-related element) of them harbored bla_{NDM}. At least 51 resistance genes, involved in resistance to 18 different categories of antibiotics and heavy metals, were identified in these 12 elements (**Figure 1** and **Table S2**).

A Novel IME Tn*6588*

Tn*6588* (100.0 kb in length) was inserted into the chromosomal *orf1407* gene (cytochrome c551 peroxidase). Tn*6588* had a 9.2-kb backbone (containing *int*) with insertion of two accessory modules: IS*1A* and a 90.1-kb multidrug resistance (MDR) region, and it had terminal 35-bp *attL*/*attR* pairs and were further bracketed by 5-bp direct repeats (DRs; target site duplication signals for transposition) (**Figure 2A**). The MDR region contained a total of 19 resistance genes including bla_{NDM-1} (**Figure 1** and **Table S2**), which were located at eight different resistance loci: In1718, IS*26*–*mph*(E)–IS*26* unit, In1247, a truncated IS*CR2*–*floR* unit, IS*CR2*–*sul2* unit, IS*Ec59*–*aph(4)-Ia*–*aacC4*–IS*26* unit, ΔTn*4352* containing *aphA1*, and a 6.8-kb In27-carrying Tn*6909*-related region (**Figure 2B**).

In1718 was a concise class 1 integron with a gene cassette array (GCA) *aacA4cr*–bla_{OXA-1}–*catB3*–*arr-3*. In1247 was a complex class 1 integron containing VR1 (variable region 1=GCA: *aadA2*–*lnuF*), five copies of 6.4-kb repeat [VR2 (IS*CR1*–ble_{MBL}–bla_{NDM-1}–*arr-3* unit) plus 3′-CS2 (a second 3′-conseved segment)], and VR3 (IS*CR1*–*aphA6* unit). In1247 plus a Tn*21* core transposition module *tnpAR*–*res*–*tnpM* in this MDR region were genetically related to the Tn*3*-family unit transposon Tn*6727* (Partridge et al., 2001), which was initially found in *Proteus vulgaris* and originally associated with Tn*21*. The 6.8-kb Tn*6909*-related region looked like a truncated version of Tn*3*-family unit transposon Tn*6909* that was originally associated with Tn*1696* and Tn*21* (Partridge et al., 2001).

Two Related IMEs Tn*6523* and Tn*6589*

Tn*6523*, a 42.4-kb IME initially found in *Salmonella enterica* serovar Typhimurium DT104 (Boyd et al., 2000), had a 27.2-kb backbone (containing *attL*, *int*, *xis*, *rlx*, *oriT*, and *attR*) with insertion of a single accessory module In127. Besides the GCA (*aadA2*), In127 captured additional two resistance loci: IS*CR3*–*tetA*(G)–*floR* unit and a bla_{CARB-2}-carrying In167. The backbone of Tn*6589* was almost identical to Tn*6523* but integrated with a 66.4-kb MDR region instead of In127 (**Figure 3A**). This MDR region contained a total of 16 resistance genes including bla_{NDM-1} (**Figure 1** and **Table S2**), which were located at seven different resistance loci: In27, IS*CR3*–*tetA*(G)–*floR* unit, Tn*2*–*rmtB* region, IS*CR2*–*floR* unit, IS*Pa13*–*erm42*–IS*26* unit, *aacC2*–*tmrB* region, and In363 with a GCA *dfrA1*–*gcuC* (**Figure 3B**). In27 in this MDR region was a complex class 1 integron, which carried VR1 (GCA: *dfrA12*–*gcuF*–*aadA2*), and two copies of 11.8-kb repeat region [VR2 (IS*CR1*–ble_{MBL}–bla_{NDM-1}–*arr-3* unit) + 3′-CS2 + VR3 (IS*CR1*–*qnrA1* unit) + 3′-CS3] (**Figure 3B**).

Four Related ICEs Tn*6512*, Tn*6575*, Tn*6576*, and Tn*6577*

Tn*6512*, an 88.5-kb ICE initially found in *Providencia rettgeri* 107 (Boltner et al., 2002), was composed of an 82.4-kb backbone with insertion of three accessory modules: IS*15DI*-composite transposon Tn*6578* (containing *aphA1*), IS*Prre1* and IS*Prre2* (**Figure S1**). Tn*6512*, Tn*6575* (146.9 kb in length) (Kong et al., 2020), Tn*6576* (534.1 kb in length), and Tn*6577* (137.5 kb in

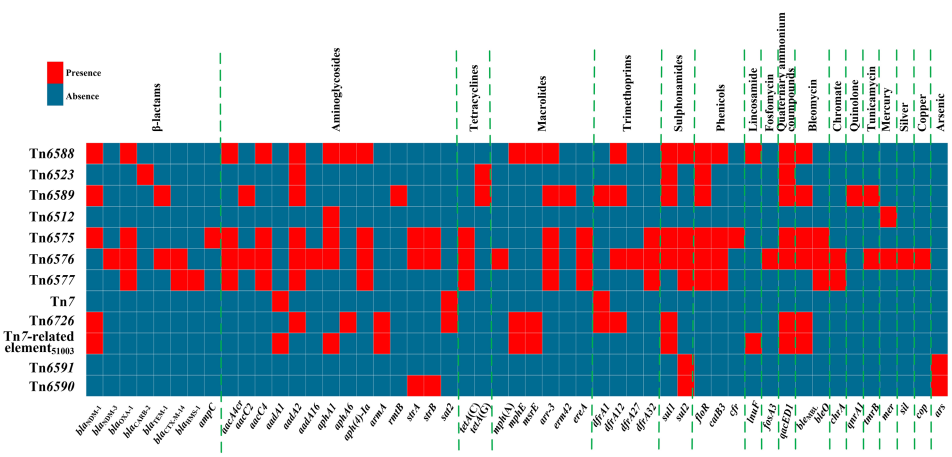

FIGURE 1 | A heatmap of prevalence of resistance genes. The original data are shown in **Table S2**.

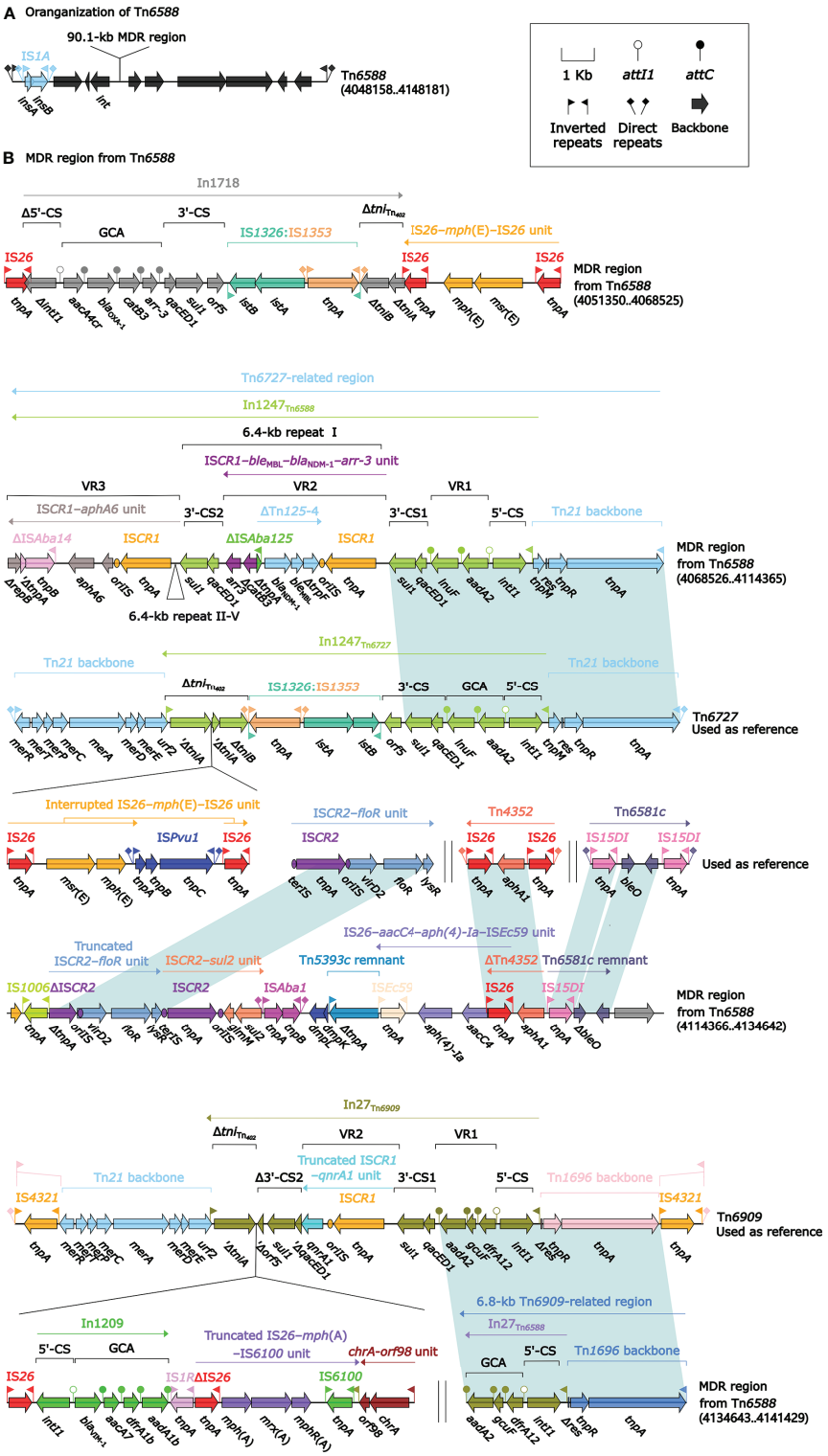

FIGURE 2 | Shown are the organization of Tn*6588* **(A)**, and MDR region from Tn*6588* **(B)**. Genes are denoted by arrows. Genes, mobile elements, and other features are colored based on their functional classification. Shading denotes regions of homology (nucleotide identity ≥95%). Numbers in brackets indicate nucleotide positions within the chromosome of strain 1701091. The accession numbers of Tn*6727*, ISCR2–*floR* unit, Tn*4352* (Wrighton and Strike, 1987), Tn*6581c*, and Tn*6909* used as reference are CP047346, CP042857, CP042858, CP042857, and CP032168, respectively.

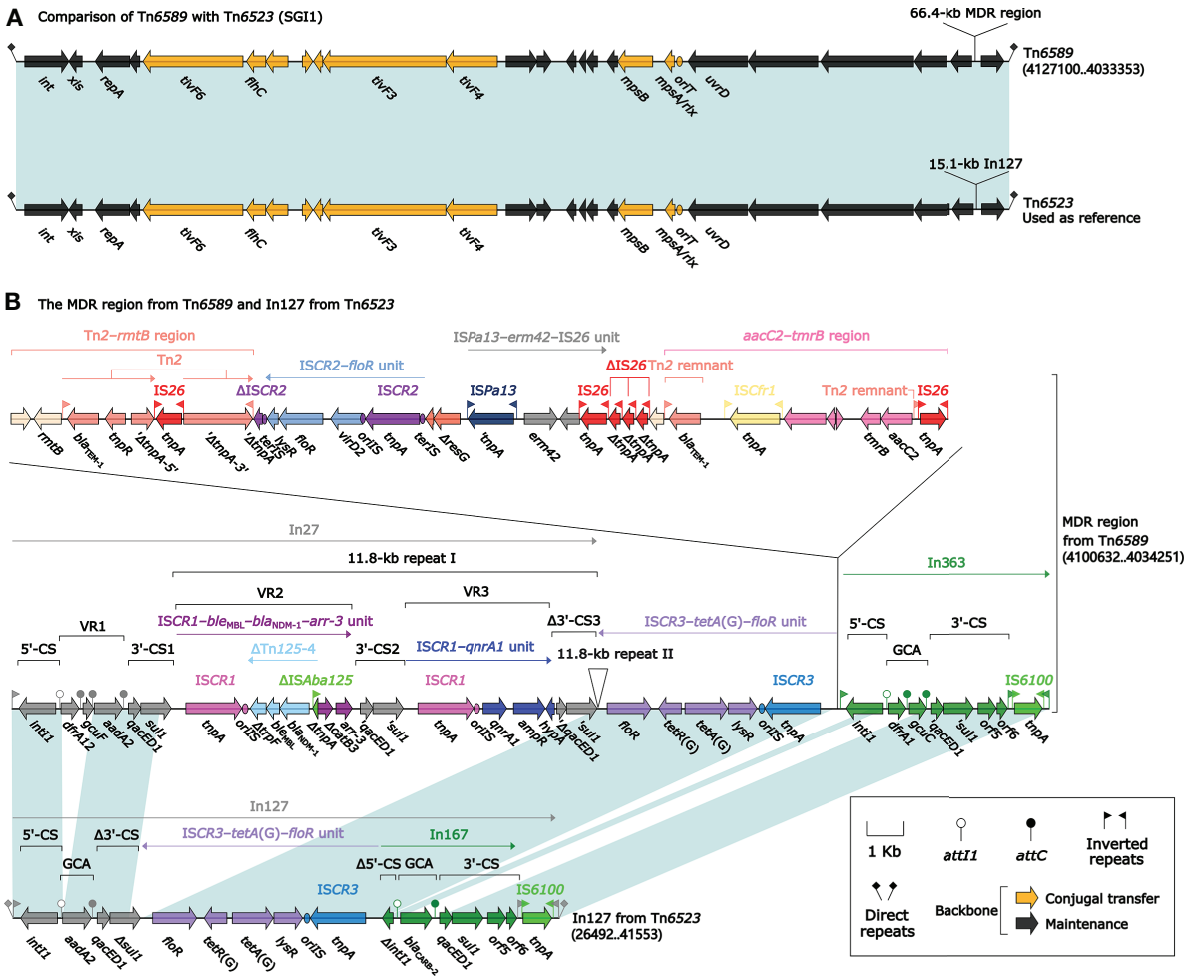

FIGURE 3 | Shown are two Tn*6523*-related transposons **(A)**, and MDR region from Tn*6589* and In127 from Tn*6523* **(B)**. Genes are denoted by arrows. Genes, mobile elements, and other features are colored based on their functional classification. Shading denotes regions of homology (nucleotide identity ≥95%). Numbers in brackets indicate nucleotide positions within Tn*6523* (Boyd et al., 2000) and the chromosome of strain 1701092. The accession number of Tn*6523* used as reference is AF261825.

length) had similar backbones and, especially, shared the core backbone genes *attL*, *int*, *xis*, *rlx*, *ori*, *cpl*, a TivF-type T4SS gene set, and *attR* (**Figure S1**). All these four ICEs were integrated at the same site within the chromosomal gene *prfC* (peptide chain release factor 3).

Each of Tn*6575*, Tn*6576*, and Tn*6577* had two accessory modules. Firstly, a 85.0-kb MDR region, a novel 406.4-kb ICE Tn*6582*, and a 11.1-kb *bla*$_{HMS}$-*sul2* region were inserted at the same site within *umuC* of Tn*6575*, Tn*6576*, and Tn*6577*, respectively, which led to different truncations of surrounding backbone regions of Tn*6575* and Tn*6577* but not Tn*6576*. Secondly, IS*Ppu12*, Tn*6580b* (55.2 kb in length), and Tn*6580a* (50.0 kb in length) were integrated at the same site downstream of *orf714* of Tn*6575*, Tn*6576*, and Tn*6577*, respectively (**Figure S1**).

The 85.0-kb MDR region of Tn*6575* contained a total of 22 resistance genes including *bla*$_{NDM-1}$ (**Figure 1** and **Table S2**), which were located at 12 different resistance loci: two copies of a truncated IS*Ec59*–*aph(4)-Ia*–*aacC4*–IS*26* unit, an unnamable In element (harboring a long GCA *aacA4cr*–*bla*$_{OXA-1}$–*catB3*–*arr-3*–*aacA4cr*–*arr-3* but lacking the whole 5′-CS), *aphA1*-containing Tn*4352*, IS*26*–*cfr*–IS*26* unit, IS*26*-composite transposon Tn*6581a* containing *bleO*, In525 (GCA: *dfrA32*–*ereA*–*aadA2*), *tetA*(C)-containing ΔTn*6309*, *bla*$_{NDM-1}$-containing ΔTn*125*-2, a 6.6-kb In0-carrying Tn*6911*-related region, a truncated IS*CR2*–*floR* unit, a truncated IS*CR2*–*sul2* unit, and *strAB*-containing ΔTn*5393c* (**Figure 4**). The Tn*6911*-related region in this MDR region looked like a truncated version of Tn*3*-family unit transposon Tn*6911* that was originally associated with Tn*21* (Partridge et al., 2001).

