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Genotypes and Phenotypes of Enterococci Isolated From Broiler Chickens

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The objective of this study was to investigate the distribution and persistence of antimicrobial resistance genotypes of enterococci from broilers fed bambermycin (BAM), penicillin (PEN), salinomycin (SAL), bacitracin (BAC), or a salinomycin/bacitracin combination (SALBAC) for 35 days. A total of 95 enterococci from cloacal ($n = 40$), cecal ($n = 38$), and litter samples collected on day 36 ($n = 17$) were isolated weekly from day 7 to 36. All isolates were identified by API-20 Strep and their antimicrobial susceptibilities were evaluated using the Sensititre system with the plates for Gram positive bacteria. Whole genome sequencing (WGS) was used to assess their intra- and inter-genetic variability, with a focus on virulence and antibiotic resistance characteristics. All isolates were further characterized for hemolysin production (HEM), bile salt hydrolysis (BSH), and gelatinase (GEL) activities. Of the 95 isolates, *Enterococcus faecium* ($n = 58$) and *Enterococcus faecalis* ($n = 24$) were the most common *Enterococcus* species identified. Significant differences in the level of resistance for the *E. faecium* isolates to ciprofloxacin, macrolide, penicillin and tetracycline were observed among treatments. The *bcrR, mefA,* and *aac*(6) genes were higher in BAM treatment than the other groups whereas *bcrR, ermA, ermB, aphA(3),* and *tetL* were more prevalent in PEN and BAC treatments. Overall, *E. faecium* isolates showed a higher prevalence of antimicrobial resistance, but *E. faecalis* from litter also exhibited a significant level of resistance. A range of 4–15 different virulence genes was detected in *E. faecalis*. All isolates from litter but one (94.1%) showed BSH activities while 52.9% of them produced GEL. HEM activity was observed only in isolates collected on Day 7 ($n = 9$) and Day 14 ($n = 1$). This study confirmed that genetically diverse AMR enterococci harboring virulence factors can be promoted by the use of certain antimicrobials in feed. Such enterococci could persist in broiler chickens and their litter, which can potentially contaminate the soil upon land application.

Keywords: enterococci, broiler chickens, antimicrobial resistance (AMR), whole genome sequencing (WGS), AMR genotypes, AMR phenotypes

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INTRODUCTION

Enterococci are Gram-positive facultative anaerobic bacteria that are part of the normal intestinal microbiota, with densities ranging from 10^5 to 10^8 CFU/g of intestinal content (Yost et al., 2011; Dubin and Pamer, 2017). Members of the genus *Enterococcus*, which includes presently about 40 recognized species, were initially classified as group D streptococci sharing several phenotypic and biochemical similarities, making their identification difficult (Yost et al., 2011). Enterococci have been proposed as fecal indicator bacteria for microbial source tracking (Byappanahalli et al., 2012; Boehm and Sassoubre, 2014) and are often used in tracking trends in resistance to antimicrobials for various resistance surveillance systems (Tyson et al., 2018a).

Enterococcus species have emerged as the cause of ∼12% of nosocomial infections, with only two species, *Enterococcus faecalis* and *Enterococcus faecium,* causing about 90% of clinical infections (Billington et al., 2014; Torres et al., 2018). Moreover, these two species are considered the third and fourth most prevalent human pathogens worldwide (ECDC, 2011) and ranked third in causing bacteremia in Europe and North America, responsible for ∼11–13% of all bacteremia cases (Ammerlaan et al., 2013; De Kraker et al., 2013). Hospital associated outbreaks linked to vancomycin-positive *E. faecium*, which belongs to MLST clonal complex 17 (CC17), have been reported in several countries (Werner et al., 2008; Pinholt et al., 2015). Genetic relatedness was found between *E. faecalis* isolates from urinary tract infection cases and those from poultry, reinforcing the zoonotic potential of this species and suggesting a possible role of poultry in its spread to humans (Poulsen et al., 2012; Bortolaia and Guardabassi, 2015; Abat et al., 2016).

