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The Impact of In-Water vs. In-Feed Chlortetracycline and Tiamulin Administration in Piglets on the Fecal Prevalence and Antimicrobial Resistance of *Salmonella*

Victor L. Ishengoma [1], Raghavendra G. Amachawadi [1,*], Mike D. Tokach [2], Qing Kang [3], Robert D. Goodband [2], Joel DeRouchey [2], Jason Woodworth [2] and Tiruvoor G. Nagaraja [4]

[1] Department of Clinical Sciences, College of Veterinary Medicine, Kansas State University, Manhattan, KS 66506, USA; victorishengoma@gmail.com
[2] Department of Animal Sciences & Industry, College of Agriculture, Kansas State University, Manhattan, KS 66506, USA; mtokach@ksu.edu (M.D.T.); goodband@ksu.edu (R.D.G.); jderouch@ksu.edu (J.D.); jwoodworth@ksu.edu (J.W.)
[3] Department of Statistics, College of Arts and Sciences, Kansas State University, Manhattan, KS 66506, USA; kangqing@hotmail.com
[4] Department of Diagnostic Medicine and Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS 66506, USA; tnagaraj@vet.ksu.edu
* Correspondence: agraghav@vet.ksu.edu; Tel.: +1-785-532-4356

Abstract: Antimicrobial Resistance (AMR) in bacteria is a growing public health concern in the US and around the world threatening the continual use of antimicrobials. In pigs, the oral route, either in-feed or in-water, is by far the most common route of administration of antimicrobials. Because the distribution of the antibiotic in the gut and the dosages are different, the impact of in-feed vs. in-water administration of antibiotics on the prevalence of pathogens, such as *Salmonella*, and the development of AMR are likely to be different. Therefore, a study was conducted to compare in-feed vs. in-water administrations of chlortetracycline (CTC) and/or tiamulin on the fecal prevalence and AMR profiles of *Salmonella* in nursery piglets. A total of 1296 weaned piglets, housed in 48 pens (27 piglets per pen), were assigned randomly to six treatment groups: Control (no antibiotic), in-feed CTC, in-water CTC, in-feed tiamulin, in-water tiamulin, or in-feed CTC and tiamulin. Fecal samples (n = 1440) were collected randomly from five piglets from each pen during the pre-treatment (days 7, 0), treatment (days 7, 14), and post-treatment (days 21, 28) phases. *Salmonella enterica* isolation and identification were completed by culture and PCR methods. The microbroth dilution method with Sensititre™ (ThermoFisher Scientific, Lenexa, KS, USA) plates was used to determine the antimicrobial susceptibility and resistance of *Salmonella* strains. The susceptibility and resistance were interpreted based on the Clinical and Laboratory Standards Institute guidelines. The overall prevalence of *Salmonella* was 3.0% (43/1440). All isolates belonged to *Salmonella enterica* subsp. *enterica* serovar Typhimurium. *Salmonella* isolates were susceptible to azithromycin and resistant (100%) to ampicillin, streptomycin, sulfisoxazole, tiamulin, and tetracycline. Neither antibiotic, CTC or tiamulin, nor the route of administration, in-feed or in-water, had an effect ($p > 0.05$) on the occurrence of resistant *Salmonella* in the feces of piglets.

Keywords: piglets; antibiotic administration route; in-feed; in-water; chlortetracycline; tiamulin; *Salmonella*

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1. Introduction

Antimicrobial Resistance (AMR) is a global public health concern and is listed among the top health challenges in the world [1]. In the United States, 2.8 million AMR bacterial and fungal infections are estimated to occur, resulting in approximately 35,900 deaths every year [2]. The public health risks associated with antimicrobial use as growth promoters in food animal production have led to regulations to restrict antimicrobial use in many

countries, including in the US. Antimicrobials have been used in swine routinely as a management tool by farmers [3]. The weaned (nursery) piglets consume most of these antimicrobials as they are more vulnerable to infectious diseases [4,5]. Several studies have shown a clear association between antimicrobial use and the development of AMR in food animals [6,7].

The use of In-feed antimicrobials for disease prevention has been an integral part of swine production and management. Tetracyclines, macrolides, and pleuromutilin are the most commonly used antimicrobials in swine [8]. Chlortetracycline (CTC), a tetracycline, is widely used in nursery pigs and is available for use by three routes of administration (in-feed, in-water, and injectable) [4,9,10]. Chlortetracycline (CTC) is used in nursery pigs to treat respiratory and enteric infections [11]. Tiamulin, a pleuromutilin and another commonly used antibiotic in nursery pigs to treat pneumonia and swine dysentery caused by *Brachyspira hyodysenteriae*, is administered in-feed or in-water [4,10]. Tiamulin is not considered medically important, does not require veterinary feed directives for its usage, and is likely to be frequently used or overused by swine producers.

Salmonella enterica is a foodborne pathogen of major public health concern. In the US, non-typhoidal *Salmonella* is estimated to cause annually about 1.35 million infections, 26,500 hospitalizations, and 420 deaths (https://wwwn.cdc.gov/norsdashboard/; accessed on 29 November 2023) and (https://www.cdc.gov/salmonella/index.html; accessed on 24 January 2024). Based on the CDC's National Outbreak Reporting System (NORS), between 2008 and 2021, a total of 2904 *Salmonella* outbreaks have been reported in the US with 68,050 illnesses, 10,839 hospitalizations, and 109 deaths [12,13]. The US Department of Agriculture (USDA) Economic Research Service (ERS) reports the total annual cost of foodborne illnesses due to *Salmonella* is $3.6 billion [14]. Antimicrobial-resistant *Salmonella* is categorized as a serious threat level by the CDC and causes about 212,500 infections, resulting in about 70 deaths every year [2]. Considering its ubiquitous nature, *Salmonella* prevalence varies across the swine production systems [15]. Bernard-Roche et al. [16] reported a 36% prevalence of Salmonella among piglets.