Tn*6582* had a complete set of core ICE backbone determinants and, moreover, a lot of accessory modules: two MDR (MDR-1 and MDR-2) regions, *sil*–*cop* region as found in IncHI2 plasmid R478 (Gilmour et al., 2004), *floR*–*strAB*–*sul2*

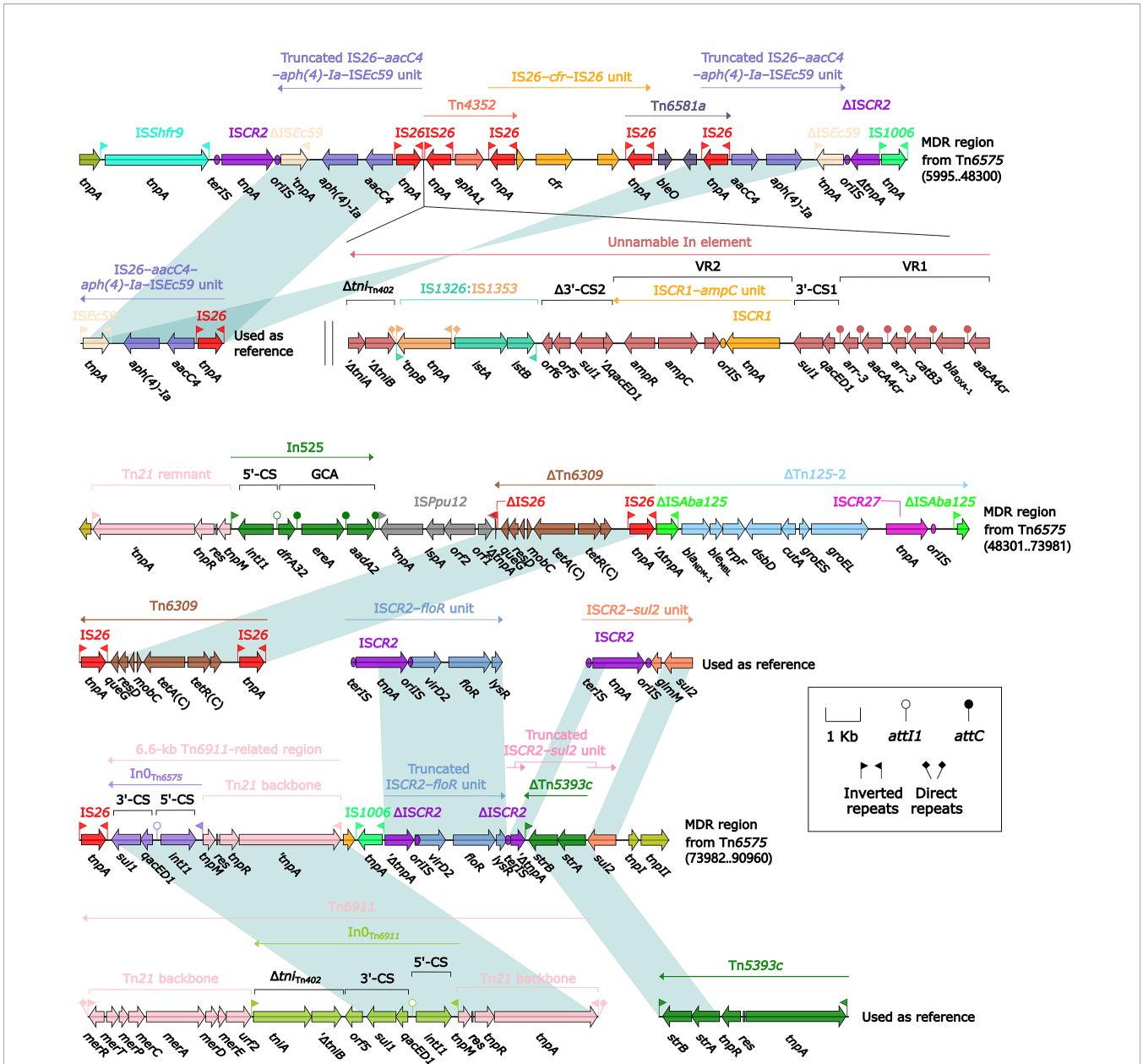

FIGURE 4 | Organization of MDR region from Tn*6575*, and comparison to related regions. Genes are denoted by arrows. Genes, mobile elements, and other features are colored based on their functional classification. Shading denotes regions of homology (nucleotide identity ≥95%). Numbers in brackets indicate nucleotide positions within Tn*6575*. The accession numbers of IS*26*–*aacC4*–*aph(4)-Ia*–IS*Ec59* unit, Tn*6309* (Sun et al., 2016), Tn*6911*, Tn*5393c* (L'Abée-Lund and Sørum, 2000), IS*CR2*–*floR* unit, IS*CR2*–*sul2* unit used as reference are CP042857, KX710094, KT033469, AF262622, CP042857, and AE014073, respectively.

region (containing ΔTn*5393c* and a truncated IS*CR2*–*floR* unit), and multiple intact or residue IS elements (**Figure S2**). The 38.9-kb MDR-1 region (**Figure 5**) comprised ΔTn*6029* (containing *sul2* and *strAB*), Tn*6581b*, an unnamable In37-like element (harboring the In37 GCA *aacA4cr*–*bla*$_{OXA-1}$–*catB3*–*arr-3* but lack of the whole 5′-CS), In27 (GCA: *dfrA12*–*gcuF*–*aadA2*), and two copies of 11.0-kb repeat (each copy harbored a truncated *aacC2*–*tmrB* region, Tn*4352*, IS*26*–*mph*(A)–IS*6100* unit, and *chrA*-*orf98* unit). The 43.7-kb MDR-2 region (**Figure 6**) comprised ΔTn*21* (containing *mer*), *aacC2*–*tmrB* region, and two copies of 21.4-kb repeat (each copy harbored IS*26*–*mph*(A)–IS*6100* unit, *chrA*-*orf98* unit, In1021, and a truncated *aacC2*–*tmrB* region). In1021 was a complex class 1 integron containing VR1 (GCA: *aacA4cr*–*arr3*–*dfrA27*–*aadA16*) and VR2 (IS*CR1*–*ble*$_{MBL}$–*bla*$_{NDM-3}$–*arr-3* unit).

The IS*Ppu12*-composite transposon Tn*6580a* contained a total of 17 resistance genes (**Figure 1** and **Table S2**), which were located at nine different resistance loci: In525 (GCA:

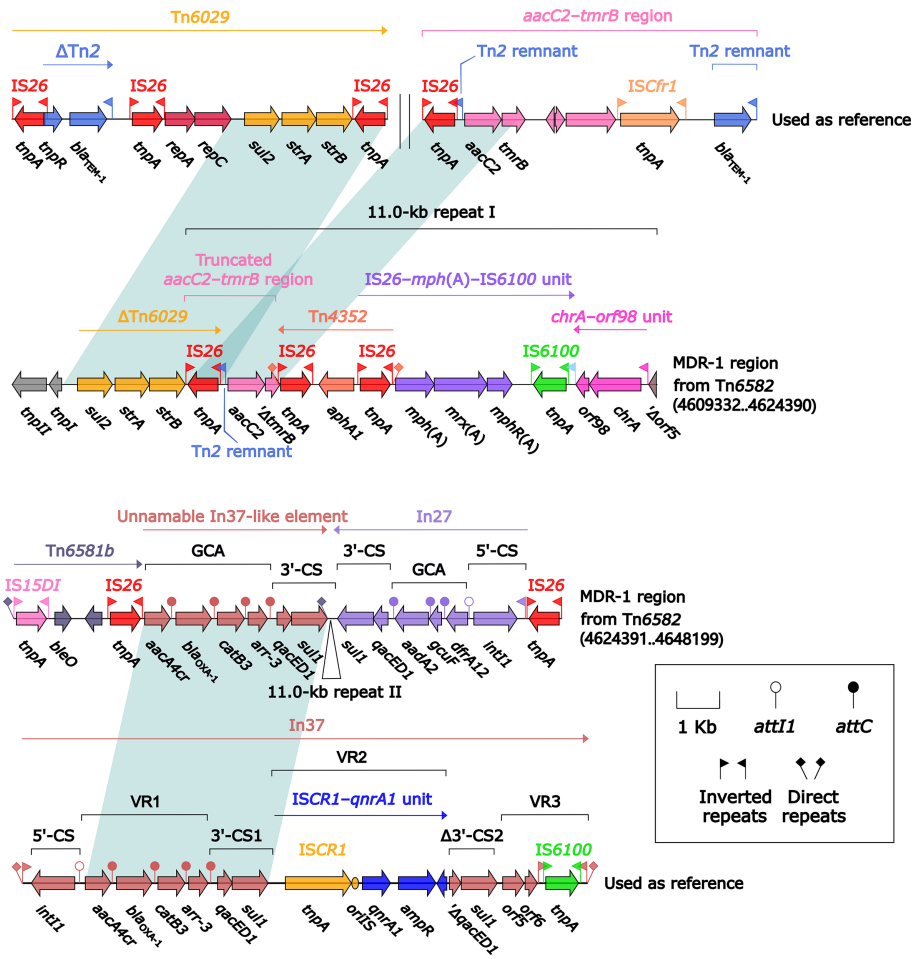

FIGURE 5 | Organization of MDR-1 region from Tn*6582*, and comparison to related regions. Genes are denoted by arrows. Genes, mobile elements, and other features are colored based on their functional classification. Shading denotes regions of homology (nucleotide identity ≥95%). Numbers in brackets indicate nucleotide positions within the chromosome of strain QD23. The accession numbers of Tn*6029* (Cain et al., 2010), *aacC2–tmrB* region (Partridge et al., 2012), and In37 (Wang et al., 2003) used as reference are GQ150541, JX101693, and AY259086, respectively.

dfrA32–ereA–aadA2), a truncated *chrA–orf98* unit, Tn*6581c*, the unnamable In37-like element as described above, IS*Ec59–aph(4)-Ia–aacC4*–IS*26* unit, IS*CR2–sul2* unit, IS*CR2–floR* unit, *bla*$_{CTX-M-14}$-containing ΔTn*6503a*, and *tetA*(C)-containing ΔTn*6309* (**Figure 7**). Three major modular differences were recognized in Tn*6580b* relative to Tn*6580a*: i) replacement of Tn*6581c* by Tn*6581b*; ii) inversion of the unnamable In37-like element; and iii) insertion of IS*26–fosA3*–IS*26* unit together with Tn*4352* at a site between ΔTn*6503a* and ΔTn*6309*.

Three Related Unit Transposons Tn*7*, Tn*6726*, and the 40.7-kb Tn*7*-Related Element

All of Tn*7* (14.1 kb in length), Tn*6726* (175.8 kb in length) (Sakamoto et al., 2018), and 40.7-kb Tn*7*-related element were integrated at a site downstream of the chromosomal gene *glmS* (glutamine-fructose-6-phosphate aminotransferase). Tn*6726* differed from Tn*7* by acquisition of In2-3 (GCA: dfrA1) instead of In2-4 (GCA: *dfrA1–aadA1*) and, moreover, a 162.6-kb IS*Kpn26*-composite transposon Tn*6728* was inserted at a site within *intI2* of In2-3 (**Figure 8**). Tn*6728* harbored a 40.9-kb MDR region as well as an array of IncHI3 core backbone genes (**Figure S3**). This 40.9-kb MDR region included a 9.4-kb Tn*6909*-related region together with ΔTn*1548* (these two shared In27), a truncated IS*Aba14–aphA6*–IS*Aba14* unit, and *bla*$_{NDM-1}$-carrying ΔTn*125*-1 (**Figure 9**).

Compared with Tn*7*, 40.7-kb Tn*7*-related element underwent two insertion events: i) *tnsABCDE* was truncated by insertion of a 33.6-kb MDR region; and ii) *tnsB* was interrupted by insertion of IS*Prst3*; this element could not be discriminated as an intact Tn*7*-like transposon due to the presence of an incomplete *tnsABCDE* module (**Figure 8**). This 33.6-kb MDR region contained In2-16, ΔTn*1548*, and *aphA1*-carrying ΔTn*4352*. In2-16 carried VR1 (GCA: *lnu*(F)–*dfrA1–aadA1a*) and two

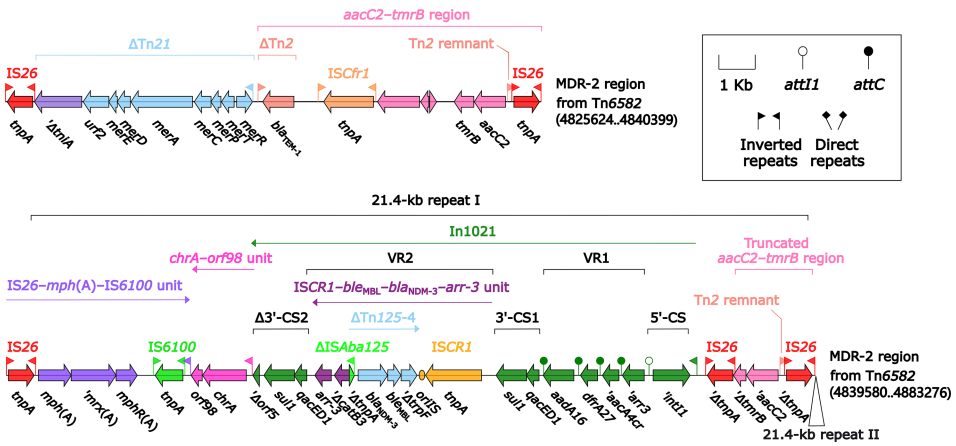

FIGURE 6 | Organization of MDR-2 region from Tn*6582*. Genes are denoted by arrows. Genes, mobile elements, and other features are colored based on their functional classification. Shading denotes regions of homology (nucleotide identity ≥95%). Numbers in brackets indicate nucleotide positions within the chromosome of strain QD23.

copies of 5.6-kb repeat [VR2 (IS*CR1*–*ble*$_{MBL}$–*bla*$_{NDM-1}$ unit) plus Δ3′-CS2] (**Figure 10**).

IS*Ec29*–*mph*(E)–*IS26*/IS*15DI* unit and IS*CR1*–*armA* unit were presented in both ΔTn*1548* from Tn*6728* and that from 40.7-kb Tn*7*-related element, whereas In27 was found in the former ΔTn*1548* rather than the later one; in addition, IS*Ec29*–*mph*(E)–*IS26*/IS*15DI* unit was interrupted by insertion of two different IS elements IS*Kpn21* and IS*Aba24*, respectively, in these two ΔTn*1548* (**Figures 9** and **10**).

Two Related IMEs Tn*6591* and Tn*6590*

The 15.5-kb IME Tn*6591* (GI*sul2*) (Wei et al., 2003), initially found in *Shigella flexneri* 2457T, was integrated into the chromosomal gene *guaA* (glutamine-hydrolyzing GMP synthase) and had a 12.6-kb backbone (containing *attL*, *int*, *oriT* and *attR*) with insertion of a single accessory module IS*CR2*-*sul2* unit (**Figure 11**). Tn*6590* (15.6 kb in length) was integrated at the same chromosomal site, and Tn*6590* differed from Tn*6591* by only truncation of IS*CR2*–*sul2* unit due to insertion of *strAB*-carrying ΔTn*5393c*.

Plasmids of the Four Strains Sequenced in This Study

Proteus mirabilis 1701092 carried no plasmids, and all accessory resistance regions (Tn*6577* and Tn*6589*) were located in the chromosome. Besides chromosome-borne accessory resistance regions, an IncFII plasmid p701091-FII (carrying no resistance genes), an IncI plasmid pQD23-CTXM [harboring *bla*$_{CTX-M-104}$ and *erm*(B)], and an IncFII plasmid p51003-FII (containing *bla*$_{TEM-1B}$ and *bla*$_{CTX-M-3}$) together with an Col3M plasmid p51003-qnrD (having *qnrD*) were identified in *Providencia* spp. 1701091, *K. pneumoniae* QD23, and *Providencia* spp. 51003, respectively. Coexistance of a large array of resistance genes in both chromosome and plasmids of a single bacterial isolate makes it tends to become extensively resistant.

Transferability and Antimicrobial Susceptibility

This work identified three ICEs Tn*6577*, Tn*6582* and Tn*6576* in total, all of which had essential conjugal transfer genes. Notably, Tn*6582* was located within Tn*6576*. As for conjugation experiments, Tn*6577* was transferred from the wild-type isolate (susceptible to rifampin) into *E. coli* EC600, generating the transconjugant Tn*6577*–TETA(C)–EC600; Tn*6582* could be transferred from the wild-type isolate (non-susceptible to rifampin but susceptible to sodium azide) in *E. coli* J53 to obtain Tn*6582*–NDM–J53, but repeated conjugation attempts failed to transfer Tn*6576* into *E. coli* J53. Tn*6577*–TETA(C)–EC600 was highly resistant to tetracycline and ceftriaxone owing to presence of *tetA*(C) and *bla*$_{CTX-M-14}$. Tn*6582*–NDM–J53 was highly resistant to ceftriaxone and imipenem resulted from production of NDM enzyme (data not shown). The Ambler class B carbapenemase activity was detected in Tn*6582*–NDM–J53 and its wild-type isolate.