In poultry, enterococci have been associated with septicemia, endocarditis, and other diseases (Gilmore, 2002). The safety issue regarding enterococci has not been recognized in poultry meat, however concerns about transmission of antimicrobial resistant enterococci to humans have been reported (Simonsen et al., 1998; Marshall and Levy, 2011). Recently, the isolation of AMR *E. faecalis* strains from broilers with vertebral osteomyelitis disease has been reported (Braga et al., 2018). Antibiotic resistant enterococci have been reported in poultry retail meats (Aslam et al., 2012). The ability of enterococci to acquire AMR through the transfer of plasmids and transposons, chromosomal exchange, or mutation presents a significant challenge to infection control (Hollenbeck and Rice, 2012). Mobile genetic elements, including transposons, play an important role in the dissemination of AMR though horizontal gene transfer in bacteria including enterococci. Transposons such as Tn*916*/Tn*1545*, Tn*917*/Tn*551,* and Tn*5397* have been reported to disseminate resistant genes, including *tetM, ermB,* and *aphA*-III, by enterococci (Hegstad et al., 2010). Furthermore, in the absence of antimicrobials, pheromone production was reported to induce a high-frequency plasmid transfer in *E. faecalis* (Hirt et al., 2018).

Due to substantial scientific evidence on the selection, distribution, and dissemination of AMR genes in broiler chicken production systems, in relation to the use of specific therapeutic agents or antimicrobial growth promoters (AGPs) (Aarestrup, 2000; Diarra et al., 2007; Nhung et al., 2016), and due to public and possible food safety and environmental health concerns, the monitoring of AMR in chicken production is imperative.

In our previous study, we described the effects of the in-feed inclusion of bambermycin (BAM), penicillin (PEN), salinomycin (SAL), bacitracin (BAC), or a salinomycin/bacitracin combination (SALBAC) on bacterial population, including enterococci, as well as the incidence and distribution of antibiotic-resistant *Escherichia coli* in broiler chickens (Diarra et al., 2007). The present study describes details of resistance and virulence genotypes of enterococci isolates in relation to their phenotypes using the whole genome sequencing (WGS) approach. WGS is being rapidly adapted in many laboratories for strain typing, outbreak investigations, and comparative genome analysis (Salipante et al., 2015; Sekse et al., 2017; He et al., 2018; Pightling et al., 2018). Several studies have highlighted the need of WGS for antimicrobial resistance (AMR) characterization (Pightling et al., 2018; Tyson et al., 2018b) and accurate prediction of AMR phenotypes from the genotype data. This has been realized to be a critical step in the event that WGS becomes the benchmark to predict the MICs in order to guide clinical decision making (Macesic et al., 2017). Currently, the majority of WGS-based studies focused on Gram-negative bacteria to study AMR phenotype-to-genotype correlations, for example *Salmonella*, *Campylobacter*, *E. coli,* and *Pseudomonas* (Tyson et al., 2015; Davis et al., 2016; McDermott et al., 2016; Zhao et al., 2016; Jeukens et al., 2017; Macesic et al., 2017; Rehman et al., 2017; Neuert et al., 2018). In contrast, relatively limited data is available on Gram-positive bacteria such as enterococci (Macesic et al., 2017; Mason et al., 2018).

The objective of the present study was to examine the complete genome sequences of enterococci isolated from broiler chickens that were fed various antimicrobial agents in order to provide a detailed genome content and to perform comparative genomic analysis of major species (*E. faecalis* and *E. faecium*). These genome sequences were used to determine phylogenetic relationships among isolates, including contemporary human isolates from various sources and days, as well as to show how genomic variations between isolates may influence phenotypic traits such as antibiotic resistance and virulence phenotypes. The sequence data were further used to determine the link between specific antimicrobials administered in the feed and the presence of specific genotypes presenting health risks to both animals and humans.