Apart from implementing management systems to monitor and minimize antimicrobial use, optimizing the dosage and the route of administration can be an effective strategy to mitigate the development and dissemination of AMR without affecting the therapeutic efficacy of the antimicrobials. In swine, the oral route (in-feed and in-water) has been the most commonly used route of antimicrobial administration to treat pigs in groups [10,13]. Based on a systematic review, the oral administration of antimicrobials is considered to be a critical risk factor in the development and propagation of AMR in swine [17]. A study conducted in mice suggested that administering antimicrobials orally caused a notable increase in AMR in the gut microbiota compared to intravenous injection [18]. Another study in humans recommended that parenteral antibiotic administration is preferred over oral antibiotic administration as the gut commensal and pathogenic bacteria are less exposed to the antimicrobials, leading to less chance of resistance selection [19]. We hypothesize that a water-soluble antibiotic, administered in drinking water, is likely to be distributed more uniformly in the gut and that it, therefore, has a greater impact on the AMR of gut bacteria than the same antibiotic administered in a dry form mixed with the feed. Therefore, the route of administration of antimicrobials in food animal production is likely to be a significant contributing factor in the development of AMR. The impact of in-water vs. in-feed antibiotic administration on the development and persistence of AMR in the foodborne pathogen, *S. enterica*, has not been studied in nursery pigs. Therefore, the objective of this study was to assess the impact of the in-feed and in-water administration of CTC and tiamulin on the prevalence and AMR profiles of *S. enterica* in the feces of piglets.

2. Materials and Methods

2.1. Animals and Experimental Design

The experimental protocols used in this study were approved by the Kansas State University Institutional Animal Care and Use Committee (IACUC # 4033). The study was

conducted at a commercial swine research facility in the Midwest. The facility is totally enclosed, environmentally controlled, and mechanically ventilated. Pens were equipped with slatted flooring and deep pits for manure storage. Each pen consisted of a six-hole stainless steel self-feeder and a pan waterer to provide ad libitum access to feed and water. A total of 1296 weaned piglets (L337 × 1050, PIC, Hendersonville, TN, USA) were used in the study. Piglets, weaned at 21 days of age, were allocated into 48 pens (27 piglets per pen) distributed in a single room so that average pen body weights were relatively equal among pens. After a 7-day acclimatization period, pens were randomly assigned to the following six treatment groups: (a) Control: piglets fed a basal diet with no antibiotic administration, (b) In-feed CTC: piglets fed the basal diet supplemented with CTC to provide 22 mg/kg body weight (BW; CTC-hydrochloride, Elanco-Eli Lilly and Company, Indianapolis, IN, USA) for 14 days, (c) In-water CTC: piglets fed the basal diet with CTC administered in drinking water to provide 22 mg/kg BW for 14 days, (d) In-feed tiamulin: piglets fed the basal diet supplemented with tiamulin to provide 5 mg/kg BW (Denagard®, Elanco Animal Health-Eli Lilly and Company, Greenfield, Indianapolis, IN, USA) for 14 days, (e) In-water tiamulin: piglets fed a basal diet with tiamulin administered in drinking water to provide 23 mg/kg body weight for 14 days, and (f) a combination of CTC and tiamulin (In-feed): supplemented with CTC to provide 22 mg/kg BW and tiamulin at 5 mg/kg BW for 14 days. Each treatment had 8 pens, and the treatment allocations to the pens followed a blocked design that ensured adjacent pens alternated among treatment groups. Also, the treatment allocation ensured that each pen treatment was in contact with an equal number of other pen treatments. The basal diet consisted of corn, soybean meal, vitamins, amino acids, and trace mineral supplements. Daily feed additions to each pen were accomplished through a robotic feeding system to provide measured amounts for individual pens. The duration of the study was 35 days, which was divided into three phases: pre-treatment (days 0 and 7), treatment (days 14 and 21), and post-treatment (days 28 and 35). In this study, all the antibiotic dosages and combinations are as per the FDA guidance for the treatment and prevention of diseases.

2.2. Fecal Sample Collection

Fecal samples were collected randomly from 5 piglets from each pen by a gentle rectal massage with proper restraining. Fecal samples were placed in individual plastic bags (Whirl-Pak® bags, Nasco, Ft. Atkinson, WI, USA) and transported on ice in a cooler to the Pre-harvest Food Safety Laboratory, Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, at Kansas State University and stored at 4 °C before processed within 24 h. The laboratory personnel were blinded to the treatments.

2.3. Isolation of Salmonella

Approximately, 10 g of fecal sample was added to 90 mL of Tetrathionate (TT) broth (Difco™, Sparks, MD, USA) with iodine added just before processing in high-temperature Nasco Whirl-Pak plastic bags (Nasco, Ft. Atkinson, WI, USA). The contents were mixed well and incubated at 37 °C for 24 h. One milliliter of TT broth was transferred to 9 mL of Rappaport Vassiliadis (RV) broth (Difco™) and incubated at 42 °C for 24 h. After enrichment, the RV broth was plated onto Hektoen Enteric (HE) agar (Difco™) and incubated at 37 °C for 24 h. A presumptive *Salmonella* colony (green colored with black center colonies) was picked and streaked onto a blood agar plate and incubated overnight at 37 °C. The *Salmonella* isolates from each sample were subjected to a rapid slide agglutination test using *Salmonella* antisera (BD Difco™ *Salmonella* O Antiserum Poly A-I & VI) for *Salmonella* identification. Fecal samples with presumptive *Salmonella* colonies and agglutination positive were considered *Salmonella* positive for further analyses.