DISCUSSION

Since the *bla*$_{NDM}$ gene was initially identified in India in 2009 (Yong et al., 2009), it spread rapidly all over the world (Dortet et al., 2014). Although *bla*$_{NDM}$ was initially discovered in a plasmid of *K. pneumoniae* (Yong et al., 2009), the chromosomal location of *bla*$_{NDM}$ in Enterobacteriaceae species has been reported in recent years (Pfeifer et al., 2011; Poirel et al., 2011; Girlich et al., 2015; Shen et al., 2017; Sakamoto et al., 2018; Reynolds et al., 2019; Kong et al., 2020). There were few reports related to a detailed genetic dissection of different kinds of *bla*$_{NDM}$-carrying accessory resistance regions in the chromosomes (Girlich et al., 2015; Sakamoto et al., 2018; Reynolds et al., 2019; Kong et al., 2020), but none of them had

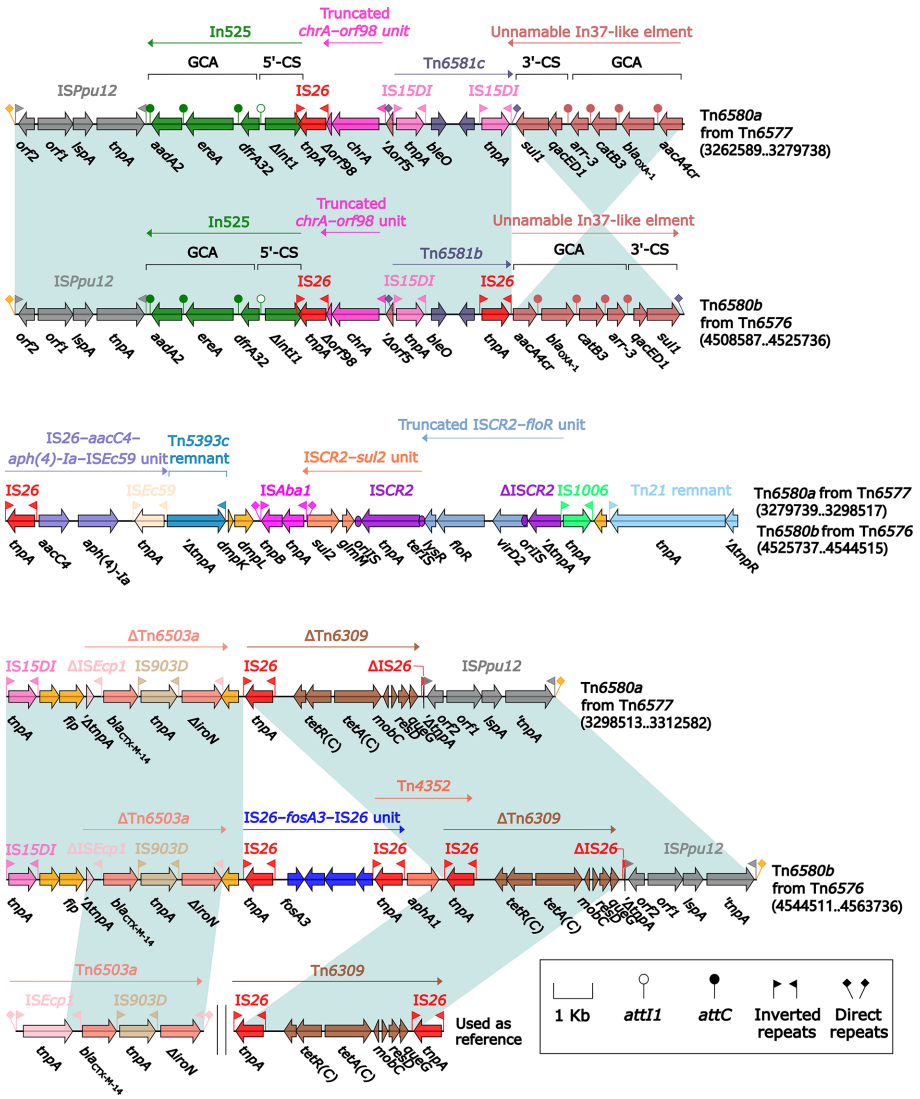

FIGURE 7 | Comparison of Tn*6580a* from Tn*6577* and Tn*6580b* from Tn*6576*, and related regions. Genes are denoted by arrows. Genes, mobile elements, and other features are colored based on their functional classification. Shading denotes regions of homology (nucleotide identity ≥95%). Numbers in brackets indicate nucleotide positions within the chromosomes of strains 1701092 and QD23. The accession numbers of Tn*6503a* (Feng et al., 2015) and Tn*6309* (Sun et al., 2016) used as reference are KP987215 and KX710094, respectively.

a systematic summary for these *bla*$_{NDM}$-carrying mobile genetic elements.

Data presented here involved a total of six chromosomal *bla*$_{NDM}$-carrying genetic elements Tn*6575*, Tn*6726*, Tn*6588*, Tn*6589*, Tn*6576*, and 40.7-kb Tn*7*-related element, and the last four were sequenced in this work. These six genetic elements belonged to three different categories: ICEs (Tn*6575* and Tn*6576*), IMEs (Tn*6588* and Tn*6589*), and two derivatives (Tn*6726* and 40.7-kb Tn*7*-related element) of Tn*7* unit transposon. Notably, Tn*6576* carried another ICE Tn*6582*. These ICEs and IMEs would have the intercellular self-mobility as they carried essential conjugal transfer genes (Bellanger et al., 2014; Botelho and Schulenburg, 2021). Tn*6726* would have the intracellular mobility as it had a complete core transposition module *tnsABCDE*, while 40.7-kb Tn*7*-related element would loss its mobility due to lesion in *tnsABCDE*.

Tn*6512*-related ICEs were frequently reported in *Vibrio*, *Proteus*, and *Shewanella* (Burrus et al., 2006; Nonaka et al., 2012; Lei et al., 2016; Fang et al., 2018). Tn*6575* and Tn*6576* were the only two *bla*$_{NDM}$-carrying Tn*6512*-related ICEs (last accessed 15 December 2019). Tn*6523*-related IMEs were frequently reported in *Salmonella* and *Proteus mirabilis* (Hall, 2010; Siebor and Neuwirth, 2013; Sung et al., 2017). This study presented Tn*6589*, the first *bla*$_{NDM}$-carrying Tn*6523*-related IME. Tn*7*, and its derivatives had the ability to integrate into

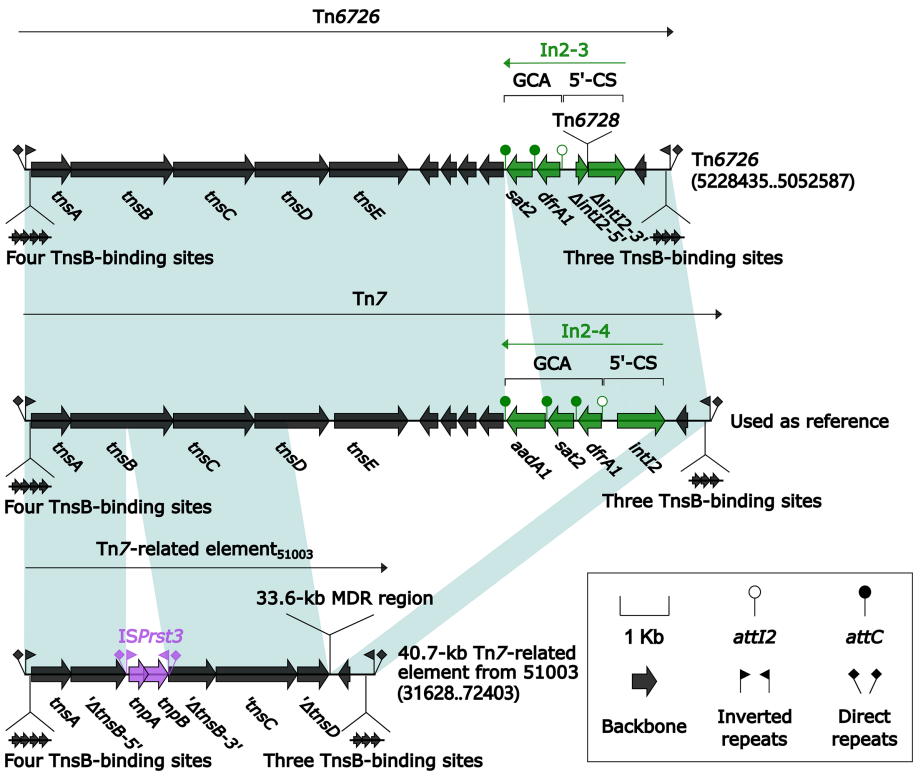

FIGURE 8 | Comparison of Tn*7* and its two derivatives Tn*6726* and 40.7-kb Tn*7*-related element. Genes are denoted by arrows. Genes, mobile elements, and other features are colored based on their functional classification. Shading denotes regions of homology (nucleotide identity ≥95%). Numbers in brackets indicate nucleotide positions within the chromosomes of strains KP64 and 51003. The accession number of Tn*7* used as reference is KX117211.

bacterial plasmids and chromosomes (Peters, 2014). There were several reports of Tn*7* derivatives located in bacterial chromosomes (Chen et al., 2018; Chen et al., 2019). To date, Tn*6726* and 40.7-kb Tn*7*-related element were the only two *bla*$_{NDM}$-carrying Tn*7* derivatives integrated into chromosomes. Different to 40.7-kb Tn*7*-related element, Tn*6726* carried a series of backbone genes of IncHI3 plasmid, which means that *bla*$_{NDM}$ together with its surrounding genetic environment in Tn*6726* might be originated from a IncHI3 plasmid. In summary, Tn*6512*-related ICEs, Tn*6523*-related IMEs, and Tn*7* derivatives recently began to be a reservoir of *bla*$_{NDM}$ genes in Enterobacteriaceae.

Each of these six *bla*$_{NDM}$-carrying genetic elements had large accessory resistance regions: i) Tn*6575*, Tn*6588*, Tn*6589*, and 40.7-kb Tn*7*-related element; each had a single MDR region, 85.0 kb, 90.1 kb, 66.4 kb, and 33.8 kb in length, respectively; ii) Tn*6726* contained a 162.6-kb IS*Kpn26*-composite transposon Tn*6728* integrated with a 40.9-kb MDR region; and iii) Tn*6576* harbored a 406.4-kb ICE Tn*6582* (containing two distinct MDR-1 and MDR-2 regions, 38.9 kb and 43.7 kb in length, respectively), and additionally a 55.2-kb IS*Ppu12*-composite Tn*6580b* that as a whole could be considered as a MDR region. Each of these large MDR regions had a very complex mosaic structure, which was composed of intact or residue mobile genetic elements including ISs, unit or composite transposons, integrons and putative resistance units, and likely assembled from complex transposition and homologous recombination.

Four different Tn*125* derivatives, namely ΔTn*125*-1, ΔTn*125*-2, ΔTn*125*-3, and ΔTn*125*-4 (**Figure 12**), were identified from the relevant MDR regions of these six *bla*$_{NDM}$-carrying genetic elements. ΔTn*125*-1 from Tn*6726* and ΔTn*125*-2 from Tn*6575* highly resembled the prototype Tn*125*: ΔTn*125*-1 resulted from the insertion of IS*Ec33* into the left copy of IS*Aba125*, while terminal truncation of both copies of IS*Aba125* generated ΔTn*125*-2. It was thought that ΔTn*125*-1 and ΔTn*125*-2 were generated from transposition of Tn*125* into Tn*6726* and Tn*6575*, followed by further modular modifications such as insertion and truncation. ΔTn*125*-3 from 40.7-kb Tn*7*-related element and ΔTn*125*-4 from Tn*6588*, Tn*6589* and Tn*6576* had very short *bla*$_{NDM-1/-3}$-carrying structures. ΔTn*125*-3 or ΔTn*125*-4 was captured by IS*CR1*, generating IS*CR1*–*ble*$_{MBL}$–*bla*$_{NDM-1}$ or IS*CR1*–*ble*$_{MBL}$–*bla*$_{NDM-1/-3}$–*arr-3* unit, respectively. Furthermore, the former unit was integrated into In2-16 (**Figure 10**) while the later one into In1247$_{Tn6588}$ (**Figure 2**), In27$_{Tn6589}$ (**Figure 3**) and In1021$_{Tn6576}$ (**Figure 6**), manifesting as the VR2 regions of these integrons. Notably, two or more copies of *bla*$_{NDM-1/-3}$ genes were found in each of Tn*6576*, Tn*6588*, Tn*6589*, and 40.7-kb Tn*7*-related element, which resulted from the presence of multiple ≥5.6-kb repeats (each harboring a

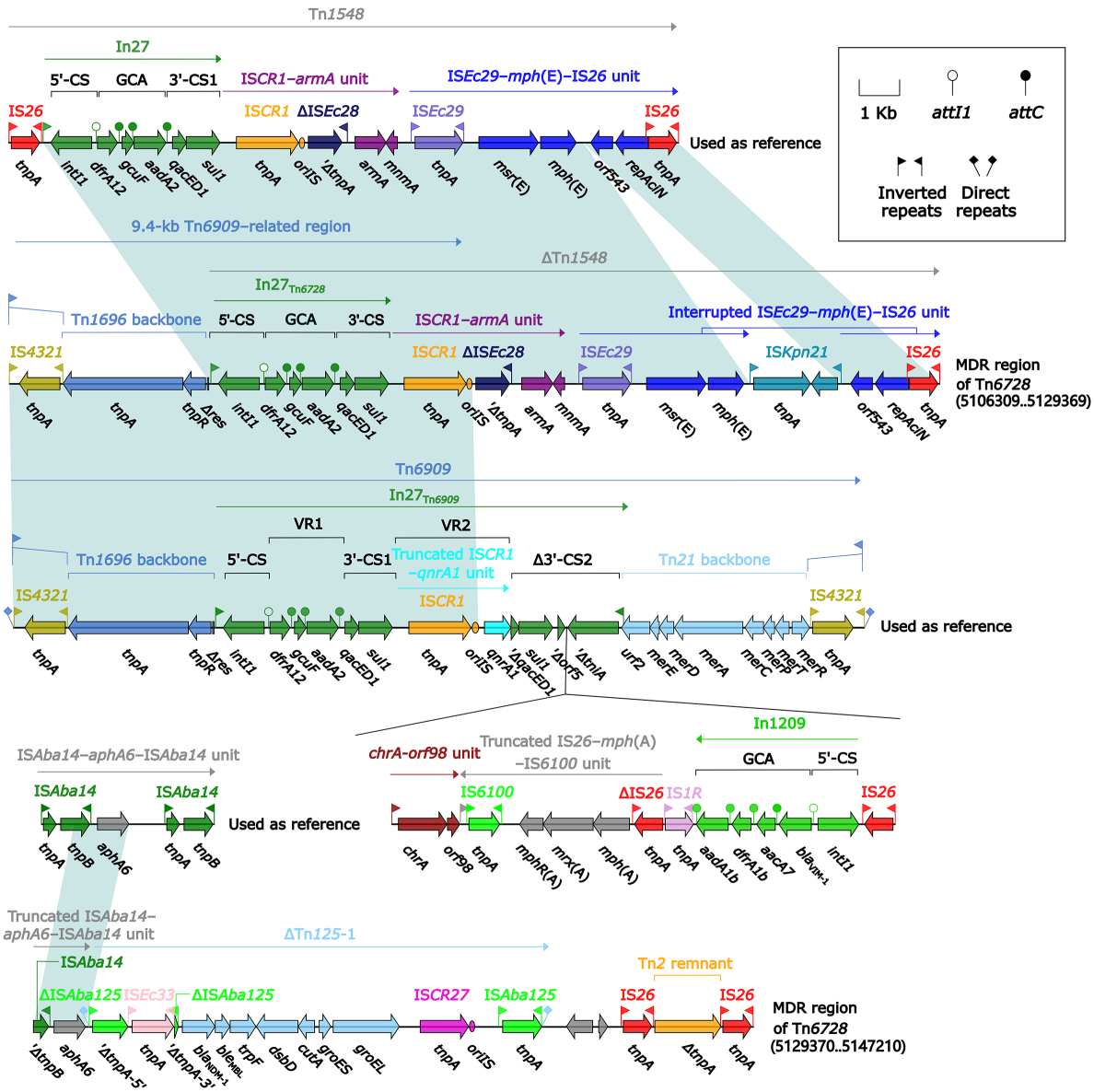

FIGURE 9 | Organization of MDR region from Tn*6728*, and comparison to related regions. Genes are denoted by arrows. Genes, mobile elements, and other features are colored based on their functional classification. Shading denotes regions of homology (nucleotide identity ≥95%). Numbers in brackets indicate nucleotide positions within the chromosome of strain KP64. The accession numbers of Tn*1548* (Galimand et al., 2005), Tn*6909*, and IS*Aba14*–*aphA6*–IS*Aba14* unit used as reference are AF550415, CP032168, and CP046406, respectively.

Tn*125* derivative and the other components) in these four genetic elements.

Multiple copies of *bla*$_{NDM}$ located in a single plasmid or chromosome were reported in previous studies (Jovcić et al., 2013; Shen et al., 2017; Feng et al., 2018), and all these *bla*$_{NDM}$ genes were around IS*CR1*. Similarly, Tn*6576*, Tn*6588*, Tn*6589*, and 40.7-kb Tn*7*-related element in this study also contained IS*CR1*-around *bla*$_{NDM}$ genes. It was confirmed that IS*CR1* captured adjacent genes (frequently including antibiotic resistance genes) at the end of its initiation of replication (*ori*IS) through rolling-circle transposition (Toleman et al., 2006). Our sequencing data suggested that IS*CR1* might experience multiple rounds of capturing *bla*$_{NDM}$ and further integrating *bla*$_{NDM}$ into the integrons, resulting in the presence of multiple copies of IS*CR1*-accociated *bla*$_{NDM}$ genes in a single genetic element.

There were eight novel (firstly identified in this study) mobile genetic elements, including three ICEs Tn*6576*, Tn*6577*, and Tn*6582*, two IMEs Tn*6588* and Tn*6589*, two composite transposons Tn*6580a* and Tn*6580b*, and one integron In1718. Additional 12 genetic elements (IME: Tn*6590*; composite

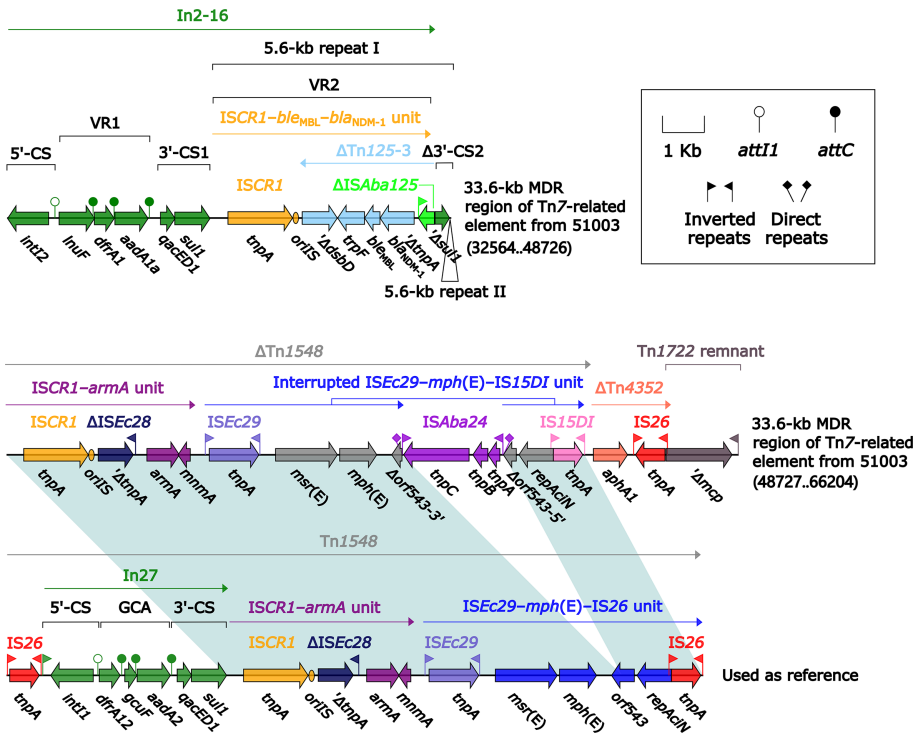

FIGURE 10 | Organization of MDR region from 40.7-kb Tn*7*-related element, and comparison to related regions. Genes are denoted by arrows. Genes, mobile elements, and other features are colored based on their functional classification. Shading denotes regions of homology (nucleotide identity ≥95%). Numbers in brackets indicate nucleotide positions within the chromosome of strain 51003. The accession number of Tn*1548* (Galimand et al., 2005) used as reference is AF550415.