MATERIALS AND METHODS

Enterococcal Strain Selection

The bacterial isolates used in this study were from a previous study conducted on broiler chicken fed with or without [per kg of feed] the following: basal level (non-antimicrobial diet) as control, 2 mg bambermycin, 2.2 mg procaine penicillin, 60 mg salinomycin, 55 mg bacitracin, and a combination of 55 mg bacitracin plus 60 mg salinomycin with each treatment being applied to three pens. Ceca, cloacae (day 7–35) and litter (day 36) samples were collected to isolate enterococci as described previously (Diarra et al., 2007). Bacteriological analyses were

performed with a total of 90 fecal, 90 cecal, and 48 litter samples. Presumptive colonies were identified previously by API and a DNA microarray carrying 70 taxonomic, 17 virulence, and 174 antibiotic resistance gene probes (Champagne et al., 2011). A total of 184 enterococci isolates were identified, however non-redundant isolates were used in this study. All experimental procedures performed in this study were approved by the Animal Care Committee of the Agassiz Research and Development Center and followed principles described by the Canadian Council on Animal Care.

Antimicrobial Susceptibility Testing

The Sensititre automated system (Trek Diagnostic Systems, Cleveland, OH, USA) using the Gram-positive antimicrobial panel CMV3AGPF plates, was used to determine the minimal inhibitory concentrations (MICs) of all isolates. The MIC data was interpreted according to the Clinical and Laboratory Standards Institutes (CLSI) breakpoints (CLSI, 2015) and the Canadian Integrated Program for Antimicrobial Resistance Surveillance guidelines (CIPARS, 2008). All enterococci isolates were tested against four major categories of antimicrobials based on their importance in human medicine (https://www.canada.ca/en/health-canada/services/drugs-health-products/veterinary-drugs/antimicrobial-resistance/categorization-antimicrobial-drugs-based-importance-human-medicine.html). The category I antimicrobials (representing very high importance in human medicine) were ciprofloxacin, daptomycin, linezolid, quinupristin/dalfopristin, and vancomycin; category II antimicrobials (high importance) included erythromycin, gentamicin, kanamycin, lincomycin, penicillin, streptomycin, tylosin; category III (medium importance) included chloramphenicol, nitrofurantoin, tetracycline, tigecycline, and bacitracin; while category IV (low importance) included flavomycin. Multidrug resistance was defined as resistance to at least three different classes of antimicrobials. *E. faecalis* ATCC 51299 and *E. faecalis* ATCC 29212 were used as quality control strains in this study.

Gelatinase (GEL), Hemolytic (HEM), and Bile Salt Hydrolyze (BSH) Activities

All enterococcal isolates were screened for GEL, HEM, and BSH activities in order to assess phenotype and genotype correlations for these characters as well as their ability to survive in the gut (BSH activity), as has previously been described (Diarra et al., 2010). Briefly, the production of gelatinase was determined using Todd-Hewitt agar (Becton Dickinson) containing 30 g of gelatin per liter and incubated overnight at 37°C. The HEM production was performed by cultivating colonies onto layered fresh horse blood agar plates for 1–2 days at 37°C. Clearing zones around colonies indicated hemolysin production. The BSH activity was determined using MRS agar plates supplemented with 0.5% (wt/vol) sodium salt of taurodeoxycholic acid (Sigma-Aldrich, Oakville, Ontario, Canada) and 0.37 g/liter $CaCl_2$. Plates were then incubated anaerobically (atmosphere of 80% N_2, 10% CO_2, and 10% H_2) at 37°C for 48 h (Diarra et al., 2010).

Pulsed Field Gel Electrophoresis (PFGE) Analysis

Selected enterococci isolates, based on resistance phenotype, were typed using the PFGE technique according to the Centers for Disease Control and Prevention (CDC) PulseNet standardized protocol using *SmaI* (Roche, Laval, QC, Canada). The PFGE cluster analysis was performed in BioNumerics software version 7.5 (Applied Maths, Austin, TX) using Dice's coefficient and the Unweighted Pair Group Method (UPGMA). Isolates with similar banding patterns were considered as closely related.