2.4. PCR Identification of Salmonella enterica

DNA was extracted using the boil-prep method by suspending two bacterial colonies in 150 µL of nuclease-free water, followed by boiling for 10 min. The boiled bacterial

lysate was centrifuged at 16,000× *g* for 2 min, and the resulting supernatant was used as a DNA template for PCR reactions. Species confirmation was completed by detecting *invA* (encodes for an inner membrane protein of the type III secretion system) and *pagC* (encodes for a periplasmic nonspecific phosphatase) genes using a duplex real-time PCR assay [20].

2.5. Antimicrobial Susceptibility Testing

Minimum inhibitory concentrations of antibiotics (MIC) to *Salmonella* isolates were determined using commercial Sensititre panel plates (Sensititre™ CMV3AGNF, Trek Diagnostic Systems, Thermo Scientific Microbiology, Table 1). Bacterial colonies grown overnight on blood agar plates were suspended in demineralized water (Trek Diagnostic Systems), vortexed, and adjusted to 0.5 McFarland turbidity standard (Trek Diagnostic Systems, Thermo Scientific Microbiology). Ten microliters of the suspension were transferred to cation-adjusted Mueller-Hinton (Trek Diagnostic Systems) broth and vortexed. Then, 50 µL of the culture was inoculated into Sensititre plates and incubated at 37 °C for 24 h. The antimicrobials tested were amoxicillin/clavulanic acid, ampicillin, azithromycin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid, streptomycin, sulfisoxazole, tiamulin, tetracycline, and trimethoprim/sulphamethoxazole. The Clinical and Laboratory Standards Institute (CLSI, 2023) guidelines for antimicrobial breakpoints were used to interpret the MIC values as either resistant (including intermediate) or susceptible. To obtain tiamulin MICs, the commercially prepared panels (Sensititre™ BOPO7F, Trek Diagnostic Systems, Thermo Scientific Microbiology Table 1) were used. The veterinary standards were used to determine the resistance breakpoint for tiamulin since there are no values established by CLSI. Isolates that showed resistance to three or more antimicrobial classes were classified as multidrug-resistant (MDR) [21].

Table 1. The antimicrobial agents, concentrations, interpretive criteria, and WHO classifications applied for the study.

Antimicrobial Agent	Concentration, µg/mL	Breakpoint, µg/mL [1]	WHO Classification [2]
Amoxicillin/Clavulanic Acid	1/0.5–32/16	≥16/8	Critically important
Ampicillin	1–32	≥16	Critically important
Azithromycin [3]	0.12–16	N/A	Critically important
Cefoxitin	0.5–32	≥16	Highly important
Ceftiofur	0.12–8	≥4	Critically important
Ceftriaxone	0.25–64	≥2	Critically important
Chloramphenicol	2–32	≥16	Highly important
Ciprofloxacin	0.015–4	≥0.12	Critically important
Gentamicin	0.25–16	≥8	Critically important
Nalidixic Acid	0.5–32	≥32	Critically important
Streptomycin [4]	2–64	≥64	Critically important
Sulfisoxazole	16–256	≥256	Highly important
Tetracycline	4–32	≥8	Highly important
Tiamulin [5]	0.5–32	≥32	Important
Trimethoprim/Sulfamethoxazole	0.12/2.38–4/76	≥4/76	Highly important

[1] Resistance breakpoints based on Clinical and Laboratory Standards Institute (CLSI) guidelines for breakpoint (CLSI, 2023) except for streptomycin, azithromycin, and tiamulin, which have no CLSI breakpoints. [2] Categorization of antimicrobials for human medicine according to the World Health Organization (WHO, 2019). [3] CLSI has not established resistance breakpoints for azithromycin. [4] CLSI has not established resistance breakpoints for streptomycin; interpretive standards used are the National Antimicrobial Resistance Monitoring System (NARMS)-established breakpoints for resistance monitoring. [5] CLSI has not established resistance breakpoints for tiamulin; veterinary standards were used instead.

2.6. PCR Detection of Tetracycline Resistance Genes

The DNA extracted from *Salmonella* was screened for *tet* (A, B, C, D, and E) genes by PCR. *E. coli* positive control strains were procured from USDA (Meat Animal Research Center, Clay Center, NE, USA) and Dr. Marilyn C. Roberts at the University of Washington. The target genes and primers used are shown in Table 2. The 20 µL reaction volume

consisted of 10 µL of the master mix, 6 µL of nuclease-free water, 1 µL each of forward and reverse primers, and 3 µL of the DNA template. Reactions were run in a Master-cycler gradient thermal cycler (Eppendorf, Hamburg, Germany), and the conditions were 1 cycle of initial activation at 95 °C for 3 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 90 s, and extension at 72 °C for 90 s, followed by a cycle of final extension at 72 °C for 10 min. Analysis of PCR products was completed by capillary gel electrophoresis in the QIAxcel Advanced system (Qiagen, Germantown, MD, USA).

Table 2. PCR target genes and primers used for detection of *tet* (A, B, C, D, and E) resistance genes in *Salmonella* isolated from piglets administered with in-feed or in-water chlortetracycline and tiamulin.