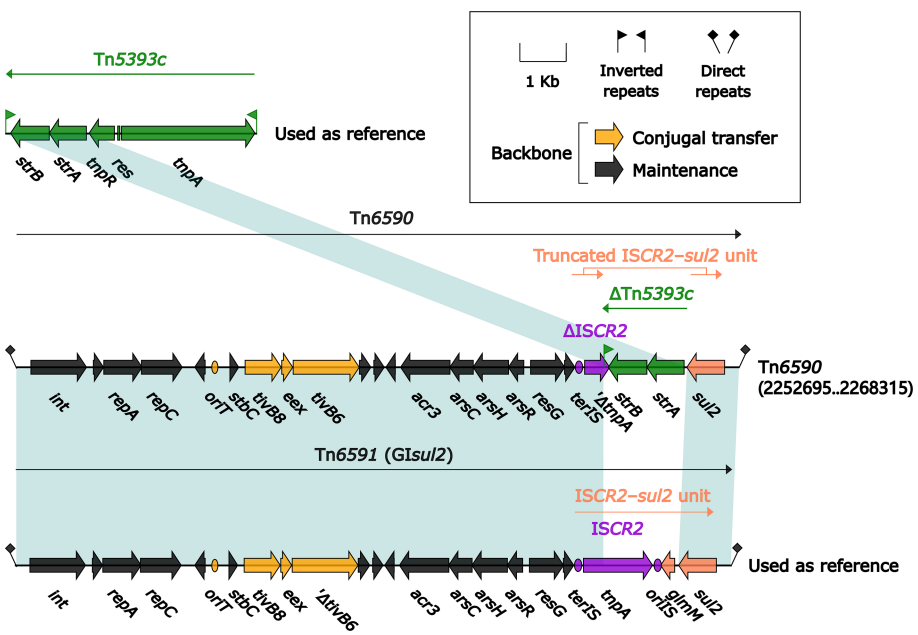

FIGURE 11 | Organization of two related IMEs Tn*6590* and Tn*6591*, and comparison to related regions. Genes are denoted by arrows. Genes, mobile elements, and other features are colored based on their functional classification. Shading denotes regions of homology (nucleotide identity ≥95%). Numbers in brackets indicate nucleotide positions within the chromosome of strain 51003. The accession number of Tn*5393c* (L'Abée-Lund and Sørum, 2000) used as reference is AF262622.

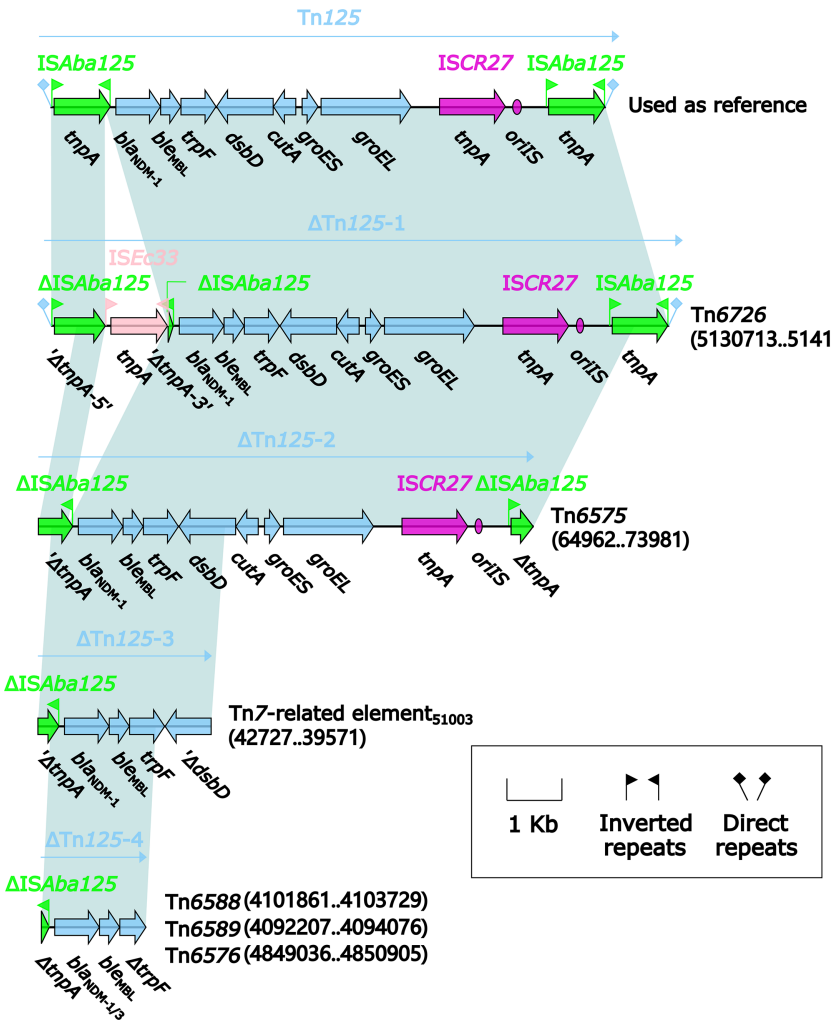

FIGURE 12 | Comparison of Tn*125* and its four derivatives. Genes are denoted by arrows. Genes, mobile elements, and other features are colored based on their functional classification. Shading denotes regions of homology (nucleotide identity ≥95%). Numbers in brackets indicate nucleotide positions within Tn*6575* and the chromosomes of strains KP64, 51003, 1701091, 1701092, and QD23, respectively. The accession number of Tn*125* (Poirel et al., 2012) used as reference is JN872328.

transposons: Tn*6578*, Tn*6581a*, Tn*6581b*, Tn*6581c*, and Tn*6728*; unit transposons: Tn*6726*, Tn*6727*, Tn*6909*, and Tn*6911*; IS: IS*Pvu1*; and 40.7-kb Tn*7*-related element) were newly designated (firstly designated in this study, but with previously determined sequences). The four previously designated ICEs/IMEs SGI1, R391, ICE*Pvu*ChnBC22, and GI*sul2* were renamed as standard Tn designations Tn*6523*, Tn*6512*, Tn*6575*, and Tn*6591*, respectively. All the putative resistance units presented in this work were annotated and collected in a custom and yet unpublished database.

CONCLUSION

This study dealt with an extensive sequence comparison of 12 chromosomal genetic elements, including six *bla*$_{NDM}$-carrying ones. All these *bla*$_{NDM}$-carrying genetic elements had huge and complex MDR regions. The core *bla*$_{NDM}$ genetic environments manifested as four different Tn*125* derivatives. Notably, two or more copies of *bla*$_{NDM}$ were found in each of the four genetic elements. Egiht novel mobile elements were firstly identified, including three ICEs Tn*6576*, Tn*6577*, and Tn*6582*, two IMEs Tn*6588* and Tn*6589*, two composite transposons Tn*6580a* and Tn*6580b*, and one integron In1718. This study would provide a deeper genetic insight into the chromosomal integration of *bla*$_{NDM}$-carrying genetic elements in Enterobacteriaceae.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the complete chromosomal nucleotide sequences of 1701091, 1701092, QD23 and 51003, which were submitted to GenBank under accession numbers CP042860, CP042857, CP042858 and CP042861, respectively.

ETHICS STATEMENT

This study uses the bacterial isolates obtained from the Chinese livestock farm and public hospitals as listed in **Table S1**. The local legislation did not require the study to be reviewed or approved by an ethics committee, because the bacterial isolates involved in this study was part of the routine laboratory procedures. The research involving biohazards and all related procedures were approved by the Biosafety Committee of the Beijing Institute of Microbiology and Epidemiology.

AUTHOR CONTRIBUTIONS

DZ and HY conceived the study and designed experimental procedures. XL, YJ, and FC performed the experiments. XL, XJ, and LZ analyzed the data. LH, DW and YS contributed to reagents and materials. XL and ZY wrote the original draft. DZ and HY reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.690799/full#supplementary-material

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REVIEW
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Significance of Endogenous Antimicrobial Peptides on the Health of Food Animals

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Acquired resistance to in-feed antibiotic growth promoters continues to be an imperative problem in the livestock industries, thereby necessitating continuous pursuit for alternatives. Antimicrobial peptides (AMPs) represent a critical part of the host's innate immune system and have been documented to have immunomodulatory activity. Increasing research evidence suggests that in contrast to antibiotics, AMPs exert broad-spectrum antibacterial activity in a manner that reduces bacterial acquisition of resistance genes. This review summarizes current knowledge on the protective effects of endogenous (natural) AMPs in the gastrointestinal tract of food animals. Factors limiting the efficacy of these AMPs were also discussed and mitigating strategies were proposed.

Keywords: antimicrobial peptide, food animals, host immune system, antimicrobial resistance, gut health

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INTRODUCTION

Antimicrobial peptides (AMPs), also referred to as host (endogenous) defense peptide (HDPs), are tiny cationic peptides that occur naturally in a variety of plants, animals, and microbes (1). Typically, AMPs have a broad-spectrum activity against microorganisms, and have the ability to kill multidrug-resistant bacteria (2). They also exert immunomodulatory activities such as recruiting and activating cells of the innate and adaptive immune system (3). The molecular and cellular mechanisms underlying the activity of AMPs involve inducing changes in membrane-associated targets (such as cell wall biosynthesis and cell division) or on cytoplasmic targets such as macromolecular synthesis in cells (4). In food animals such as pigs and poultry, the antimicrobial and immunomodulatory activities of AMPs synergistically culminate in beneficial physiological effects such as improvements in growth performance, nutrient digestibility, and intestinal morphology, in addition to a diversified healthy gut microbiota (2).

Scientists have classified AMPs into five major families based on their structural compositions and amino sequence: defensins, cathelicidins, hepcidins, histone-derived peptides, and the fish specific piscidins (5, 6). Several reports show that AMPs from fish exhibit similar antimicrobial and immunomodulatory properties to those found in other organisms (7).

Until recent, conventional antibiotics were included in feed for food animals as prophylactics to prevent and control foodborne and disease-causing pathogens, and to promote growth (8). However, the ability of bacteria to be intrinsically resistant to certain antibiotics and/or acquire resistance to antibiotics *via* mutations in chromosomal genes and by horizontal gene transfer has dampened the effectiveness of antibiotics (9). The consequent evolution of antibiotic-resistant bacterial strains and their transmission to humans, has regrettably threatened food safety and public health (10–13). Therefore, governmental legislation(s) have been enacted to phase out (or halt) the incorporation of antibiotics (and other antimicrobial drugs) in animal feed (14). The use of antimicrobials in food animal production worldwide is geographically heterogeneous due

to the existence of different regulations governing their use (15). For instance, incorporation of antibiotics into animal feed has been banned in the European Union and USA since 2006 and 2017 respectively, while countries such as China, Vietnam, Brazil, and Bangladesh have only limited their use (15–17). In spite of these efforts, it has been projected that worldwide antimicrobial consumption by animals will rise by 67% between 2010 [from 63,151 (±1,560) tons] and 2030 [to 105,596 (±3,605) tons] to sustain animal health (18). Presently, the consequential rise of antibiotic-resistant bacteria annually results in the death of about 700,000 people worldwide, and this catastrophe has been projected will be killing about 10 million people yearly by 2050, on a global basis (19). Accordingly, continuous effort is being made to identify alternative non-antibiotic effective strategies for controlling enteric pathogens in food animals.

Studies have shown that AMPs could control and prevent infectious diseases, particularly against antibiotic-resistant bacteria (20, 21). This review therefore explores the role(s) of various classes of AMPs in maintaining the health of food animals (such as pigs, cattle, fish, and poultry), with more emphasis on gut health.

ENDOGENOUS ANTIMICROBIAL PEPTIDES THAT ENHANCE PORCINE HEALTH

Cathelicidins

Cathelicidins exhibit both pro-and anti-inflammatory activity through complex interactions that modulate various immune processes such as apoptosis, inflammasome activation, and phagocytosis (22). Proline-Arginine-39 (PR-39) is a small cationic cathelicidin that is found in the pig's intestinal cells, bone marrow, lymphoid tissues (e.g., thymus and spleen), and leukocytes (23). PR-39 has broad-spectrum antimicrobial activity against enteric pathogenic bacteria such as *Enterococcus faecalis*, *E. coli*, and *Bacillus subtilis* (24), and may play a role in mitigating intestinal inflammation and diarrhea in pigs (25). Enterocolitis in piglets is a diarrheal disease caused by multiple bacteria including *Escherichia coli* and *Salmonella* spp. The disease has endangered the sustainability of the pork sector through production losses such as reduced feed efficiency, increased mortality, treatment costs, and lack of confidence by consumers on the safety of the meat (pork) products. Enterocolitis results in intestinal inflammation that is often characterized by massive infiltration of neutrophils, followed by septicemia and death (23). Cathelicidins probably mitigate enterocolitis by reducing neutrophil adhesion and rolling by ***blocking the recruitment of neutrophils*** (26). ***This effect is accomplished through the inhibition of ubiquitin proteasome-mediated $I_kB\alpha$ degradation on endothelial cells, which in turn downregulates the expression of ICAM-1 and VCAM-1*** (26). ***Thus, PR-39 could alleviate excessive inflammation during intestinal diseases by limiting the influx of neutrophils, and may partially replace current expensive therapies*** such as fluid therapy, antibiotic and anti-inflammatory therapy. Furthermore, PR-39 may re-establish epithelial integrity and intestinal barrier function by promoting intestinal wound healing and angiogenesis.

β-Defensins

Defensins are AMPs that are cysteine-rich with six conserved cysteine residues that form three pairs of disulfide bridges (6). In vertebrate animals, defensins are classified into three sub-groups, namely α-defensins, β-defensins, and θ-defensins, based on distribution of cysteine residue that forms the disulfide bridges and the length (27). Defensins produced by cells in the course of innate immune response serve as signals that initiate, mobilize, and amplify adaptive immune host defenses (28). β-defensins are known to play an essential role in innate and adaptive immunity due to their antimicrobial, chemotactic, and regulatory activities (28). β-***defensins exert inhibitory effects on pathogenic bacteria, fungi, mycobacteria, and enveloped viruses, particularly by creating pores on the microbial membrane surface to increase cellular permeability*** (29). They are also considered chemotactic for T-lymphocytes and immature dendritic cells (30).

The synthesis and secretion of AMPs is triggered by molecules such as cytokines, lipopolysaccharide, β-glucans, and bacterial DNA that signal the presence of potentially pathogenic microorganisms (31). Expression of β-defensins is prevalent in the tissues that control the immune system, such as the spleen, thymus, and lymph node (28). Certain nutrients such as isoleucine, arginine, glucose, Ca^{2+}, and zinc are able to regulate the expression and synthesis of β-defensins (31–33). For instance, Mao et al. (31) supplemented pig diets with 0.5% L-arginine and observed a significant increase ($P < 0.05$) in the expression of porcine β-defensin-2 gene in the oral epithelium, tongue, ileum and inguinal lymph node, and that of porcine β-defensin-3 gene in the ileum and inguinal lymph node. Supplementing swine diets with amino acid and cation mixtures that can optimally enhance the expression and synthesis of intestinal β-defensins will likely improve gut health and resistance to pathogens, and consequently reduce (or eliminate) the use of antibiotics in swine diets.

Cecropins

Cecropins are naturally occurring AMPs in the small intestine of pigs. This AMP possess bactericidal activity against both Gram-negative and Gram-positive bacteria, fungi, and viruses (34). Their physiological and pathophysiological relevance is inherent in their ability to modulate membrane permeability. ***The mechanisms of action involve creopins forming partially selective ion channels or binding to negatively charged membrane lipids to form a closely packed layer that renders membranes permeable*** (35). Weaning is a critical stage for piglets because it is associated with changes in the architecture and function of the gastrointestinal tract, as well as changes in enteric microbiota and immune responses (36). A common disease of piglets is post-weaning diarrhea which is characterized by watery feces and reduced performance, thereby causing economic loss to farmers (37). Wu et al. (35) demonstrated that cecropins improved the performance of piglets challenged with *E. coli* and increased the population of *lactobacilli* strains—a healthy bacteria population in the intestine. Furthermore,

the study revealed that creopins increased the concentrations of serum immunoglobulins and inflammatory cytokines [such as interleukin (IL)-1 and IL-6] which are indicators of an activated immune system (35). The ability of Creopins to act as adjuvants that stimulate humoral and antigen-specific cytotoxic-T-cell responses positions them as an alternative to antibiotics in diets for weaned piglets.

ENDOGENOUS ANTIMICROBIAL PEPTIDES THAT ENHANCE BOVINE HEALTH

Bovine Cathelicidins

Cattle are raised as livestock for meat, milk, and other multi-purpose uses. Infectious diseases, including respiratory, intestinal and reproductive maladies, lameness, and mastitis are major concern (38). Because of the ability of cathelicidins to recognize and kill invading pathogens and stimulate immune defenses (39), they are promising alternatives to antibiotics and drugs for the control of diseases in cattle. Bovine mastitis is a disease condition in which the udder of the cow is infected by a wide range of bacteria, including *S. aureus*, *E. coli*, *S. uberis*, non-aureus *Staphylococci*, *Klebsiella* sp., *Streptococcus dysgalactiae*, and *Mycoplasma bovis* (40). Production-related symptoms associated with mastitis include decreased milk quality and yield. The family of bovine myeloid antimicrobial peptides (BMAPs) BMAP−27, BMAP-28, and BMAP-34 are synthetic host defense peptides derived from naturally occurring bovine cathelicidins, and ***they have shown antibacterial activity against pathogens*** **such as *S. aureus, B. megaterium, E. coli, P.aeruginosa***, and *S. enterica* serotype Typhimurium that cause mastitis (41). Increased levels of cathelicidins in milk were found in 75% of cows naturally infected with *S. uberis*, whereas cathelicidins were absent in healthy cows (42). Therefore, cathelicidins have been proposed as alternative diagnostic markers for mastitis compared to the expensive and sometimes inaccurate traditional bacteriological culture methods to detect somatic cell counts.