Genome Sequencing, Assembly, and Comparative Genome Analysis

DNA Extraction

To perform whole genome sequencing (WGS), genomic DNA was extracted from overnight cultures in 5 mL of Brain Heart Infusion (BHI) broth (BD, New Jersey, USA) using the DNeasy Blood & Tissue Kits (QIAGEN) following the protocol as described (Beukers et al., 2017). The extracted DNA was stored in 10 mM Tris-HCl buffer (pH 8.0) and quantified by Invitrogen Qubit® 2.0 Fluorometer (Life Technologies). The quality of DNA was visualized by electrophoresis on a 1% agarose gel and stored at −20°C until genomic library construction.

Sequencing and Assembly Statistics

Sequencing libraries were prepared from 1 ng of genomic DNA with an Illumina Nextera XT DNA sample preparation kit (Illumina, Inc., CA, USA), and paired-end sequencing was performed on an Illumina MiSeq platform (Illumina Inc.) using a 600 cycle MiSeq reagent kit (v3), generating 2 × 300 bps paired-end reads. Sequences were analyzed and quality checked using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The reads were combined using [FLASH] Fast Length Adjustment of SHort reads v1.2.9 (Magoc and Salzberg, 2011). High-quality reads were then assembled *de novo* using SPAdes genome assembler version 3.9.0 software (Bankevich et al., 2012). On average the genome coverage, genome size, number of contigs and G+C contents were 88.6-fold ± 32.3-fold, 22.95 ± 0.089 Mbp, 70 ± 13 (N50 37.9 ± 5.3 kbp) and 37.0 ± 1.5%, respectively. Additionally, two representative isolates, one each of *E. faecalis* and *E. faecium*, were selected for PacBio long read sequencing, and their genomes were assembled in the Hierarchical Genome Assembly Process (HGAP) (Chin et al., 2013), producing an estimated depth of coverage 317-fold, generating two contigs and a N50 of 31.9 kbp. The assembly statistics of all sequenced genomes in this study were comparable to those previously published using similar sequencing technologies (Bertels et al., 2014; Beukers et al., 2017). The assembled genomes were annotated using Prokka version 1.11 (Seemann, 2014), which on average identified a total of ∼2,900 coding sequences, five to seven rRNA, 53–56 tRNA, and 67 miscellaneous RNA.

Genomes From Public Repositories

The genomes of 81 *E. faecalis* and 73 *E. faecium* isolates arising from bloodstream and urinary infections, animals (chicken and

cow), and environmental sources (aquatic, metal, wood, plastic) collected from 1960 to 2015 were obtained from NCBI database GenBank (Benson et al., 2013) (accessed on February 06, 2018) and used as references in phylogenetic and comparison studies. The list of selected reference genomes, with their accession numbers, source and host of isolation and geographical location, is presented in **Tables S1**, **S2**.

WGS-Based Species Identification

An *in silico* ribosomal multi-locus sequence typing (rMLST) approach that indexes the variation of the 53 genes that encode the bacterial ribosome protein subunits (*rps* genes) was used as a means of integrating microbial taxonomy and typing of *Enterococcus*-assembled genomes using the Bacterial Isolate Genome Sequence Database (BIGSdb) (Jolley et al., 2012). MLST from the assembled genomes was determined based on *in silico* analysis of seven housekeeping genes, including *gdh*, *gyd*, *pstS*, *gki*, *aroE*, *xpt*, *yqil* for *E. faecalis* and *adk*, *atpa*, *ddl*, *gdh*, *gyd*, *psts*, *purk* for *E. faecium* (Larsen et al., 2012).

Bioinformatics Analysis

The detection of a comprehensive set of full length ARGs in the assembled genomes was performed using a combination of ResFinder v3.0 (Zankari et al., 2012) and the comprehensive Antimicrobial Resistance Database (CARD), the Resistance Gene Identifier (RGI) version 4.0.3 (McArthur et al., 2013). Additionally, two resistance genes (streptothricin N-acetyltransferase (*Sat4*) and bacitracin (*bcr*) were manually screened among all genomes using the National Center for Biotechnology Information (NCBI), Basic Local Alignment Search Tool (BLAST), BLASTn and BLASTp (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Virulence factors were identified using the VirulenceFinder v1.5 (Joensen et al., 2014). The plasmids were identified using PlasmidFinder version 1.5 (Carattoli et al., 2014).