Target Gene	Primer Sequences (5′ to 3′)	Amplicon Size (bp)	Source
tet(A)	TET A1: CGA GCC ATT CGC GAG AGC TET A2: CGA AGC AAG CAG GAC CAT G	2027	[22]
tet(B)	TET BF: CAG TGC TGT TGT TGT CAT TAA TET BR: GCT TGG AAT ACT GAG TGT AA	576	[22]
tet(C)	TET C: TTG CAT GCA CCA TTC CTT GCG TET CR: TGG TCG TCA TCT ACC TGC C	521	[22]
tet(D)	TET D FW2: GGA TAT CTC ACC GCA TCT GC TET D(RV1): CAT CCA TCC GGA AGT GAT AGC	436	[22]
tet(E)	TET EF: TCC ATA CGC GAG ATG ATC TCC TET ER: CGA TTA CAG CTG TCA GGT GGG	442	[22]

2.7. Whole Genome Sequencing and Bioinformatics

DNA libraries from *Salmonella* isolates (n = 43) were prepared using the Illumina Nextera XT library preparation kit, with a modified protocol. The library quantity was assessed with Qubit (ThermoFisher). The libraries were then sequenced on an Illumina HiSeq platform 2 × 150 bp. Raw sequencing reads were trimmed and processed using BBDuk with a read quality trimming parameter of 22 for further downstream analysis [23]. The trimmed fastqs were assembled using SPAdes with the careful parameter [24]. The assembled contigs were then processed through the CosmosID core-genome SNP typing pipeline to evaluate the phylogenetic placement and SNP differences for meaningful epidemiological inferences. The CosmosID SNP typing pipeline uses Parsnp as the core-genome aligner to align the core genome of multiple microbial genomes [25]. While generating the core-genome alignment with the default parameters, Parsnp considers the recombination events and the variation that might exist within the microbial genomes. The final set of core-genome SNPs is then used by Parsnp to reconstruct the phylogenomic relationship among the genome using FastTree2 [26]. Bioinformatics tools from the Center for Genomic Epidemiology (CGE) were used to detect AMR genes (ResFinder; http://genepi.food.dtu.dk/resfinder, accessed on 20 January 2024) [27,28] and plasmids (PlasmidFinder; https://cge.food.dtu.dk/services/PlasmidFinder/, accessed on 20 January 2024) in the genomes [29].

2.8. GenBank Accession Numbers

Assembled genomes from 43 *Salmonella* isolates have been deposited in the National Center for Biotechnology Information (NCBI) under Bioproject Accession number PRJNA663574.

2.9. Statistical Analysis

Statistical analysis was performed using the Statistical Analysis Software (SAS version 9.4; Cary, NC, USA) LOGISTIC procedure with a sampling day in the STRATA statement and treatment group in the EXACT statement. Prevalence data were organized as a binomial response on the pen basis, i.e., the number of '+' out of 5 samples per pen per sampling day. Because of the low prevalence rate, the exact conditional logistic regression approach was used to evaluate the treatment effect for Day 7 and Day 14 only. The block

served as the stratifying variable for the conditional inference. Fixed effects of the model included treatment and the number of '+' samples in the pen on Day 0. All tests were conducted at the 0.10 significance level. Comparisons between the treatment and control were carried out using the 2-sided exact test when the treatment group had '+' samples and the 1-sided exact test when the treatment group had no '+' samples [30]. The MIC data categorized as resistant or susceptible to 15 different antimicrobials were considered as binary responses and multidrug profiles. The MICs were expressed as median after the assumption of normality. A likelihood ratio chi-square test was performed to assess the relationship between the *Salmonella* prevalence and MDR profiles.

3. Results

3.1. Fecal Prevalence of Salmonella

A total of 1440 fecal samples were collected (240 fecal samples per week) during the study period. The overall prevalence of *Salmonella* was 3.0% (43/1440). The fecal prevalence of *Salmonella* within the treatment groups, treatment phases, and sampling week are displayed in Table 3. Neither antibiotic route of administration nor sampling week had an effect ($p > 0.10$) on the fecal prevalence of *Salmonella*. The prevalence of *Salmonella* was numerically higher among piglets in the in-water tiamulin (4.2%, n = 240) group compared to the control (2.5%), in-feed tiamulin (1.7%), in-feed CTC (2.1%), in-water CTC (3.8%), and in-feed combination of CTC and tiamulin (3.8%). The treatment phase had the numerically highest prevalence of *Salmonella* (5.0%, 24/480) when compared to the pre-treatment (3.5%, 17/480) and post-treatment phases (0.42%, 2/480), but the differences were not significant.

Table 3. Animal-level fecal prevalence of *Salmonella enterica* in piglets administered with in-feed or in-water chlortetracycline (CTC) and/or tiamulin (n = 1440).

Treatment Group	Treatment Phases						Total (%)
	Pre-Treatment		Treatment		Post-Treatment		
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	
Control	1	1	3	1	0	0	6 (2.5)
In-feed CTC	1	2	2	0	0	0	5 (2.1)
In-water CTC	0	3	4	1	0	1	9 (3.8)
In-feed Tiamulin	0	3	1	0	0	0	4 (1.7)
In-water Tiamulin	0	3	5	2	0	0	10(4.2)
In-Feed CTC + Tiamulin	0	3	3	2	0	1	9 (3.8)
Weekly Total (%)	2 (0.8)	15 (6.3)	18 (7.5)	6 (2.5)	0	2 (0.8)	
Total (%)	17 (3.5)		24 (5.0)		2 (0.42)		43 (3%)

3.2. Prevalence of Tetracycline Resistance Genes

A tetracycline resistance gene PCR was conducted to see if chlortetracycline (CTC), one of the treatment groups in our experimental study, had any effect (positive or negative) on the prevalence of *Salmonella*. *Salmonella* isolates (n = 43) were screened for *tetA*, *tetB*, *tetC*, *tetD*, and *tetE* genes. The *tetB* gene was detected in all 43 isolates (100%). Only 11 (25.6%) and 4 (9.3%) isolates were positive for *tetD* and *tetA* genes, respectively, while all isolates were negative for *tetC* and *tetE* genes. Fifteen *Salmonella* isolates were positive for two tetracycline resistance genes (*tetA* and *tetB*), with 11 positives for *tetB* and *tetD* and four isolates positive for *tetA* and *tetD*. Only one isolate harbored three tetracycline-resistant genes (*tetA*, *tetB*, and *tetD*).