Another important cathelicidin in animal health is the synthetic Bac2A that is derived from bovine bactenecin, a 12-amino acid cyclic cationic antimicrobial peptide that contains one intramolecular disulfide bond, through the substitution of two cysteine residues for two alanine residues (43). Synthetic Bac2A exhibits antibacterial activity with minimum inhibitory concentrations (MICs) ranging between 2 and 32 μg/mL against Gram-negative bacteria, and between 0.25 and 16 μg/mL against Gram-positive bacteria (43). Innate defense regulator (IDR)-1018 is another synthetic cathelicidin derived from bovine cathelicidin bactenecin (44). At concentrations of 20 μg/mL, IDR-1018 decreased LPS-induced TNF-α pro–inflammatory response in monocytes by 89% (44). It also has considerable activity against *S. aureus* (MIC 5 μg/mL), although little activity against *P. aeruginosa* was reported (44). Such innate immune regulatory characteristics make IDR-1018 an ideal alternative to traditional in-feed prophylactic antibiotics.

β-defensins

Bovine respiratory disease (BRD) also known as bacterial pneumonia disease, affects production in the beef industry with a significant economic loss (45). Predisposing factors for BRD in beef cattle include viral infections and the stresses of weaning, transportation, castration and inclement weather conditions (45). Under predisposing conditions, the innate immune system is compromised and bacterial composition in the nasal cavity is altered in a manner that increases in the population of pathogenic bacteria (46). Typically, cattle BRD is controlled through metaphylactic use of antibiotics, but this intervention does not consider the underlying roles of stress and viral infection in the disease (45). ***Tracheal antimicrobial peptide (TAP), a 38-amino acid cationic peptide, is a β-defensin produced by epithelial cells lining of the respiratory tract and other mucosal surfaces in cattle and protect the respiratory mucosal surfaces from infection*** (46). Research findings suggest that antibacterial activity of TAP during BRD is compromised by stress-induced elevations in cortisol concentration (46). Similarly, Vulikh et al. (45) reported that the bactericidal activity of β-defensin naturally produced in bovine airways during pneumonia is suppressed by glucocorticoid (stress) and viral infection. We propose that effective control of BRD will require the implementation of management strategies that reduce animal stress, and administration of potent endogenous-source AMPs.

ENDOGENOUS ANTIMICROBIAL PEPTIDES THAT ENHANCE POULTRY HEALTH

Most studies documenting the antimicrobial activity of AMPs in poultry species has been done with chickens. Therefore, the role of AMPs in chicken health is discussed in this section.

Cathelicidins

Modulation of inflammatory response *via* the activation of a wide variety of TLRs during infections is regulated by cathelicidins (47). Three main cathelicidins, namely chCATH-1, −2 −3 (also called fowlicidin-1, −2, and −3), have been identified in chickens and confirmed to have antimicrobial activity against Gram-negative and Gram-positive bacteria, including antibiotic-resistant strains (48). The presence of cathelicidins in lymphoid tissues suggest a possible involvement of AMPs in the maturation and development of adaptive immunity (3). A study by Veldhuizen et al. (49) revealed that chicken cathelicidins have antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA). Specifically, Veldhuizen et al. observed about two log reduction in MSRA count when a concentration of 0.6 mM of cathelicidins was used, and found a complete eradication of the bacteria when the concentration of cathelicidins was increased to 2.5 mM. Goitsuka et al. (50) concluded that chCATH-B1 is an antimicrobial defense element whose cellular localization is pivotal to protection against invasion by viable microbes *via* the mucosal M cell gateway. Furthermore, Bommineni et al. (51) showed that CATH1 possess excellent immunomodulatory properties with a strong capacity

to ***specifically chemo-attract neutrophils without affecting the migration of monocytes or lymphocytes***.

Most studies have shown that Avian AMPs are active against a broad-spectrum of bacteria. The AMPs have a strong ability to modulate the host response to infection and inflammation. However, inability of host organism to produce adequate quantity of AMPs (also known as host defense peptides, HDPs) could limit the functions of HDPs. Bioactive compounds such as butyrate and vitamin D_3 could be used as dietary supplements to increase the production of AMPs. In chickens, butyrate has been proven to be a strong inducer of HDP expression *in vitro* and *in vivo*. A study conducted by Sunkara et al. (52), revealed that supplementing chicken diets with butyrate and a plant extract containing forskolin, which is an adenylyl cyclase agonist, showed a strong synergy in augmenting HDP expression in the crop and jejunum of chickens. We propose that supplementing the diets of poultry with bioactive volatile fatty acids such as butyrate and vitamin D3 could promote HDP synthesis, host immunity, and disease resistance.

B-Defensins

Avian β-defensins (AvBD) are characterized based on their antimicrobial capability against a broad spectrum of pathogens including bacteria and fungi (53). In chickens, 14 AvBD genes have been identified (AvBD1 to AvBD14), and their expression has been confirmed in various tissues including the bone marrow, respiratory tract, skin, digestive tract, and reproductive organs, and in cells like heterophils (54, 55). β-defensin gallinacin-6 (Gal-6) has been reported to show *antimicrobial activity against food-borne pathogens* such as *Campylobacter jejuni*, *Salmonella enterica* serovar Typhimurium, *Clostridium perfringens*, and *E. coli* (54). There is also some evidence that the expression of some AvBDs is enhanced with oviductal growth during sexual maturation by the effects of estrogen (56). Accordingly, as hens age, the increasing expression of AvBDs as the oviduct grows may be important in protecting egg contents from pathogenic microorganisms.

Ovodefensins and Gallin

Ovodefensins belongs to the family of β- defensins antimicrobial peptides containing conserved glycine and six cysteine residues (57). Ovodefensins are expressed in large amount in many parts of the chicken oviduct (57). The ovodefensins differ from classical vertebrate defensins in the spacing of amino acids within the six-cysteine sequence motif, and are slightly shorter in length having only 39–41 amino acids. Whenham et al. (56), indicated that ***ovodefensins caused a 98% reduction in Escherichia coli CFU/mL at 100 lM, and about 40 and 90% reduction in the viability of avian pathogenic E.coli 078:H9 and S. aureus, respectively***.

Gallin, a peptide with a 41-residue protein is a composition of hen egg white that helps to protect the chicken embryo during its development in the egg (57). Gallin is synthesized in the magnum and shell gland of the oviduct, and is deposited into the egg albumen (58). Antibacterial assays confirmed that ***gallin at a concentration of 0.25 μM was active against E. coli***, but no additional antibacterial activity was observed against the other Gram-positive or Gram-negative bacteria tested (57, 58).

ENDOGENOUS ANTIMICROBIAL PEPTIDES THAT ENHANCE FISH HEALTH

Antimicrobial peptides have been found to play immunomodulatory roles in fish species to provide defense against pathogenic attack (7). Natural AMPs such as piscidins, defensins, hepcidins and histone-derived peptides have been found in fish, thereby making fish a major source for AMPs (59).

Piscidins

Piscidins are natural AMPs in the fish which are generally active against various microorganisms (mostly bacteria) and also possess anti-fungal, anti-viral and anti-parasitic activities (60, 61). The ***mode of action by piscidins is to inhibit further growth and development of pathogens by penetrating and destroying the spores*** (62). Other findings have indicated piscidins expression contributes to phagocytic activities. Thus, the intracellular release of piscidins by granulocytes aids in the gut health and defense against pathogenic attack (63). Furthermore, piscidins can mitigate pathogenic activities in extreme conditions due to the hemolytic activity conferred by the amphiphilic α-helical cationic structure (64). Research evidence has also suggested that the immunomodulatory effects of piscidins observed in fish are similar to those exhibited in mammalian species such as mice (65).

Defensins and Cathelicidins

β-defensins remain the only type available in fish species (66). β-defensins are also active against some fish specific-viruses like the Singapore grouper iridovirus (SGIV) and the viral nervous necrosis virus [VNNV; (67)]. Research reports have indicated that β-defensins found in Atlantic cod can influence ***antimicrobial activity in phagocytes***, thereby providing multiple functions to the innate host defense (7, 68). Cathelicidin play significant role in immunity as an *anti-bacterial* host defense. Its expression has been observed during embryonic development where it provides some fish species with a first line of response against pathogenic bacteria such as *P. aeruginosa*, *V. anguillarum*, *E. coli*, and *Lactobacillus* spp. (69).

Hepcidins

Hepcidins are normally expressed in the liver in the adult fish albeit its expression occurs early during development in some fish species (70). Fish have two types of hepcidins: hamp1 and hamp2 (7). Research reports suggest that the role of hamp1 focuses on iron metabolism regulation, whilst that of hamp2 is mostly antimicrobial in nature (71). Hypoxia in fish hinder hepcidin to actively fight against microorganism attack (72). On the other hand, hepcidin expression is induced by high levels of iron in both zebrafish and sea brass (73, 74). Hepcidin is believed to ***increase resistance to microbial infection by preventing the release of iron from macrophages, and preventing the absorption of iron in the small intestine***.

Histone-Derived Peptides

Histone-derived peptides ***exhibit a variety of host defense mechanisms against broad spectrum of both Gram-positive***

and Gram-negative bacteria, and have been shown to be secreted in fish species when epidermal damage occurs before (75). Some histone-derived peptides require binding with other antimicrobial peptides in order to express their maximum antimicrobial potential. For instance, histone-derived peptides containing NETs (neutrophil extracellular traps) exhibit strong potential to trap and kill bacteria in some fish species. However, very little information is known about the mode of action of histone-derived peptides against pathogenic attack and immunomodulatory responses in fish, compared to other antimicrobial peptides (7).

MITIGATING MICROBIAL RESISTANCE TO ENDOGENOUS ANTIMICROBIAL PEPTIDES IN FOOD ANIMALS

The antimicrobial activities of endogenous AMPs include inactivation of pathogenic microbes, reinforcing the antimicrobial barrier function of epithelial cells particularly in the gut, and linking innate immunity to adaptive immunity (76, 77). The cationic nature of endogenous AMPs facilitate their ability to exert antimicrobial effect(s) on pathogens. Although the precise mechanism(s) by which AMPs cause bacterial cell death is still poorly understood, The amphipathic interaction between the AMPs (net-positively charged) and the negatively charged microbial cell surfaces allow the insertion of AMPs into the microbe's cell membrane (78). In bacterial, the net-negative charge in the cell membrane is due to their constituent phospholipids (like cardiolipin, phosphatidylserine, and phosphatidyl glycerol), lipopolysaccharide, and lipotechoic acids (79). To cause cell death, AMPs bind directly to the lipopolysaccharides of Gram-negative bacteria and lipoteichoic acids of Gram-positives, and then depolarize the cell membranes to make them permeable (80, 81). Regardless, mounting research evidence show that pathogenic microbes can develop the ability to evade antimicrobial effects of AMPs. Microbes utilize various mechanisms to evade the antimicrobial activity of AMPs. For instance, bacterial resistance mechanisms to AMPs include (i) membrane modification through electrostatic repulsion of AMPs by alanylated teichoic acids, aminoacylated peptidoglycan, or amine compound-added lipid A, (ii) binding of AMP and

TABLE 1 | Endogenous antimicrobial peptides in food animals.

Species	Antimicrobial peptide	Mode of action	Endogenous source
Porcine	Cathelicidin—Proline-Arginine-39 (PR-39)	Block the recruitment of neutrophils through the inhibition of ubiquitin proteasome-mediated IkBα degradation on endothelial cells	Intestinal cells, bone marrow, lymphoid tissues, and leukocytes
	β-Defensins	Creating pores on the microbial membrane surface to increase cellular permeability	Tissues and cells of the immune system
	Creopins	Modulate membrane permeability by forming partially selective ion channels, or binding to negatively charged membrane lipids to form a closely packed layer that renders membranes permeable	Small intestine
Bovine	Cathelicidins (synthetic endogenous-source)—Bovine myeloid antimicrobial peptides (BMAPs), Bac2A, and IDR-1018	Antibacterial activity against pathogens	Different tissues of the body and milk
	β-Defensin—Tracheal antimicrobial peptide (TAP)	Prevent infection at the respiratory mucosal surfaces through bactericidal activities	Epithelial cells lining the respiratory tract and other mucosal surfaces
Poultry	Cathelicidins, namely chCATH-1, −2 −3 (also called fowlicidin-1, −2 and −3),	Exert antibacterial activity by serving as a chemo-attractant to neutrophils, without affecting the migration of monocytes or lymphocytes	Lymphoid tissues in chickens
	β-Defensins—AvBD1 toAvBD14	Provides broad-spectrum antimicrobial activity	Expressed in various tissues, including the reproductive organs, bone marrow, respiratory tract, skin, digestive tract, and in cells like heterophils
	Ovodefensins	Antibacterial activity against *E. coli* and *S. aureus*	Expressed throughout the chicken oviduct
	Gallin	Antibacterial activity against *E. coli*	Found in egg albumen
Fish	Piscidins	Bacteriostatic—inhibit further growth and development of pathogens by penetrating and destroying the spores	Various tissues in the fish
	β-Defensins	Contributes to antimicrobial activity in phagocytes	Various tissues in the fish
	Cathelicidin	Antibacterial	Embryonic tissue in fish
	Hepcidins—Hemp1 and Hemp2	Increase resistance to microbial infection by preventing the release of iron from macrophages, and preventing the absorption of iron in the small intestine	Predominantly expressed in the liver of adult fish
	Histone-derived peptides	Broad-spectrum antibacterial activity	In Fish

inactivation by mechanisms such as surface shedding, (iii) active removal of AMPs from the bacterial cell by efflux pumps, (iv) proteolytic degradation of AMPs by extracellular proteases such as elastase and gelatinase, (v) upregulation of bacterial AMP resistance genes by global transcriptional regulators, and (vi) downregulation of AMP expression or upregulation of host AMP-degrading proteases such as cathepsins (81–83). However, compared to antibiotics, it is believed that endogenous AMPs contribute less to microbial resistance to drugs because of their stable structure, antimicrobial activity, selective toxicity, wide-spectrum and high-efficiency, and minimal side effects (84, 85).

Microbial resistance to endogenous AMPs can be mitigated through dietary modulation strategies that increase the expression of AMPs, enhance the stability of AMPs at epithelial surfaces, and upregulate redox proteins present at the epithelial surfaces. In a recent review, Wu et al. (85) summarized the nutrients that have been established to upregulate the expression of AMPs in the gut mucosa. These include (i) amino acids such as branched-chain amino acids, Arginine, and tryptophan that upregulate the expression of AMPs in the gut *via* the Sirt1–ERK1/2–90RSK (sirtuin-1-extracellular regulated protein kinase1/2- 90-kDa ribosomal S6 kinase), GPCR–MAPK (G protein-coupled receptor-mitogen-activated protein kinase), and NO signal or mTOR (nitric oxide signal- mammalian target of rapamycin) pathways; (ii) fatty acids such as short-chain fatty acids, medium-chain fatty acids, and long-chain fatty acids contribute to the expression of AMPs by directly influencing histone acetylation and the GPCR–MAPK signal pathway; (iii) lactose from plants, and polysaccharides from plants and bacteria, and (iv) trace elements and vitamins such as zinc, lactoferrin, cholecalciferol (vitamin D3) and its metabolite 1,25-dihydroxycholecalciferol [1,25(OH)2-D3], vitamin B-3, and vitamin A. Furthermore, the presence of thioredoxin, a redox protein present at the intestinal epithelial surface, is known to facilitate the antimicrobial activity of AMPs (86). A recent report by has also suggested that redox active AMPs can undergo reversible oxidation after interaction with some electron transport chain proteins and their products, or interaction with the periplasmic redox system of Gram-negative bacteria (77). Further research is required to identify molecular-based strategies that will exploit the reversible oxidation state of endogenous AMPs, for the synthesis of AMPs that will have improved stability in the gut mucosa and other epithelial membranes.

CONCLUSION

Considerable research has been done to identify endogenous AMPs in food animals, and their mechanisms of antimicrobial activity (**Table 1**). The main advantages of using AMPs include their broad spectrum of activity, fast action against pathogenic bacteria, and probably decrease in bacterial acquisition of resistance genes contrary to the situation often seen with the use of conventional antibiotics. Different approaches for using AMPs to manage animal health include (i) combined dietary administration of antimicrobial peptides and conventional antibiotics, and (ii) dietary supplementation of their precursor nutrient molecules (such as amino acids, fatty acids, and some micronutrients) at optimum concentrations that can increase AMP expression in the animal's body. The possibility to manipulate the oxidative state of some AMPs to achieve increased stability at in the gut mucosa and other epithelial membranes require further investigation. It was concluded that AMPs can could at least partially replace conventional antibiotics in food animal production, thereby improving the quality and microbiological safety of animal meat and egg products intended for human consumption.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Role of Recent Therapeutic Applications and the Infection Strategies of Shiga Toxin-Producing *Escherichia coli*

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Shiga toxin-producing *Escherichia coli* (STEC) is a global foodborne bacterial pathogen that is often accountable for colon disorder or distress. STEC commonly induces severe diarrhea in hosts but can cause critical illnesses due to the Shiga toxin virulence factors. To date, there have been a significant number of STEC serotypes have been evolved. STECs vary from nausea and hemorrhoid (HC) to possible lethal hemolytic-based uremic syndrome (HUS), thrombotic thrombocytopenic purpura (TTP). Inflammation-based STEC is usually a foodborne illness with Shiga toxins (*Stx 1* and *2*) thought to be pathogenesis. The STEC's pathogenicity depends significantly on developing one or more Shiga toxins, which can constrain host cell protein synthesis leading to cytotoxicity. In managing STEC infections, antimicrobial agents are generally avoided, as bacterial damage and discharge of accumulated toxins are thought the body. It has also been documented that certain antibiotics improve toxin production and the development of these species. Many different groups have attempted various therapies, including toxin-focused antibodies, toxin-based polymers, synbiotic agents, and secondary metabolites remedies. Besides, in recent years, antibiotics' efficacy in treating STEC infections has been reassessed with some encouraging methods. Nevertheless, the primary role of synbiotic effectiveness (probiotic and prebiotic) against pathogenic STEC and other enteropathogens is less recognized. Additional studies are required to understand the mechanisms of action of probiotic bacteria and yeast against STEC infection. Because of the consensus contraindication of antimicrobials for these bacterial pathogens, the examination was focused on alternative remedy strategies for STEC infections. The rise of novel STEC serotypes and approaches employed in its treatment are highlighted.