The sequenced genomes were investigated for the presence of transposons or integrative conjugative elements (ICEs) by homology search using BLAST against 460 ICEs in the ICEberg database version 1.0 (Bi et al., 2012). In combination with their respective reference genome sequences downloaded from the NCBI, GenBank database (Benson et al., 2013), the whole genomes of *E. faecium* and *E. faecalis* sequenced in this study were comparatively analyzed with the CGView comparison tool (CCT) (Grant et al., 2012). All phylogenetic analyses were conducted using the single nucleotide variant phylogenomics (SNVphyl) (Petkau et al., 2017) and/or Reference sequence Alignment-based Phylogeny builder (RealPhy) version 1.12 pipelines (Bertels et al., 2014) with default parameters. The resulting tree was visualized in FigTree software version 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree).

Statistical Analysis

Data were analyzed using a completely randomized design with the GLM procedure of SAS (SAS Institute, 2016). This generated the total number of antibiotics against which isolates were resistant and the number of resistance genes found in isolates. The association test of Cochran-Mantel-Haenszel and Fisher's exact test were used to determine the relationship between the presence of resistance phenotypes and genotypes using the FREQ procedure. Logistic analysis (proportional odds model) was used to determine the relationship between resistance phenotypes and genotypes (Diarra et al., 2007). The *P*-value of 0.05 was used to declare significance.

Genome Sequence Accession Numbers

The draft whole genome sequences of the 95 enterococci have been deposited in GenBank under Bio Project no. PRJNA273513 with the submission ID SUB4666681.

RESULTS

Antimicrobial Susceptibility of *Enterococcus* spp.

A total of 95 identified enterococci isolates were screened for antimicrobial susceptibility testing (AST) and WGS (**Table 1**). These isolates derived from diverse sources, including ceca, cloaca and litter samples of broiler chickens fed with and without antibiotics. The percentages of resistant isolates to each class of antimicrobial, and their associated MIC values for each species, are presented in **Table 2a**.

All *E. faecium* ($n = 58$) isolates were susceptible to vancomycin, gentamicin, and tigecycline, while some isolates showed intermediate resistance to chloramphenicol (5.1%) and nitrofurantoin (96.5%). The majority of *E. faecium* showed a high prevalence of resistance to lincomycin (96.5%), flavomycin (89.7%), bacitracin (81%), tetracycline (68.9%), ciprofloxacin (55.2%), erythromycin (51.7%), kanamycin (44.8%), penicillin (37.9%), tylosin (34.5%), and streptomycin (27.5%). Intermediate resistance to daptomycin (22.4%) as well as a low frequency of resistance to linezolid and quinupristin/dalfopristin (10.3%) were observed among the *E. faecium* isolates. The most common resistance phenotypes in *E. faecium* were ciprofloxacin-lincomycin-bacitracin-tetracycline-flavomycin in combination with resistance to other antimicrobials at varying frequencies. Significant effects of antimicrobial feeding were observed for levels of resistance to ciprofloxacin, macrolide, penicillin, and tetracycline. For example, the lowest resistance to ciprofloxacin was observed in birds that received diets with SAL and BAC, while the highest resistance to penicillin was observed in isolates from birds fed diets containing BAC and SAL + BAC ($P < 0.05$) (**Table 2b**).

The *E. faecalis* ($n = 24$) isolates were susceptible to vancomycin, tigecycline and daptomycin, while some isolates showed decreased susceptibility to chloramphenicol (12.5%) and nitrofurantoin (25%). As expected, intrinsic resistance to lincomycin (100%) and quinupristin/dalfopristin (83.3%) in all *E. faecalis* isolates was observed. Of these isolates, a high level of resistance to tetracycline (95.8%), bacitracin (87.5%), erythromycin (83.3%), and tylosin (79.2%) was observed, while resistance to other antimicrobials such as ciprofloxacin (8.3%), linezolid (12.5%), gentamicin and penicillin (8.3%) was less frequent. The most frequently observed profile among *E. faecalis* was resistance to quinupristin/dalfopristin-erythromycin-lincomycin-tylosin-bacitracin-tetracycline in combination with

TABLE 1 | Characteristics of the 95 Enterococci selected for whole genome sequencing (WGS).