3.3. Antimicrobial Susceptibility Phenotypes

The phenotypic resistance and MIC distributions are summarized in a squashtogram as shown in Table 4. Neither CTC nor tiamulin treatment affected AMR profiles of Salmonella strains. All isolates were resistant (100%) to ampicillin, streptomycin, sulfisoxazole, tetracy-

cline, and tiamulin, and the majority were resistant to ciprofloxacin (95.4%) and nalidixic acid (74.4%). All isolates were susceptible to azithromycin (100%) and cefoxitin (100%). A low level of resistance to trimethoprim/sulphamethoxazole (4.7%), ceftriaxone (7.0%), and ceftiofur (7.0%) was observed among the isolates. Resistance to most antimicrobials decreased ($p < 0.10$) over time. All isolates expressed resistance to at least three antimicrobial classes and, hence, were classified as MDR. Six unique resistance phenotypes were identified among the isolates. No isolate was resistant to all antimicrobials used in the susceptibility testing. The most common phenotype belonged to MDR strains, which were resistant to six antimicrobial classes (90.7%; 39/43), followed by resistance to seven antimicrobial classes (7.0%; 3/43), and only one isolate exhibited resistance to five antimicrobial classes (2%; 1/43). The most frequently observed (48.8%; n = 43) AMR phenotype was hepta-resistance pattern AMP_CIP_FIS_NAL_STR_TET_TIA (ampicillin, ciprofloxacin, streptomycin, sulfonamides, and tetracycline).

Table 4. The MIC * distribution and percentage of resistance among *Salmonella* strains isolated from piglets administered with in-feed or in-water chlortetracycline and/or tiamulin (n = 43).

Antimicrobials	Resistant Breakpoint	% Resistant	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256
Amoxicillin/ Clavulanic Acid	≥16/8	20.9									2	32	2	7			
Ampicillin	≥16	100												43			
Azithromycin	≥32	0									31	3	9				
Cefoxitin	≥16	0								30	11	2					
Ceftiofur	≥4	7				3	1	34	2	1	2						
Ceftriaxone	≥2	7					40	0	0	0	0	0	1	2			
Chloramphenicol	≥16	2.3									7	35	1				
Ciprofloxacin	≥0.12	95.4		1	1	0	0	21	20								
Gentamicin	≥8	20.9						27	3	2	2	2	7				
Nalidixic Acid	≥32	74.4									1	2	8	32			
Streptomycin	≥32	100													43		
Sulfisoxazole	≥256	100															43
Tetracycline	≥8	100												43			
Tiamulin	≥32	100												43			
Trimethoprim/ sulphamethoxazole	≥4/76	4.7				34	3	1	2	1	2						

* MIC = Minimum inhibitory concentration. Note: The top dilution tested for each drug should be interpreted as ≥ and the lowest dilution tested for each drug as ≤. Phenotypes: AMP = ampicillin, CIP = ciprofloxacin, AUG = amoxicillin/clavulanic acid, AXO = ceftriaxone, CHL = chloramphenicol, FIS = sulfisoxazole, GEN = gentamicin, NAL = nalidixic acid, STR = streptomycin, SXT = trimethoprim/sulfamethoxazole, TET = tetracycline, TIA = tiamulin, XNL = ceftiofur.

3.4. Whole Genome Sequence Results

The number of contigs ranged from 61 to 403, with the N50 ranging from 172,736–325,586 bp. The genome size ranged from 4,906,044 to 5,174,184 bp, with the GC content averaging 52.26%. The Parsnp in the CosmosID core-genome SNP typing pipeline identified all 43 isolates to be *Salmonella enterica* subsp. *enterica* serotype Typhimurium. ResFinder detected the presence of amikacin and tobramycin (aminoglycosides) (aac(6′)-Iaa), amoxicillin, ampicillin, cephalothin, piperacillin and ticarcillin ($bla_{\text{TEM-1B}}$), ciprofloxacin (*qnr*B19), streptomycin (*strA* and *strB*), sulfamethoxazole and sulfisoxazole (*sul2*), and tetracycline (*tetB*) genes in all 43 isolates. None of the strains carried genes that encode for clavulanic acid, azithromycin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, gentamicin, nalidixic acid, tiamulin, and trimethoprim. The correlation between the phenotypic and genotypic resistance to antimicrobials was over 90% for ampicillin, azithromycin, cefoxitin, ceftiofur,

ceftriaxone, chloramphenicol, ciprofloxacin, streptomycin, sulfisoxazole, tiamulin, and tetracycline, as shown in Table 5. Furthermore, Plasmid Finder revealed the presence of Col (pHAD28) and IncQ1 in all strains as shown in Table 6. The low-level resistance to fluoroquinolone was attributed to no mutation in the plasmid-mediated quinolone resistance in the chromosome region. A low correlation was observed between amoxicillin/clavulanic acid (20.9%) and trimethoprim/sulfamethoxazole (4.7%). Despite having resistant genes to amoxicillin and sulfa, the isolates were rendered susceptible by the presence of clavulanic acid and trimethoprim in the two antimicrobials.