Keywords: Shiga toxin-producing *Escherichia coli* (STEC), Shiga toxin, infection, symbiotic, antimicrobial agents, therapies

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INTRODUCTION

Shiga Toxin-Producing *Escherichia coli* (STEC) Gastroenteritis and Hemolytic Uremic Syndrome

Enteropathogens induce numerous diseases, most of them featuring colon distress symptoms. Enteropathogenic bacteria such as diarrhea-causing *Escherichia coli* (*E. coli*) and species of the genera *Salmonella, Shigella*, *Klebsiella*, and *Yersinia* are responsible for different types of gastrointestinal disorders. STEC is a prominent bacterial pathogen reported globally (Bhunia, 2018). Some *E. coli* naturally reside in animals and humans' colonic tract and are considered beneficial gut bacteria. However, most of the pathogenic strains of *E. coli* such as STEC are responsible for several colon infections (Bron et al., 2017). STEC is one of the six major classifications (pathotypes) of diarrheagenic *E. coli*. This differentiation depends on medical syndromes, symptoms, epidemiology, the presence of antigen type Stx1 and Stx2 virulence factors, and interaction with epithelial cells (Hwang et al., 2018). Infection with most types of enteropathogenic *E. coli* causes watery diarrhea (**Figure 1**). Many enteric infections lead to a short-lived dysfunction of the gastrointestinal system. In extreme cases, a severe disorder can occur based on specific pathogenic infections such as that with STEC (Bron et al., 2017; Hwang et al., 2018).

STEC serves as a source of food and water-borne outbreaks that contribute to life-threatening infections. STEC infection outcomes can range from mild to significant symptoms of hemorrhagic colitis (HC) and hemolytic-based uremic syndrome (HUS). Certain STEC strains are also designated as enterohemorrhagic *E. coli* (EHEC) due to their human virulence factors (Rivas et al., 2016). EHEC strains belong to the STEC subtype and are distinguished by specific serotypes, often correlated with epidemic and severe clinical disease (Mora et al., 2012). EHEC-based O157:H7 strain was reported in the center for disease control and prevention (CDC) study on microbiological findings of raw ground beef products, which was linked to several cases of HC and HUS (Fedio et al., 2011). As a result, public health and regulatory responses were primarily based on this serogroup. Cumulative evidence from various countries has shown in recent years that up to 40 to 70 percent of human EHEC diseases are induced by non-O157 EHEC (Delannoy et al., 2013).

EMERGING STEC SEROTYPES

Recent research has shown that the number of STEC infections other than O157 often exceeds the number of STEC O157 infections (Gould et al., 2009; Gould et al., 2013). The HUS-associated STEC list and their non-motile derivatives were therefore expanded in **Table 1A**. These are the seven STEC priority serotypes most often associated with HC and HUS infections and sporadic cases worldwide (Stanford et al., 2018); USDA study on the classification of non-O157 STEC from meat products are reported in **Table 1B**.

MODE OF TRANSMISSION OF STEC AND THE EFFECT OF SHIGA TOXIN IN HUMANS AND ANIMALS

STEC leads to fatal inflammation in the host as a sign of Shiga-based toxins' expression. STEC, comprising strains of the serogroups (**Table 1**), causes severe diarrhea, hemorrhagic colitis (HC), and

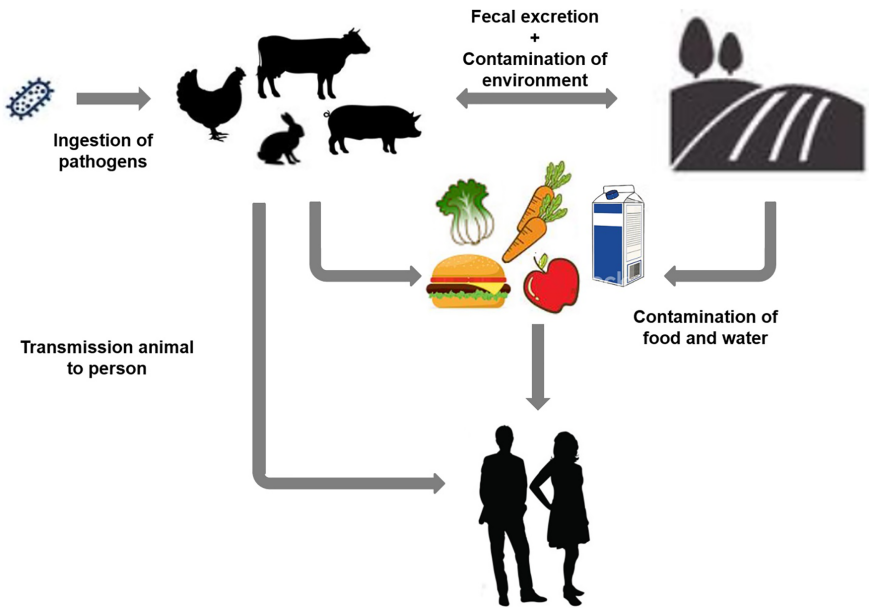

FIGURE 1 | Schematic representation of different contamination sources and transmission of Shiga toxin-producing *Escherichia coli* infection based on various environmental factors.

TABLE 1A | A description of the global reports of outbreaks of two cases or more of non-O157 strains of Shiga toxin-producing *E. coli* along with the reported frequency of dysentery and hemolytic uremic syndrome where these data were available, and the implicated vehicle of transmission, 1995¸017. (Copyright obtained from Valilis et al., 2018).

Years	Number of confirmed cases	Serogroups/types	Median number of people per outbreak (range)	Number reporting dysentery (%)	Implicated vehicle of transmission	Number reporting HUS (%)
1995–1999	**183**	**O26:H11, O111:(H-,H8), O118:H2**	**25/(2–131)**	**6/57 (11%)**	**Ice in open barrels, serving utensil, dry fermented sausage**	**16/183 (8.7%)**
1990	5	O111	1/5	1/5	Private home/Family cluster	
1994	18	O104	0/18	0/18	Pasteurized Milk	
1998	8	O121	unknown	unknown	Camp	
1999	55	O111	2/55	2/55	Salad Bar, Ice from barrel	
1999	11	O121	3/11	3/11	Lake Water	
1999	2	O121	0/1	0/1	Daycare	
2000–2004	**26**	**O26:H11, O148:H8**	**11/(2–13)**	**0/26 (0%)**	**Mutton, beef**	**2/26 (7.7%)**
2000	61	O111	0/59	0/59	Animal contact (calves)	
2000	18	O145	2/18	2/18	Water-based punch	
2001	4	O26	0/4	0/4	Lake Water	
2001	31	O111,O-rough	0/25	0/25	Animal Contact (Calves)	
2001	3	O111	3/3	3/3	Family cluster (animal exposure reported for one patient)	
2001	3	O111	0/3	0/3	Daycare	
2004	213	O111	0/212	0/212	Unpasteurized Apple Cider	
2005–2009	**221**	**O26:(H11), O45, O103: H25, O104:H4, O111, O145:H28**	**16/(3–156)**	**93/137 (68%)**	**Ice cream, farm animals, eating outside of home, restaurant, beef sausage, mutton**	**34/91 (37%)**
2005	52	O45	0/52	0/52	Ill Food Worker(s)	
2005	4	O26	unknown	unknown	Daycare	
2006	42	O121	3/42	3/42	Lettuce	
2006	5	O26	0/4	0/4	Berries	
2006	5	0121	4/5	4/5	Daycare	
2006	11	O45	0/11	0/11	Animal contact (goats)	
2006	3	O165	0/3	0/3	Correctional facility	
2007	23	O111	0/23	0/23	Private home (ground beef)	
2007	8	O111	0/8	0/8	Daycare	
2010–2014	184	O26:(H11), O103:H2, O104:H4, O111:H8, O121, O145:(NM)	25/(2–35)	20/184 (11%)	Raw clover sprouts, Farm Rich brand frozen products, dairy products, cattle, person-to-person, venison, romaine lettuce	26/184 (14%)
	3816	O104:H4a	3816	141/161 (88%)	Sprouts	845/3816 (22%)
2015–2017	60	O26	30/(5–55)	0/60 (0%)	Multiple restaurant chains	0/60 (0%)

**Excludes any isolates for which serogroup could not be determined (including isolates in unknown, undetermined, and rough categories). HUS, hemolytic uremic syndrome; STEC, Shiga toxin-producing E. coli.*

TABLE 1B | Non-O157 STEC isolates characterized at the National *Escherichia coli* Reference Laboratory, by serogroups.

Serogroup	Number of isolates reported, 1995-2020	Percentage of total isolates serogroup
14	7	0.2%
22	7	0.2%
88	7	0.2%
91	60	1.5%
76	52	1.3%
165	45	1.1%
228	28	0.7%
174	27	0.7%
123	23	0.6%
177	22	0.6%
153	21	0.5%
28	20	0.5%
178	10	0.3%
63	9	0.2%
7	8	0.2%

(Continued)

TABLE 1B | Continued

Serogroup	Number of isolates reported, 1995-2020	Percentage of total isolates serogroup
2	7	0.2%
26	**918**	**23.2%**
103	**806**	**20.4%**
111	**643**	**16.3%**
45	290	7.3%
121	248	6.3%
145	179	4.5%
69	71	1.8%
118	71	1.8%
117	6	0.2%
175	6	0.2%
84	19	0.5%
128	19	0.5%
146	18	0.5%
113	17	0.4%
119	15	0.4%
8	14	0.4%
55	14	0.4%
172	12	0.3%
130	10	0.3%
156	10	0.3%
126	7	0.2%
9	6	0.2%
110	6	0.2%
112	6	0.2%
179	6	0.2%
6	5	0.1%
43	5	0.1%
71	5	0.1%
141	5	0.1%
181	5	0.1%
1	4	0.1%
33	4	0.1%
50	4	0.1%
80	4	0.1%
98	4	0.1%
116	4	0.1%
132	4	0.1%
166	4	0.1%
51	3	0.1%
60	3	0.1%
73	3	0.1%
79	3	0.1%
82	3	0.1%
86	3	0.1%
109	3	0.1%
125	3	0.1%
162	3	0.1%
163	3	0.1%
168	3	0.1%
5	2	0.1%
11	2	0.1%
18	2	0.1%
20	2	0.1%
21	2	0.1%
25	2	0.1%
38	2	0.1%
42	2	0.1%
49	2	0.1%
53	1	0.0%
61	1	0.0%
70	1	0.0%
87	1	0.0%
96	1	0.0%

(Continued)

TABLE 1B | Continued

Serogroup	Number of isolates reported, 1995-2020	Percentage of total isolates serogroup
101	1	0.0%
105	1	0.0%
115	1	0.0%
131	1	0.0%
74	2	0.1%
75	2	0.1%
77	2	0.1%
85	2	0.1%
100	2	0.1%
104	2	0.1%
124	2	0.1%
136	2	0.1%
137	2	0.1%
143	2	0.1%
149	2	0.1%
158	2	0.1%
160	2	0.1%
3	1	0.0%
4	1	0.0%
12	1	0.0%
19	1	0.0%
24	1	0.0%
27	1	0.0%
52	1	0.0%
134	1	0.0%
135	1	0.0%
140	1	0.0%
150	1	0.0%
151	1	0.0%
152	1	0.0%
154	1	0.0%
180	1	0.0%

**Data represented from CDC Bacterial Foodborne and Diarrheal Disease National Case Surveillance Annual Reports, 2003-2020.*

can also lead to life-threatening diseases like hemolytic uremic syndrome (HUS) (Singh et al., 2015) (**Table 1**). The frequency of non-O157 STEC-based cases in the United States (U.S) was unclear; based on the symptoms, the researchers have quantified the level of infection in the human feces (Newell and La Ragione, 2018). Mostly, non-STEC strains (specifically O26,O45,O103, O111, and O145 serogroups) showed similar virulence and biochemical characteristic with the O157 strain as per US research reports (Gould et al., 2013). In 2020 as per the Centers for Disease Control and Prevention (CDC) FoodNet Data and Reports indicated that the level of non –STEC O157 was found to be significantly higher than the STEC *E. coli* (Joseph et al., 2020). Besides, the food Net report states that among 451 non-O157 STEC reported cases 80 percent was children, and the rest 20% was adult (Joseph et al., 2020). From 2000-2010, FoodNet reported 1,842 instances of non-STEC O157 infection were (**Table 1A**).

Based on the epidermic of non-O157 STEC reported by outbreak Surveillance System (FDOSS) which identified over 1,500 illnesses confirmed cases of non-O157 STEC outbreaks on November 4, 2020 (Thierry et al., 2020). Most of the epidemic was caused by non 0157 serotype strains but correlated with other enteropathogens (EFSA BIOHAZ Panel et al., 2020) . The most frequent outbreak of non 0157 STEC serotype reported among 120 serogroups was determined as follows O26, O111, and O121 (**Table 1B**) (Blankenship et al., 2020). The pathogenicity with *Shigella species* and STEC *E. coli* was almost similar but they are varied in the symptoms, metabolic traits, and severity of illness (Bommarius et al., 2013; Chou et al., 2013; Merkx-Jacques et al., 2013; Michelacci et al., 2017; Smati et al., 2017; Yun et al., 2017). The entero-aggregative STEC outbreak reported with 790 cases of HUS and 3128 non- HUS cases in Germany (May 2011) indicated a lethal HUS percentage (Cheung et al., 2011). Noval STEC strains (new serotypes) were reported for HUS cases (Bae et al., 2006). Among the previous outbreak, reports consisted that the major non-O157 STEC such as O26, O45, O103, O111, O121, and O145, likewise among the STEC the most frequent reports in 2020 such as O26: H11, O111:H8, and O121:H19 serogroups (Taylor et al., 2013) (**Supplementary Table 1**).

The pathogenic mechanisms of STEC merited further investigation. As per the proven theory, it was predicted that *Shigella* strains were evolved and it forms the ancestral for infectious virulent *E. coli* (Welch et al., 2002; Paauw et al., 2015; Njamkepo et al., 2016; Dunne et al., 2017). In contrast, Enteroinvasive *E. coli* (EIEC) are thought to have evolved later than *Shigella* and from widely diverged strains of *E. coli* (Lan et al., 2004). Additional research is required to characterize the virulent effects of STEC, and it is hoped this could prevent the evolution of novel strains that are more virulent or difficult to

treat and could pose a serious human health threat. Several animal models have been proposed for studying EHEC infection (Panda et al., 2010; Golan et al., 2011; Mohawk and O'Brien, 2011). Some of the infectious mechanism and virulence factors are yet to be determined for the EHEC due to the lack of an efficient animal model system which hampers the pathway mechanism (Franz et al., 2014) (**Supplementary Table 2**).

STEC ATTACHMENT AND PATHOGENICITY IN THE INTESTINAL ENVIRONMENT

Diverse adhesive assemblies connected to *E. coli* O157:H7 cells influence the bacteria's adhesion to intestinal epithelial cells. These morphological attachment-based structures include fimbria, which is responsible for binding and multiplication. These adhesions of STEC bacteria facilitate the surface attachment of bacteria to human intestinal epithelial cells, which possess glycoprotein as an associate protein (Suzaki et al., 2002; Uchida, 2003). The adhesion mechanism is directly linked to virulence factors of *E. coli* OH157:H7 and leads to inflammation (lesions in colon inner wall). EHEC harbors a Type 3 secretion system (*T3SS*) and its secreted proteins, including *EspD, EspB, EscF,* and *EspA*. The *T3SS* and its secreted proteins are encoded on the locus of enterocyte effacement (LEE) pathogenicity. The over-expression was regulated by *LEE*-encoded regulator (Ler) upregulates LEE-encoded virulence genes. Some of the effector proteins imitate host ligands and receptors involved in attachment with epithelial cells. The primary function of the translocated proteins, which trigger activation of neural syndrome protein (N-WASP) such as the translocated intimin receptor (Tir), and an adaptor-like protein *EspFU* (Gao et al., 2009). The solid human immune response generated against intimin receptor, *EspA*, and *EspB* has led to these bacterial proteins being considered potential vaccine candidates (**Figure 2**).

CORRELATION OF ANTIMICROBIAL RESISTANCE WITH INCREASED TOXIN GENE EXPRESSION IN STEC

Previous reports have described resistance mechanism in *E. coli* O157:H7 and other STEC strains mainly evolved from animal reservoir-based environmental sources (**Supplementary Table 4**). The principal reason for increasing reports on antibiotic resistance is the overuse of antibiotics in agricultural-based regions, which leads to the development of multidrug resistance in bacteria. In STEC, the *Stx* gene is responsible for the production of Shiga toxin (**Supplementary Table 5**). Chloramphenicol was commonly used to suppress the growth of STEC, but along with other drugs such as sulfonamides, quinolones, and fluoroquinolones leads to enhanced toxin production as a result of over-expression of the *Stx* gene (Schroeder et al., 2002; Amézquita-López et al., 2016). Besides, STEC was commonly found to be resistant to certain antibiotics such as tetracyclines and aminoglycosides (Uemura et al., 2003; Bai et al., 2016) (**Supplementary Table 6**).