Unique ID	Sample #	Organism (Genus, species)	Day	Treatment Group[a]	Isolation Source	MLST	Plasmids	Virulence phenotypes			
								β-Hem[b]	BS[c]	BS Class[d]	Gel[e]
2812	P10 CL A7	*E. faecium*	7	CONTROL	CLOA	ST-157	rep2	1	12	L	0
2823	P17 C A7	*E. gallinarum*	7	CONTROL	CECC	N/A	No plasmid replicons found	0	13	L	0
2828	P2 C A14	*E. faecium*	14	CONTROL	CECC	ST-32	rep2	0	11	L	0
2829	P2 CL A14	*E. faecium*	14	CONTROL	CLOA	ST-236	repUS15	1	17	M	0
2843	P10 C A14	*E. faecium*	14	CONTROL	CECC	ST-32	repA(pB82)	0	15	M	0
2844	P10 CL A14	*E. faecium*	14	CONTROL	CLOA	ST-32	rep2	0	18	M	0
2857	P17 C A14	*E. faecium*	14	CONTROL	CECC	ST-9	repUS15	0	18	M	0
2879	P10 CL A21	*E. faecalis*	21	CONTROL	CLOA	ST-36	pTEF2, repA(p703/5)	0	12	L	1
2893	P17 CL A21	*E. faecalis*	21	CONTROL	CLOA	ST-249	pTEF2	0	13	L	1
2897	P2 CL A28	*E. faecium*	28	CONTROL	CLOA	ST-26	rep2, repUS15	0	13	L	0
2947	P10 C A35	*E. faecium*	35	CONTROL	CECC	ST-157	rep2	0	12	L	0
2948	P10 CL A35	*E. faecium*	35	CONTROL	CLOA	ST-329	pAM-β-1	0	13	L	0
2967	LIT2 A36	*E. faecium*	36	CONTROL	LIT	ST-490	repUS15, rep(pUB110), repS(pBT233)	0	23	H	0
2968	LIT2 A36′	*E. faecalis*	36	CONTROL	LIT	ST-82	No plasmid replicons found	0	11	L	1
2983	LIT10 A36	*E. faecalis*	36	CONTROL	LIT	ST-249	No plasmid replicons found	0	12	L	1
2997	LIT17 A36	*E. faecium*	36	CONTROL	LIT	ST-843	pAM-β-1, repUS15	0	12	L	0
2804	P5 CL A7	*E. faecalis*	7	BAM	CLOA	ST-202	No plasmid replicons found	1	12	L	0
2824	P18 C A7	*E. avium*	7	BAM	CECC	N/A	No plasmid replicons found	1	12	L	0
2825	P18 CL A7	*E. faecium*	7	BAM	CLOA	Unknown ST	pAM-β-1	0	14	M	0
2861	P1 CL A21	*E. faecium*	21	BAM	CLOA	Unknown ST	repUS15	0	17	M	0
2868	P5 C A 21	*E. faecium*	21	BAM	CECC	ST-841	pAM-β-1, repUS15	0	12	L	0
2902	P5 C A 28	*E. hirae*	28	BAM	CECC	N/A	No plasmid replicons found	0	12	L	0
2903	P5 CL A 28	*E. faecium*	28	BAM	CLOA	Unknown ST	repUS15	0	14	M	0
2927	P18 C A28	*E. faecium*	28	BAM	CECC	ST-54	pAM-β-1, repUS15	0	12	L	0
2928	P18 CL A28	*E. faecium*	28	BAM	CLOA	ST-26	rep2, repUS15	0	22	H	0
2937	P5 C A 35	*E. faecium*	35	BAM	CECC	ST-329	pAM-β-1, repUS15	0	13	L	0
2938	P5 CL A 35	*E. faecium*	35	BAM	CLOA	ST-329	No plasmid replicons found	0	12	L	0
2963	P18 C A35	*E. faecium*	35	BAM	CECC	Unknown ST	No plasmid replicons found	0	14	M	0
2965	LIT1-A37′	*E. faecium*	36	BAM	LIT	ST-54	pAM-β-1, repUS15, repUS1	0	14	M	0
2973	LIT5-A37	*E. faecalis*	36	BAM	LIT	ST-202	pTEF2	0	11	L	1
2999	LIT18-A37	*E. faecium*	36	BAM	LIT	ST-196	repUS15, rep(pUB110), repS(pBT233)	0	20	H	0
3000	LIT18-A37′	*E. faecium*	36	BAM	LIT	ST-196	repUS15, rep(pUB110), repS(pBT233)	0	14	M	0
2809	P9 C A7	*E. gallinarum*	7	PEN	CECC	N/A	No plasmid replicons found	0	11	L	0
2810	P9 CL A7	*E. faecalis*	7	PEN	CLOA	ST-4	pTEF2	0	12	L	1
2853[$]	P15 C A14	*E. gallinarum*	14	PEN	CECC	N/A	No plasmid replicons found	0	13	L	0