Table 5. The association between the phenotypic resistance profiles and the resistance genes detected by whole genome sequencing of the *Salmonella* strains (n = 43) isolated from piglets administered with in-feed or in-water chlortetracycline and/or tiamulin.

	Number of Resistant Strains			
Antimicrobial	**Phenotype +/ Genotype +**	**Phenotype +/ Genotype −**	**Phenotype −/ Genotype +**	**Phenotype −/ Genotype −**
Amoxicillin/Clavulanic Acid	9 (20.9)		34 (79.1)	
Ampicillin	43 (100)			
Azithromycin				43 (100)
Cefoxitin				43 (100)
Ceftiofur		3 (7)		40 (93)
Ceftriaxone		3 (7)		40 (93)
Chloramphenicol		1 (2.3)		42 (97.7)
Ciprofloxacin	41 (95.3)		2 (4.7)	
Gentamicin		9 (20.9)		34 (79.1)
Nalidixic Acid		32 (74.4)		11 (25.6)
Streptomycin	43 (100)			
Sulfisoxazole	43 (100)			
Tetracycline	43 (100)			
Tiamulin		43 (100)		
Trimethoprim/sulphamethoxazole	2 (4.7)		41 (95.3)	

Table 6. A summary comparison of the phenotypic resistance profiles, PCR detection of the selected tetracycline genes, and the resistance genes detected by whole genome sequencing of *Salmonella* isolates (n = 43) recovered from piglets administered with in-feed or in-water chlortetracycline and/or tiamulin.

Salmonella Strain	*** Phenotypic Resistance Profiles**	**PCR Detection of Resistance Genes**	**Resistance Genes Detection by WGS**
120 A_2019-5-*Salmonella*	amp_axo_cip_fis_gen_nal_str_sxt_tet_tia_xnl	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
157 A_2019-5-*Salmonella*	amp_fis_str_tet_tia	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
265 A_2019-5-*Salmonella*	amp_cip_fis_gen_str_tet_tia	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
268 A_2019-5-*Salmonella*	amp_axo_cip_fis_gen_nal_str_sxt_tet_tia_xnl	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
286 A_2019-5-*Salmonella*	amp_cip_fis_str_tet_tia	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
288 A_2019-5-*Salmonella*	amp_cip_fis_nal_str_tet_tia	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
309 A_2019-5-*Salmonella*	amp_aug_axo_cip_fis_gen_str_tet_tia_xnl	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
315 B_2019-5-*Salmonella*	amp_cip_fis_gen_str_tet_tia	*tetB*, *tetD*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B

Table 6. *Cont.*

Salmonella Strain	* Phenotypic Resistance Profiles	PCR Detection of Resistance Genes	Resistance Genes Detection by WGS
317 A_2019-5-*Salmonella*	amp_cip_fis_str_tet_tia	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
325 A_2019-5-*Salmonella*	amp_aug_cip_fis_nal_str_tet_tia	*tetB*, *tetD*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
329 A_2019-5-*Salmonella*	amp_cip_fis_gen_str_tet_tia	*tetB*, *tetD*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
333 A_2019-5-*Salmonella*	amp_cip_fis_nal_str_tet_tia	*tetB*, *tetD*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
337 A_2019-5-*Salmonella*	amp_aug_cip_fis_nal_str_tet_tia	*tetA*, *tetB*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
350 A_2019-5-*Salmonella*	amp_cip_fis_str_tet_tia	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
352 A_2019-5-*Salmonella*	amp_cip_fis_nal_str_tet_tia	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
356 A_2019-5-*Salmonella*	amp_cip_fis_str_tet_tia	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
397 A_2019-5-*Salmonella*	amp_cip_fis_gen_str_tet_tia	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
502 A_2019-5-*Salmonella*	amp_aug_cip_fis_nal_str_tet_tia	*tetB*, *tetD*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
503 B_2019-5-*Salmonella*	amp_chl_fis_gen_str_tet_tia	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
541 A_2019-5-*Salmonella*	amp_cip_fis_nal_str_tet_tia	*tetB*, *tetD*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
565 A_2019-5-*Salmonella*	amp_cip_fis_nal_str_tet_tia	*tetB*, *tetD*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
569 A_2019-5-*Salmonella*	amp_cip_fis_gen_nal_str_tet_tia	*tetB*, *tetD*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
602 A_2019-5-*Salmonella*	amp_aug_cip_fis_nal_str_tet_tia	*tetB*, *tetD*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
612 A_2019-5-*Salmonella*	amp_aug_cip_fis_nal_str_tet_tia	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
624 A_2019-5-*Salmonella*	amp_cip_fis_nal_str_tet_tia	*tetA*, *tetB*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
633 A_2019-5-*Salmonella*	amp_cip_fis_nal_str_tet_tia	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
637 A_2019-5-*Salmonella*	amp_cip_fis_nal_str_tet_tia	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
642 A_2019-5-*Salmonella*	amp_cip_fis_nal_str_tet_tia	*tetA*, *tetB*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
643 A_2019-5-*Salmonella*	amp_cip_fis_nal_str_tet_tia	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
647 A_2019-5-*Salmonella*	amp_cip_fis_nal_str_tet_tia	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
648 A_2019-5-*Salmonella*	amp_cip_fis_nal_str_tet_tia	*tetB*, *tetD*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
669 A_2019-5-*Salmonella*	amp_cip_fis_nal_str_tet_tia	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
685 A_2019-5-*Salmonella*	amp_cip_fis_nal_str_tet_tia	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
693 A_2019-5-*Salmonella*	amp_cip_fis_nal_str_tet_tia	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
720 A_2019-5-*Salmonella*	amp_cip_fis_nal_str_tet_tia	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B
815 A_2019-5-*Salmonella*	amp_cip_fis_nal_str_tet_tia	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, $bla_{\text{TEM-1B}}$, *qnr*B19,*sul*2, *tet*B