The horizontal gene transfer of resistance is a multidrug resistance (MDR) dissemination mechanism since virulence and antibacterial factors-based genes can be found in clusters and transmitted together to the recipient (Bello-López et al., 2019). Various theories of recombination, transition, or intracellular transduction may transfer these genes. Under environmental circumstances, the most effective transfer mechanisms in bacteria appear to be conjugation and transduction (Muniesa et al., 2013). Such tools often involve integrons, which are mobile genetic elements that acquire gene cassettes for antimicrobial resistance. Integrons are described as

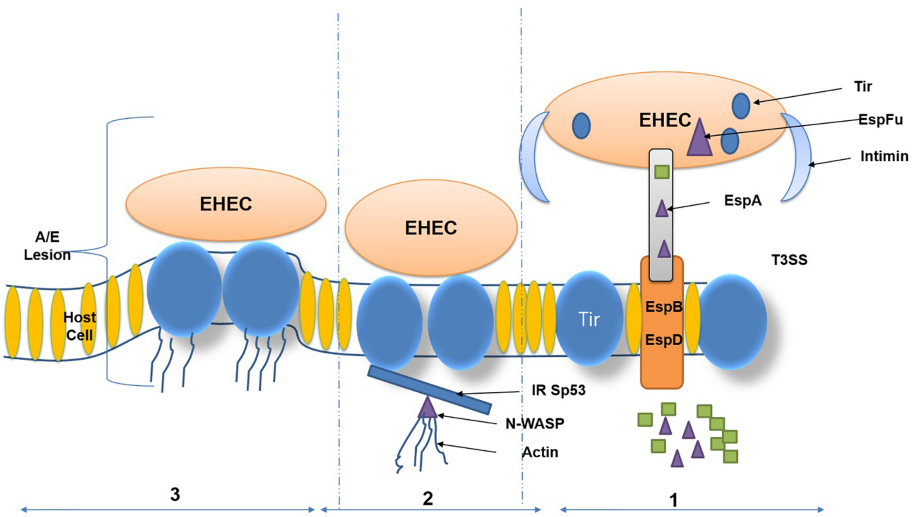

FIGURE 2 | Virulence factors and attaching and effacing (A/E) lesions of Enterohemorrhagic *Escherichia coli* (EHEC). (Copyright obtained from Saeedi et al., 2017).

genetic units that include site-specific recombination component factors that contribute to capturing portable gene cassettes (Hall and Collis, 1995). In Enterobacteriaceae, the existence of class 1 integrons was highly linked with MDR (Kor et al., 2013).

The discovery in TET-resistant isolates of a high proportion of *tetA* and *tetB* genes indicates t the key TET resistance mechanism in isolated calf *E. coli* occurs by active efflux (Chirila et al., 2017). Among TET-doxycycline-resistant E, a predominance of the *tetB* gene was observed. Diarrheal *E. coli* isolated from calves correlated with prior results obtained in different countries (Chirila et al., 2017). Phenotypically susceptible strains of bacteria were carrying resistance genes, which may have contributed to their expression not yet occurring. Other experiments have also shown that certain bacteria lack the expression of resistance genes (O'Brien, 2002).

Integron genes are common in Enterobacteriaceae and contribute to MDR (Kang et al., 2005). Class 1 and class 2 integron genes were discovered in isolated *E. coli*. Their incidence was lower than that reported in *E. coli* isolated from poultry and pigs (Lapierre et al., 2008). Total 13 percent of *stx*-positive strains were also positive for integrons in the above study. A higher prevalence of integron-positive STEC strains isolated in the USA and derived from human patients (n=81) and domestic animals (n=193; Livestock) was observed in previous studies (Chirila et al., 2017).

Total 18 percent of 50 analyzed STEC strains originating from humans, livestock, and food (EFSA BIOHAZ Panel et al., 2020) Integron class 1 predominance reported strains possessed genes that coded for spectinomycin (*aadA1*) and trimethoprim resistance (*dfrA1*). Research conducted in Brazil on thirty-two STEC strains showed that in 22 percent of isolates, the integrase gene associated with Class 1 integrons, all of which had a uniform size and contained a single cassette gene (Chirila et al., 2017). Another aspect of all integron-positive strains showed factors of virulence that are important because of neonatal diarrhea. The strains of *E. coli* are a significant cause of economic losses on farms. These virulence factors draw attention not only to *E. coli* but also more resistant and more aggressive to antimicrobials.

TREATMENT STRATEGIES FOR INFECTIONS CAUSED BY SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI*

The inevitability of a STEC infection treatment strategy has a major issue in public safety and human health. Current treatment measure depends on hydration and antibiotic therapy (Ciccarelli et al., 2013). The frequent application of antimicrobial compounds towards the disease caused by STEC infections currently developed the risk of HUS association (Wong et al., 2000; Panos et al., 2006; Rahal et al., 2015).

NOVEL AND ALTERNATIVE STEC TREATMENT STRATEGIES

Different alternative therapies have been increased by a debatable use of antimicrobials in the management of STEC infection (**Table 1**). This ranges from the use of novel secondary metabolite towards different therapies that revisit antibiotics (Grisaru et al., 2017).

SHIGA TOXIN ANALOG RECEIVER

Different drugs have been developed that imitate and bound Stx receptors, minimizing their accessibility to effector cells. Gb3-held carbosilane dendrimers deactivate Shiga toxins *in vitro* and have been shown to treat the impaired mice by intravenous administration (Jacobson et al., 2014). Conjugated carbohydrates-based compounds, such as STARFISH and DAISY, based on *in vitro* analysis, confirmed that the conjugated multivalent compound neutralizes Shiga toxins (Chabre and Roy, 2010; Bhatia et al., 2014). Highly clustered *Gb3* polymers bind with Shiga toxins with strong binding efficacy and treat the impaired mice when ingested orally (Tesh, 2010; Jacobson et al., 2014). Previous reports indicated that oral ingestion of synthetic version of verotoxin (*VT*, Shiga-like toxin) Pk-oligosaccharides-based receptor sequences attached to Chromosorb (Synsorb-Pk) by healthy adult volunteers. Sensor-based-Pk reclaimed from volunteer stool samples was also analyzed to determine if its *VT*-binding activity was affected by exposure to the pH extremes and digestive processes of the human gastrointestinal tract, but most of the compounds were found to be not providing promising results in the clinical studies (Armstrong et al., 1995).

SHIGA TOXINS - INTRACELLULAR INTERFERENCE

It has been stated that cell permutant agents are capable of binding Shiga toxin 2 (*Stx2*) and probably interfering with its traffic. The two agents were evaluated in animals, and *Stx2* inhibitor skills were demonstrated by both acetyl groups to all the amino termini of PPP-tet (yielding Ac-PPP-tet) (Rahal et al., 2015) and using baboons as an animal model, the cell-penetrating peptide (TVP) dock with *Stx2*, this leads to the reduction of toxin lethality with (55 ng/kg) (Stearns-Kurosawa et al., 2011) The intracellular transmission of the B subunits of *Stx* was also reported to interfere and defend against Stx1 and Stx 2 in Mice (Watanabe-Takahashi et al., 2010; Jandhyala et al., 2016). The *Retro-1* and *2* with small molecular inhibitors have also shown to be agents that interfere with *Stx* trafficking employing high-performance testing (Wahome et al., 2011; Abdelkafi et al., 2020).

ANTIBODY-BASED THERAPY

Antibodies have been identified that can bind and nullify the Shiga toxins effects (Reid and Burton, 2002), and monoclonal *anti-Stx* subunit A showed major benefits through invitro and *in vivo* (mouse) models. Anti-lipopolysaccharide anti corporations were shown to be protective (Yamagami et al., 2001; Mejías et al., 2016). An immunoglobulin-rich bovine colostrum preparation containing a high titer of anti-*Stx1* and antic *Stx 2* was also tested,

and 13 patients and 14 placebo controls were compared with a colostrum-treated group. In colostrum-treated patients, the median level of stolen excretion was reduced but not significantly affected by the infection therapy presence in the subject's stolen stools. This therapy's effects on HUS development or other possible infection complications were not controlled by study subjects (Huppertz et al., 1999). The humanized monoclonal antibody Eculizumab in contrast to the complement component 5 (*C5*) has shown beneficial effects of STEC-associated HUS rehabilitation including clinical trials (Grisaru, 2014; Mahat et al., 2019).

NATURAL PRODUCTS (SECONDARY METABOLITES)

Numerous metabolic products have been considered as possible STEC-based natural therapeutic drugs. Which include, in addition to plants, fruit and herbal products, grains and organic acid (Anand et al., 2019), and fruit drinks [Citrus limon (Rutaceae)] (Nogueira et al., 2003; Lacombe et al., 2010). These drugs were promising *in vitro* or *in vivo* (mice) models but were not tested in clinical trials. It is important to note a study showing a synergistic treatment effect with mice with STEC infection (Lee and Stein, 2011; Rahal et al., 2015; Ma et al., 2019) between processed tea leaves and an antibiotic, levofloxacin, which indicates that the significant threat of the treatment of antibiotics may decrease with the addition of another agent.

ANTIMICROBIAL DRUGS

The application in the treatment of STEC infections of antimicrobial agents was controversial and is under vigorous debate. Although certain studies showed that the ingestion of specific agents may increase the chance of Hemolytic Uremic Syndrome Risk (HUS), some observed a decline in this risk after antimicrobial application. Whereas some drugs may be specific at a certain dose, the significant threat of antibiotic therapy triggering HUS has led to a large contraindication of these agents (Safdar et al., 2002; Puño-Sarmiento et al., 2020). The threat of HUS by increasing Shiga toxins from the bacterial cells in a variety of ways is presumed to be increased by antimicrobial agents. The bacterial SOS response is the important signaling pathway for high-level production and release of *Stx1/2* prophages in STEC bacterial strain may be caused by the DNA damage which certain antimicrobials can cause. To activate the host DNA damage response pathway (SOS response), which is to cope with nucleic acid damage, leads to the production of different proteins that can encode an Stx and enhance its development by triggering the lytic cycle of the bacteriophages. Besides, some form of antibacterial agents stress may trigger lead to increased expression of toxins (Łoś et al., 2009; Kimmitt et al., 2000). In comparison, *Stx1* is known to be deposited in the cytoplasmic membrane of STEC strains, which can lead to increased liberation from cell lysis triggered by an antibacterial property in the specific Stx form (Vázquez-Laslop et al., 2006; Harms et al., 2016).

As with other prophages, the *Stx*-encoding prophets (e.g. quinolone antibiotics) induce by activating a host DNA damage reaction pathway (SOS reaction). Thus, quinolone antibiotics are associated with complications for EHEC infections. While transcriptional and translational inhibitors can demonstrate the possibility of inhibiting the Stx production, several studies indicate that antibiotic therapy raises the chances for EHEC-associated severe infection. It is not well explored the mechanism of *Stx1/2* expression can be blocked by SOS response (such as administration of quinolone). It was therefore attempted to decide whether antibiotics to stop Stx development of pre and post activation of the host SOS mechanism that suppresses bacterial toxin gene expression can be applied (Zhang et al., 2016).

Nevertheless, in recent decades, has been developed an interest in the management of STEC-based infections with antibacterial drugs. The threat of HUS (caused STEC *E. coli*) subjects has subsequently been decreased by ciprofloxacin and subjects treated with azithromycin were also observed during the 2011 outbreak (Freedman et al., 2016; Berger et al., 2019). It was evaluated that the use of rifampicin decreases the toxin synthesis, but with *E. coli* O157 serotype appears to be sustainable, followed by gentamicin treatment at a lethal stage of infection. Especially in comparison with the bactericidal gentamicin dose, this technique was effective in reducing the production of toxins (McGannon et al., 2010; Puño-Sarmiento et al., 2020). A similar approach should be applied in an *E. coli* O157:H7 mice inflammation model that contributed to a higher survival rate for animals (Rahal et al., 2012; Fadlallah, 2014; Rahal et al., 2015). The findings indicated that various STEC serotypes significantly respond to the therapy of antimicrobial agents.

PHAGE BASED PREVENTION

The application of lytic phages is another preventive measure suggested as a way to monitor STEC. Lytic phages have shown that they may be reduced by the amount of STECs *in vitro* (Rivas et al., 2010; Chen et al., 2020). Phage-containing materials for the management of STEC species can be spread in animal fur or processed meats, are commercially available, and are licensed by the Food and Drugs Administration (FDA) (Anany et al., 2011a; Anany et al., 2011b; Tolen, 2018; Endersen and Coffey, 2020). Nevertheless, the effectiveness of oral treatment on domesticated animals with lytic phages was shown to be effective, and an improved method or delivery method is required (Pinto et al., 2020). In the case of human safety and effectiveness, the application of phages acts as a therapeutic utility against STEC *E. coli*.

Numerous vaccine-based scenarios have been attempted to establish the antimicrobial strategies that include bacterial secondary metabolite-based peptides and virulence factors like (Jamalludeen, 2006; Nonis, 2016; Tolen, 2018; Akindolire, 2019).

These vaccine formulations have been tested with some positive findings in mice models (Garcia-Angulo et al., 2013).

DNA BASED VACCINES TOWARDS PREVENTION OF STEC

Antibiotic treatment of STEC-infected patients increases the incidence of infection rather than amelioration, possibly due to cell wall damage of STEC *E. coli* and the liberation of more Shiga toxins. Consequently, there remains a need to develop a technique to generate antibodies against *E. coli* to prevent and alleviate STEC infections. Hence, vaccination is considered an appealing strategy to reduce STEC colonization. Vaccines are substances that interact with the immune system to trigger antibodies' production, which subsequently provides immunity against serious, life-threatening diseases (Nabel, 2013; Greenwood, 2014; Gómez and Oñate, 2018). The notion of vaccination was revealed 200 years ago when Jenner demonstrated that former acquaintance to cowpox could avert infection by smallpox (Gurunathan et al., 2000).

DNA vaccines comprise a bacterial plasmid with a robust viral promoter, the gene of interest, and a transcriptional stop sequence (Snedeker et al., 2012; Zhang and Sack, 2015). The genetically engineered DNA-based vaccine is taken up by host cells where the encoded protein is made. Recent research has explored the prevention of STEC infections using DNA-based vaccines (Bourgeois et al., 2016; Harding and Feldman, 2019; Jeshvaghani et al., 2019; Rodrigues-Jesus et al., 2019). Animal sources are one of the main reservoirs for STEC and a leading cause of STEC infections in cattle. Vaccination is a promising strategy to reduce the prevalence of STEC in cattle and significantly reduce the incidence of disease in humans (Cox et al., 2014; Saeedi et al., 2017). Four immunologically significant genes, *stx, espA, eae*, and *tir* are the leading DNA vaccine candidates to prevent STEC infections (**Supplementary Table 7**). Many researchers have reported on DNA-based vaccines targeting these leading candidates (Bentancor et al., 2009; Cai et al., 2011; Gu et al., 2011; Mehr et al., 2012). Gao et al. (2009) described DNA vaccination targeting stx_1 and stx_2 using the mouse model. A novel fusion protein was developed and induced a high level of humoral IgG in mice. This fusion protein elicits a high level of neutralizing antibodies and protected mice from the lethal dose challenge of STEC. Mehr et al. (2012) generated a protective immune response against EHEC by producing antibodies targeting *Esp*A and *Tir* proteins. DNA vaccine could induce protective immunity in BALB/c mice against *E. coli* O157:H7. A DNA vaccine has been reported in another study that targets *stx*, *esp*A, and intimin virulence factors. The vaccine-induced a strong humoral response and protected mice against infection with live EHEC or EHEC sonicated lysate. Besides, the vaccine enhanced evacuation of gut colonized *E. coli* (Gu et al., 2009). Meanwhile, many of the alternative virulence factors such as *EspB, EspD, NleA, TccP*, and *NleB* of EHEC have attracted vaccine designers' attention (Creuzburg and Schmidt, 2007; Roe et al., 2007; Misyurina et al., 2010). Asper et al. (2011) recently constructed a vaccine generating antibodies against *EspB, EspD*, *NleA*, and *EspA*. These factors contributed to the future candidates for the treatment of STEC infections.

DNA vaccination is a novel, economic, and effective strategy to prevent various infectious diseases, with additional advantages over live attenuated bacteria including the ease of design and construction, low cost, safety, and long-lived responses (Creuzburg and Schmidt, 2007; Roe et al., 2007; Gao et al., 2009; Gu et al., 2009; Fioretti et al., 2010; Misyurina et al., 2010; Asper et al., 2011; Mehr et al., 2012). Furthermore, DNA vaccines are a promising strategy to decrease STEC infection and spread in animals, the environment, and eventually humans. However, many challenges remain in developing a vaccine for humans. The success of a DNA vaccine depends on the nature of the host to be immunized, optimization of the DNA vaccine, an appropriate choice of a plasmid vector, and the type of immune response generated (Smith et al., 2006; Tsuji et al., 2008; Cai et al., 2011).

CURRENT SCENARIO OF PROBIOTIC THERAPY IN ERADIATION OF STEC TOWARDS A REPLACEMENT OF ANTIBIOTIC THERAPY

Lactic Acid Bacteria (LAB) are Gram-positive, non-motile, non-spore-forming, facultative, or obligate anaerobes with a spherical or rod-like shape. LAB can stimulate numerous immune responses by distressing specific receptors in the host's gut or immune cells (Bene et al., 2017; Kim et al., 2017). Activation of these receptors leads to the production of widely used effectors, such as cytokines or T cells (Frederick et al., 2017). These LAB, which have a beneficial effect on health, are often referred to as probiotics. Probiotics are live microbes which promoted for health benefits and help to restore the microbiota. Probiotics are safe to consume but the specific functional metabolic activity has to be determined for a particular health effect (Georgalaki et al., 2017) (**Figure 3**). Effective probiotics that are nonpathogenic and non-toxic were also needed to be proficient in exerting a beneficial effect on the host. Probiotic organisms can help control pathogenic bacteria by producing a wide range of antimicrobial substances such as acidophil, bacitracin, bacteriocin and some minor short-chain fatty acid, which binds with the bacterial cell wall-based lipoprotein leads to damage the cell wall or it may bind with the topoisomerase enzyme, which intends to inhibit the replication process further it may inhibit the protein synthesis, further some of the polyphenolic compounds such as quaracitin or tannin compounds which may also act as an inhibitory compound. The Lactic acid produced by probiotics decreases the pH of the host intestine and inhibits bacterial pathogens such as the genera *Escherichia*, *Clostridium*, *Salmonella*, and *Shigella*. Furthermore, effective probiotic organisms reduce the production of various toxic or carcinogenic metabolites and competitively block adhesion sites of intestinal epithelium inside the host (Singh et al., 2013; Rodjan et al., 2018) (**Supplementary Table 8**).