(Continued)

TABLE 1 | Continued

Unique ID	Sample #	Organism (Genus, species)	Day	Treatment Group[a]	Isolation Source	MLST	Plasmids	Virulence phenotypes			
								β-Hem[b]	BS[c]	BS Class[d]	Gel[e]
2871	P6 CL A21	*E. faecium*	21	PEN	CLOA	ST-9	repUS15	0	21	H	0
2876	P9 C A21	*E. casseliflavus*	21	PEN	CECC	N/A	No plasmid replicons found	0	12	L	0
2877	P9 CL A21	*E. faecalis*	21	PEN	CLOA	Unknown ST	repD(pK214), pTEF2	0	11	L	1
2904	P6 C A28	*E. faecalis*	28	PEN	CECC	ST-475	pTEF2	0	11	L	1
2905	P6 CL A28	*E. faecium*	28	PEN	CLOA	ST-490	rep(pUB110), repS(pBT233), repUS15	0	21	H	0
2911	P9 CL A28	*E. faecium*	28	PEN	CLOA	Unknown ST	No plasmid replicons found	0	12	L	0
2939	P6 C A35	*E. faecalis*	35	PEN	CECC	ST-475	pTEF2	0	11	L	1
2945	P9 C A35	*E. faecium*	35	PEN	CECC	ST-32	rep2	0	14	M	0
2946	P9 CL A35	*E. faecium*	35	PEN	CLOA	ST-32	No plasmid replicons found	0	12	L	0
2954	P15 CL A35	*E. avium*	35	PEN	CLOA	N/A	No plasmid replicons found	0	18	M	0
2981	LIT9-A36	*E. faecalis*	36	PEN	LIT	ST-249	pTEF2	0	13	L	1
2993	LIT15-A36	*E. faecalis*	36	PEN	LIT	ST-249	pTEF2	0	13	L	1
2994	LIT15-A36′	*E. faecalis*	36	PEN	LIT	ST-249	pTEF2	0	12	L	1
2799	P3 C A7	*E. faecium*	7	SAL	CECC	ST-14	repUS15	0	13	L	0
2819	P14 CL A7	*E. hirae*	7	SAL	CLOA	N/A	No plasmid replicons found	1	14	M	0
2831	P3 CL A14	*E. faecalis*	14	SAL	CLOA	ST-249	pTEF2	0	12	L	1
2832	P4 C A14	*E. faecium*	14	SAL	CECC	ST-32	*rep2*	0	17	M	0
2833	P4 CL A14	*E. faecium*	14	SAL	CLOA	ST-32	repUS15, rep2	0	13	L	0
2851	P14 C A14	*E. faecium*	14	SAL	CECC	ST-9	repUS15	0	19	H	0
2865	P3 CL A21	*E. faecium*	21	SAL	CLOA	Unknown ST	rep2	0	19	H	0
2866	P4 C A21	*E. hirae*	21	SAL	CECC	N/A	No plasmid replicons found	0	13	L	0
2867	P4 CL A21	*E. faecium*	21	SAL	CLOA	ST-54	pAM-β-1, repUS15	0	12	L	0
2886	P14 C A21	*E. faecium*	21	SAL	CECC	ST-32	repA(pB82), rep2	0	16	M	0
2898	P3 C A28	*E. faecium*	28	SAL	CECC	ST-32	rep2	0	18	M	0
2899	P3 CL A28	*E. faecalis*	28	SAL	CLOA	ST-245	pTEF2, pTEF3, repA(p703/5)	0	13	L	1
2900	P4 C A28	*E. faecium*	28	SAL	CECC	ST-54	pAM-β-1, repUS15	0	12	L	0
2933	P3 C A35	*E. faecium*	35	SAL	CECC	Unknown ST	pAM-β-1, repUS15	0	14	M	0
2934	P3 CL A35	*E. faecalis*	35	SAL	CLOA	ST-21	No plasmid replicons found	0	12	L	1
2956	P14 CL A35	*E. faecium*	35	SAL	CLOA	ST-22	repUS15	0	16	M	0
2807	P7 C A7	*E. faecalis*	7	BAC	CECC	ST-116	pTEF2, pTEF3, pAD1	1	12	L	1
2814	P111 CL A7	*E. faecium*	7	BAC	CLOA	ST-329	repA(pB82), p703/5 RepA,pAM-β-1	0	14	M	0
2821	P16 C A7	*E. gallinarum*	7	BAC	CECC	N/A	No plasmid replicons found	1	12	L	0
2822	P16 CL A7	*E. faecium*	7	BAC	CLOA	Unknown ST	repUS15	1	23	H	0