Table 6. *Cont.*

Salmonella Strain	* Phenotypic Resistance Profiles	PCR Detection of Resistance Genes	Resistance Genes Detection by WGS
867 A_2019-5-*Salmonella*	amp_cip_fis_nal_str_tet_tia	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, bla_{TEM-1B}, *qnr*B19,*sul*2, *tet*B
884 A_2019-5-*Salmonella*	amp_cip_fis_nal_str_tet_tia	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, bla_{TEM-1B}, *qnr*B19,*sul*2, *tet*B
888 A_2019-5-*Salmonella*	amp_cip_fis_nal_str_tet_tia	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, bla_{TEM-1B}, *qnr*B19,*sul*2, *tet*B
900 A_2019-5-*Salmonella*	amp_cip_fis_nal_str_tet_tia	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, bla_{TEM-1B}, *qnr*B19,*sul*2, *tet*B
931 A_2019-5-*Salmonella*	amp_aug_cip_fis_nal_str_tet_tia	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, bla_{TEM-1B}, *qnr*B19,*sul*2, *tet*B
1387 A_2019-5-*Salmonella*	amp_aug_cip_fis_nal_str_tet_tia	*tetB*	*aac(6′)*-Iaa, *str*A, *str*B, bla_{TEM-1B}, *qnr*B19,*sul*2, *tet*B
1399 A_2019-5-*Salmonella*	amp_aug_cip_fis_nal_str_tet_tia	*tetA*, *tetB*	*aac(6′)*-Iaa, *str*A, *str*B, bla_{TEM-1B}, *qnr*B19,*sul*2, *tet*B

* Phenotypes: amp = ampicillin, cip = ciprofloxacin, aug = amoxicillin/clavulanic acid, axo = ceftriaxone, chl = chloramphenicol, fis = sulfisoxazole, gen = gentamicin, nal = nalidixic acid, str = streptomycin, sxt = trimethoprim/sulfamethoxazole, tet = tetracycline, tia = tiamulin, xnl = ceftiofur.

4. Discussion

In this study, we investigated the impact of in-feed and in-water CTC and/or tiamulin administrations on the fecal prevalence and AMR profiles of *Salmonella enterica* in weaned piglets. *Salmonella* is a major foodborne pathogen that colonizes the hindgut of swine and sheds in the feces and is a widely known cause of non-typhoidal infections in humans [31]. Antimicrobials are used to treat *Salmonella* infections in both humans and animals; therefore, an increase in AMR is of major concern. In the present study, we explored the possibility of using two methods of oral administration of antimicrobials in nursery piglets as a potential strategy to mitigate AMR. We tested our hypothesis using the two most commonly used antimicrobials in the swine industry, tetracyclines and tiamulin. Tetracyclines and tiamulin are administered mainly to treat respiratory diseases in nursery and grower pigs [10]. Chlortetracycline has more broad-spectrum activity, whereas tiamulin has a limited spectrum of activity against Gram-positive bacteria and mycoplasma [32]. At the global level, the swine industry utilizes the highest number of antimicrobials when compared to other food animals [33]. In the USA, the swine industry utilizes 42% by weight of all medically important antimicrobials approved for use in food-producing animals in 2019 [34]. Over 90% of these antimicrobials are administered orally in swine (both in-feed and in-water), making the oral route the most commonly used route of administration of antimicrobials in swine [31]. The oral administration of antimicrobials has a greater impact on the gut bacteria because of the direct exposure to antibiotics.

The effects of antimicrobials on gut bacteria are dependent on the dose administered, distribution of the antimicrobial, and concentration in the gut. Chlortetracycline forms insoluble complexes with aluminum, calcium, iron, and magnesium and also binds to lipids and proteins in the gut, thus reducing its absorption [35,36]. In an experimental study, the bioavailability of orally administered CTC in pigs was only 6% [37,38]. The low bioavailability indicates that most of the antibiotic remains in the gastrointestinal tract, at a concentration high enough to act on the microbes. In contrast, tiamulin bioavailability is up to 90% when administered orally (https://www.hpra.ie/img/uploaded/swedocuments/LicenseSPC_10825-017-001_06012016144953.pdf; accessed on 29 November 2023). Because oral bioavailability has an inverse relationship with intestinal concentration of the antibiotic [38], tiamulin was expected to be available at a minimal concentration (<10%) in the gut content. Interestingly, tiamulin is quickly absorbed into the blood, gets metabolized in the liver, and is excreted via bile into the gut to accumulate in the ileum and colon contents at concentrations sufficient to exert its effect on gut microbes

(http://www.octagon-services.co.uk/articles/therapeutics.pdf; accessed on 29 November 2023). Similar to CTC, increasing the amount of tiamulin provided orally enhances bioavailability and its ultimate concentration in the gut contents [39]. As per the FDA's regulations, one of the antibiotics (CTC) can be fed for only 14 days either in-water or in-feed. So, this study mimics what actual producers practice in a real-world setting here in the US.