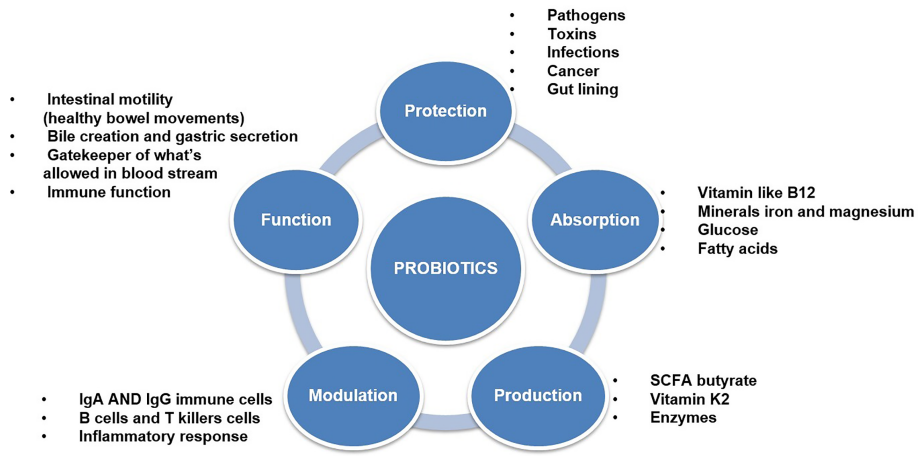

FIGURE 3 | Efficacy of probiotics and different types of functional properties.

EFFECT OF PROBIOTIC YEAST THERAPY AGAINST STEC

Yeasts are eukaryotic microbes widely found in natural environments, such as animal microbial flora, soil, plants, water, airborne particles, food, and other niches (Kurtzman et al., 2011; Hatoum et al., 2012). Yeasts play a significant role in complex ecosystems (Möndel et al., 2009). They also interact with numerous defined microorganisms in a range of processes including mutualism, symbiosis, parasitism, and antagonism. Yeasts are a significant component of the micro-flora of different fermented foods and beverages; the yeast habitat of human and animal origins has a significant impact on their food safety and nutritive features. Brewer's yeasts (*Saccharomyces* spp.) are most commonly available as dietary supplements due to their enhanced nutritional and mineral content. Despite their non-human origin, such non-pathogenic yeasts fulfill the key criteria for probiotic definition as follows; the World Health Organization defines probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (WHO, 2002); to be labeled probiotic, scientific evidence for the health benefit would have to be documented. The most common probiotics are Gram-positive LAB of the genera *Lactobacillus sensu lato* and *Bifidobacterium*, but yeasts such as *Saccharomyces boulardii* (Terciolo et al., 2019) are also used as dietary supplements or as a pharmaceutical aid for therapeutic agents (Möndel et al., 2009). *S. boulardii* is non-pathogenic yeast that has been used internationally and extensively as a probiotic since the 1950s. Lyophilized yeast (*S. boulardii*) is available as a dietary supplement for children and adults in 250 mg capsules and may be prescribed as 1–2 capsules to be taken 1–2×/day by Biocodex, USA. The product package shows the following claims for structure/function: a) retains the intestinal flora balance, b) keeps the intestines working well, and c) helps intestinal health. Using a gnotobiotic mouse model of *Shigella flexneri* infection, (Rodrigues et al., 2000) proved that *S. boulardii* cultures protected mice from pathogen-associated tissue harm without lowering the levels of *Shigella flexneri* in the bowel. The direct impacts of *S. boulardii* on the intestinal mucosa, specifically the stimulus of enzymatic activity and the enhancement of the host's intestinal mucosal immune response, are the likely mechanisms by which *S. boulardii* protects the host from diarrheal pathogens (Barc et al., 2008). *S. boulardii* differs significantly from *S. cerevisiae* in metabolic and physiological terms, especially in terms of growth yield and resistance to temperature and acid stress. While the majority of strains of *S. cerevisiae* grows and metabolize at 30°C, *S. boulardii* acts as thermophilus yeast, growing at 37°C, the physiological temperature of the host. Recent research showed that *S. boulardii* was much more resistant to a simulated gastrointestinal condition than *S. cerevisiae* strain W303 (Tiago et al., 2015). The improvement of microbial diversity in the intestines is one of the main modes of action for *S. boulardii* as demonstrated by the use of monogastric experimental designs for improved intestinal health (Möndel et al., 2009).

Interest in probiotic yeast has been raised predominantly in domestic animal feed preparation, and human applications because yeasts are rarely correlated with food-borne illness. Based on their history, most yeast species are recognized as safe by the European Food Safety Authority (EFSA, 2014). Studies reported that some *S. boulardii* strains originally selected using empirical methods, can act as an antidote against various gastrointestinal diseases (Buts, 2009; Vandenplas et al., 2009), hence *S. boulardii* is recognized as prototype of non-bacterial probiotics. Several mechanisms have been suggested for the broad health-promoting effects of consuming food-grade yeasts (Czerucka and Rampal, 2002; Czerucka et al., 2007). Some of the reported effects of yeasts as probiotic organisms in clinical trials are (i) Antibiotic-associated diarrhea; (ii) Infectious diarrhea (including that caused by recurrent *Clostridium difficile* infection); (iii) irritable bowel syndrome; and (iv) Inflammatory

bowel diseases (IBD) (Foligne J. et al., 2010). *S. boulardii* efficacy was both in preventing and treating diarrhea and colitis in humans associated with antibiotics (Guslandi et al., 2003).

EFFECT OF PREBIOTICS (OLIGOSACCHARIDES) WITH SYNBIOTIC ACTIVITY TOWARDS REDUCTION OF STEC INFECTION

The pectic-based oligosaccharide from the plant-based origin has been previously reported to control STEC pathogens; the pectin consists of homogalacturonan as a backbone and arabino-galacto oligosaccharide, which is enzymatically treated and methylated and protects the human colonic HT29 cells from the Shiga toxin-producing *E. coli* at 10mg/mL. Previous reports suggest that galacturonic acid disaccharides supported the anti-adhesion activity and trisaccharides against *E. coli*; further, oligosaccharides' concentration was mainly correlated with the anti-adhesion activity. Likewise, a study conducted by Hotchkiss (2015). Indicated that Xyloglucon extracted from the cranberry act as an effective inhibitory to the adhesion of STEC strain in the HT29 cells at a lower concentration, and it was also concluded that the adhesion mechanism was mainly due to the fimbriated *E. coli*. Pectin is extracted from the root of Panax ginseng, primarily consisting of galacturonic and glucuronic acids with rhamnose, arabinose, and galactose present as minor components exerted selective anti-adhesive effect against pathogenic bacteria *E. coli* and *Staphylococcus aureus*.

Prebiotic oligosaccharides, including FOS, XOS, and GOS, are classified as non-digestible dietary ingredients that benefit the host gastrointestinal tract (**Figure 4**). Initial non-intimate adherence is therefore an essential aspect of STEC pathogenesis because it is the first infection stage. Preventing this first adherence step will eventually hinder the cycle of infection. Oligosaccharides can stimulate the growth of beneficial intestinal microbial groups such as *Lactobacillus* spp. and *Bifidobacterium* spp., reduce constipation, and decrease colon cancer risk, promote immune-stimulation in the intestinal tract, and improve the function and health of the intestinal tract (**Figure 5**). Some intestinal pathogens, such as STEC, express multifarious proteins that allow them to adhere to separate receptor sites of oligosaccharides located on the host cell surface (Baker et al., 2016; Saeedi et al., 2017; Valilis et al., 2018). FOS is composed of glucose and fructose molecules linked by a degree of polymerization (DP) of 2-9 (DP is the number of glucose and fructose molecules) (Soleimani et al., 2012). Detailed investigations have demonstrated that oligosaccharides have functional effects in lipidemia and cholesterol decreases (Manosroi et al., 2014), inhibition of aberrant crypt foci formation, prevention of osteoporosis due to increased bone strength, inhibition of diarrhea (Akrami et al., 2013), and reduction of the risk of atherosclerotic cardiovascular disease (Ahmed, 2014). Different oligosaccharides have also shown anti-adhesive activity. Dietary oligosaccharides have been isolated from natural sources such as food grains, agricultural waste products, human breast milk, while others have been synthesized based on the known components of glycolipid oligosaccharides and glycoproteins that border the gastrointestinal tract cell surface (Baker et al., 2016). Numerous reports support the role of glycolipids, glycoproteins, and soluble oligosaccharides as molecular decoys to host cell surface oligosaccharides (**Figure 6**).

Probiotics based on beneficial microbial strains have additional health benefits (Chelliah et al., 2018). Some probiotics are effective against specific toxin-producing enteropathogens such as emetic toxin-producing *Bacillus cereus*, STEC, enterohemorrhagic *E. coli* (EHEC), and enteropathogenic *E. coli* (EPEC), which cause urinary tract infections (UTIs) and gastrointestinal infections (Hwang et al., 2018). Most probiotics were found in the gut microbiota, which mainly originates from our food intake and lifestyle of fermentation-based food products, dairy-related products, and

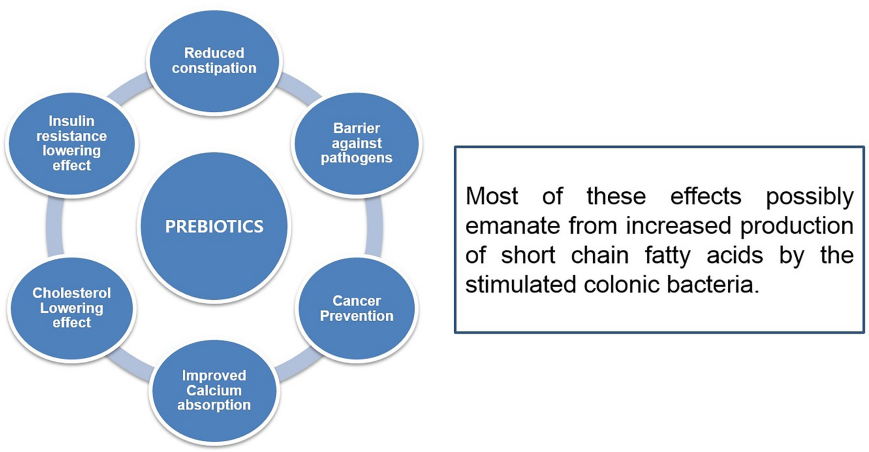

FIGURE 4 | Efficacy of prebiotics and different types of functional properties.

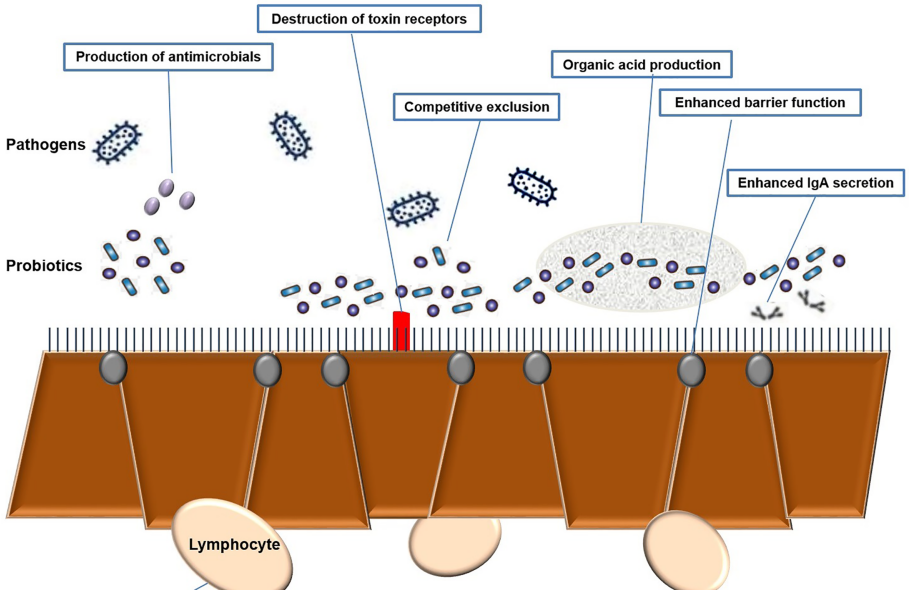

FIGURE 5 | Mechanism of probiotics towards host-pathogen [Shiga toxin-producing *Escherichia coli*, Enterohemorrhagic *Escherichia coli* (EHEC)] interaction.

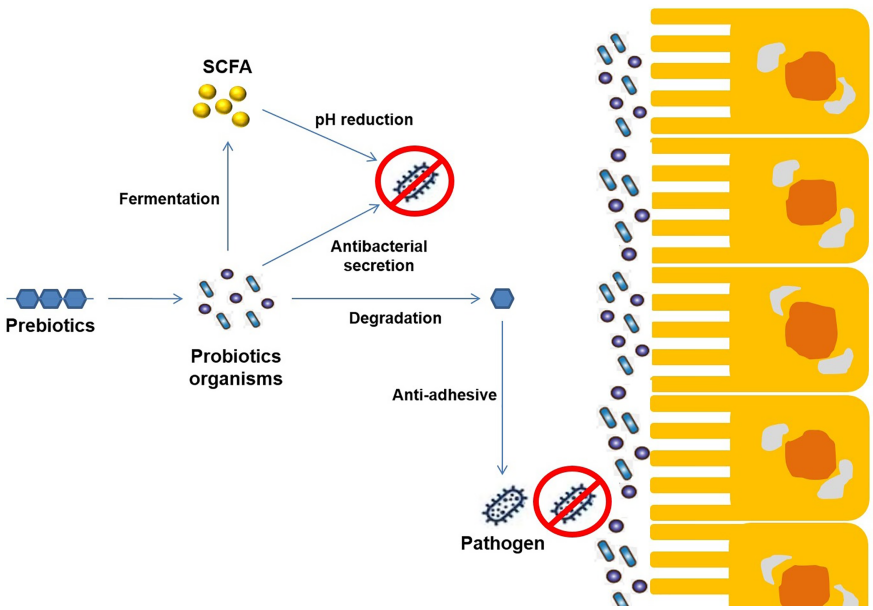

FIGURE 6 | Schematic representation of the mechanism of synergetic activity (Probiotics+ Prebiotics) towards different enteropathogenic infections.

traditional food products based on cereals and pulses (McLellan and Hunstad, 2016; Chelliah et al., 2018). The probiotics vary based on their specificity on functional activity (Gibson et al., 2017). Indirectly specific prebiotic compounds (galactooligosaccharide – GOS, fructooligosaccharide – FOS, xylooligosaccharide XOS, and inulin) encompassed functional food was mainly responsible for the gut microbiota modulation in which the phylum *Bacteroidetes* were enhanced while there was a corresponding decrease in the phylum *Firmicutes* (Chaparro, 2017; Castaner et al., 2018; Collado et al., 2019).

CONCLUSION

Among global foodborne bacterial pathogen outbreaks, the main cause of Gastroenteritis in adults and children is STEC infection. Despite the key improvements in sympathetic of STEC mechanism, no explicit effective management is presently available. The consolidated results in the review open a novel concept towards controlling the STEC infection. Further, based on the *in-vivo* and *in-vitro* data, clinical trial in humans helps us to determine the efficiency of symbiotic treatment (Probiotic+ prebiotics) and a simple cost-efficient reliable methodology were determined to understand and to differentiate the mechanism of STEC and non-STEC infection. Further, a similar methodology can be applied to understand host-pathogen interaction. To sum up, a widely accepted effective therapeutic procedure for the species remains undocumented, despite more than five decades after STEC strain was initially identified with clinical studies. Fortunately, a variety of methods have been pursued, including those to rethink the application of antimicrobial agents; benefits to certain agents, findings with antimicrobial-based results, their dose, and STEC itself, have been recorded. Additional tests of antimicrobial agents for the therapy of infection with STEC in animals should be carried out to select the best and most effective diet to be tested in the clinical trials.

AUTHOR CONTRIBUTIONS

The manuscript was written in detail and sectioned for specialized discussion with the respective authors in the field of research. Designing the outline of the Review manuscript [Shiga-toxin producing *E. coli* (STEC) Gastroenteritis management: Is there a role for Probiotics?: A Systematic Review], Visualization, Conceptualization – (S-bH, RC, D-HO). Mode of transmission of *Escherichia coli* (STEC) and the effect of shiga toxin in humans and animals– JK. 2. Survival efficacy of *Escherichia coli* (STEC) in the intestine environment, Correlation of antimicrobial resistance towards increased toxin gene expression of *Escherichia coli* (STEC) – RC, EB-M. DNA vaccines towards prevention of *Escherichia coli* (STEC), Toxic Effect of *Escherichia coli* (STEC) - *In-vivo* model (Caenorhabditis elegans), Current scenario of probiotic therapy in eradiation of *Escherichia coli* (STEC) towards replacing of antibiotic therapy – RC, S-bH. Effect of probiotic (yeast) therapy against *Escherichia coli* (STEC), Effect of probiotics against *Escherichia coli* (STEC) - *In-vivo* model (Caenorhabditis elegans), Prebiotic based oligosaccharides reduce adherence of enteropathogenic *Escherichia coli* (STEC) – FE, RC. All authors contributed to the article and approved the submitted version. First Author: S-bH, RC (Equal Contribution). *Corresponding author: D-HO (deoghwa@kangwon.ac.kr) *Co-Corresponding author: RC (ramachandran865@gmail.com).

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SUPPLEMENTARY MATERIAL

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