Salmonella infections in pigs vary from subclinical to clinical enteritis, which are often self-limiting. Estimates on the fecal prevalence of *Salmonella* in pigs of different ages differ considerably by production and management types, ranging from 3.5 to 33% in the USA [40,41]. This variation is also observed across different stages of development of pigs, where piglets have a low level of *Salmonella* shedding when compared to sows [42,43]. In our study, the prevalence of *Salmonella* in nursery piglets was 3%. All isolates belonged to the *S.* Typhimurium serotype. This is the first time a single serotype of *Salmonella* has been reported in a prevalence study. The study was conducted in an enclosed commercial farm, which explains the possibility of a single serotype circulating within farm piglets. *S.* Typhimurium is the most widespread serotype with zoonotic importance in swine production systems according to the study [44,45]. We performed a PCR analysis of tetracycline-resistant determinants to assess whether the use of CTC, one of the treatment groups in our study design, had any effect on the prevalence of tetracycline-resistant *Salmonella*. Five genes that encode resistance to tetracycline, *tet* (A, B, C, D, and G), have been identified in *Salmonella* [46]. The *tetB*, which encodes for an efflux pump, was present in all *Salmonella* isolates recovered from this study. Similar findings were reported in previous studies, where *tetB* and *tetC* were the most dominant tetracycline resistance determinants in *Salmonella* [47]. The *tetB* gene has the widest distribution among Gram-negative bacteria [32]. Moreover, it was observed in this study that CTC, tiamulin, or a combination of both had no effect on AMR in *Salmonella*. The reason is all strains obtained in this study were resistant to tetracycline and tiamulin. Several other studies have recorded high resistance to tetracyclines [46,48,49]. In this study, both CTC and tiamulin did not influence the prevalence and AMR in *Salmonella* in piglets. Similarly, the study found that pigs treated with CTC showed constant high shedding of *S.* Typhimurium during the treatment and post-treatment periods [50]. They concluded that CTC helped the establishment of *S.* Typhimurium in the gastrointestinal tract of pigs, making it a persistent shedder throughout the post-treatment phase. Moreover, there was no significant difference in the resistance to CTC when *Salmonella* strains were compared between antibiotic-free swine farms and farms using antimicrobials [51,52]. The lack of difference in resistance observed in those studies could be due to the effect of carryover from earlier times when antibiotics were used. Information on the influence of tiamulin on AMR *Salmonella* is limited, and this study provides the first insight.

Salmonella tends to persist and spread resistance to other bacteria through plasmids, making the spread of resistance possible even without the selection pressure of antimicrobials, according to the study [53]. Resistant *Salmonella* could have been passed vertically from sows, or horizontally from farm personnel and the environment, and persisted throughout the length of the experiment. Moreover, all *Salmonella* strains recovered in the present study were MDR. One previous study observed persistence and increase in MDR *S.* Typhimurium in the food chain in the analysis of the prevalence data between 1996 and 2016 [54]. This increased resistance to multiple antimicrobials could potentially cause *Salmonella* to evolve into strains that are difficult to treat [55]. Pigs infected with *S.* Typhimurium are often asymptomatic or develop mild enteritis but can transmit the bacteria to humans through direct and indirect contact according to [56]. The high occurrence of MDR *Salmonella* recorded in this study warns of the possible risk of human exposure. Third-generation cephalosporins, fluoroquinolones, and macrolides are recommended for antimicrobial therapy against salmonellosis in humans [57]. However, a high level of resistance to most of these important classes of antimicrobials observed in this study poses concern. Most of the *Salmonella* strains exhibited no to low-level resistance to ceftiofur and ceftriaxone (third-generation cephalosporins) and azithromycin (a macrolide) in our study.

Resistance to fluoroquinolones is low only due to an acquired resistance gene or genes and not attributed to mutations.

In recent years, WGS has emerged as an essential tool to identify AMR determinants. The tool has played a key role in providing information on epidemiological surveillance of AMR pathogens and studying the emergence of AMR in these pathogens, building new and improving present diagnostic tools, and developing novel antimicrobials of therapeutic importance [58]. Based on WGS, our results suggest that a similar clone of *Salmonella enterica* subsp. *enterica* serotype Typhimurium was circulating in the farm. The clone may have been dispersed among piglets and between pens through farm personnel and equipment. The persistence of the *Salmonella enterica* serotype Typhimurium DT12 in the pig farm environment for a long period of time that posed a risk of transmission to animals and humans was observed in [59]. *S.* Typhimurium has replaced *S.* Choleraesuis as the predominant serotype in pigs in the USA according to [60]. When genome sequences were compared with their expected AMR genotypes obtained by PCR, only *tetB* matched at 100%. Both *tetA* and *tetD* genes were not found in their genomes. The observed discrepancy might be due to the fact that PCR amplifies a small region in DNA that might be similar to the gene of interest but different from the actual entire sequence identified by WGS. Also, the plasmid carrying the resistant genes could have been lost through multiple re-streaking of the bacterial cells. High correlations between phenotypic and genotypic resistance to other antimicrobials were observed in this study. The discrepancy observed between some of the phenotypic and genotypic resistances could be attributed to a non-functional resistance gene, intrinsic resistance of the bacterial strains to some antimicrobials, and the inclusion of intermediate MIC values as resistant.

In summary, our results revealed that in-feed and in-water routes of antimicrobial administration did not influence the fecal prevalence and antimicrobial susceptibilities of *Salmonella* in this population of nursery piglets. Interestingly, the only serotype prevalent was *S*. Typhimurium. The lack of an AMR response was likely because of the widespread prevalence of AMR during the pretreatment phase. The MDR *S.* Typhimurium was resistant to several important and critically important antimicrobials to humans. Among the swine production stages, the nursery stage is a high-susceptibility period necessitating the implementation of an effective mitigation strategy to prevent the transmission of *Salmonella* infection. Our research findings do support the efforts of pork producers in enhancing the economic well-being of their herds and advancing the welfare of pigs across the swine production systems, thereby contributing to both public health and industry.

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