(Continued)

TABLE 1 | Continued

Unique ID	Sample #	Organism (Genus, species)	Day	Treatment Group[a]	Isolation Source	MLST	Plasmids	Virulence phenotypes			
								β-Hem[b]	BS[c]	BS Class[d]	Gel[e]
2837	P7 C A14	*E. faecium*	14	BAC	CECC	ST-540	repUS15	0	14	M	0
2855[$]	P16 C A14	*E. faecium*	14	BAC	CECC	Unknown ST	pAM-β-1	0	11	L	0
2856	P16 CL A14	*E. faecium*	14	BAC	CLOA	Unknown ST	repUS15, rep2	0	20	H	0
2872	P7 C A21	*E. faecalis*	21	BAC	CECC	Unknown ST	rep(pUB110),repUS15	0	12	L	0
2873	P7 CL A21	*E. faecium*	21	BAC	CLOA	ST-26	rep(pUB110),repUS15	0	18	M	0
2881	P11 CL A21	*E. faecium*	21	BAC	CLOA	ST-26	rep(pUB110),repUS15	0	19	H	0
2891	P16 CL A21	*E. faecalis*	21	BAC	CLOA	Unknown ST	rep(pUB110),repUS15	0	21	H	0
2906	P7 C A28	*E. hirae*	28	BAC	CECC	N/A	No plasmid replicons found	0	12	L	0
2907	P7 CL A28	*E. faecium*	28	BAC	CLOA	ST-26	rep(pUB110), repUS15	0	17	M	0
2924	P16 CL A28	*E. durans*	28	BAC	CLOA	N/A	No plasmid replicons found	0	20	H	0
2978	LIT7 A36′	*E. faecium*	36	BAC	LIT	ST-26	rep(pUB110), repUS15	0	17	M	0
2815	P12 C A7	*E. faecalis*	7	SAL + BAC	CECC	ST-249	pTEF2	1	12	L	1
2817	P13 C A7	*E. faecium*	7	SAL + BAC	CECC	ST-9	repUS15	1	24	H	0
2839	P8 C A14	*E. gallinarum*	14	SAL + BAC	CECC	N/A	No plasmid replicons found	0	13	L	0
2874	P8 C A21	*E. faecium*	21	SAL + BAC	CECC	ST-9	*rep2*	0	24	H	0
2882	P12 C A21	*E. faecium*	21	SAL + BAC	CECC	ST-54	pAM-β-1, repUS15	0	12	L	0
2883	P12 CL A21	*E. faecium*	21	SAL + BAC	CLOA	ST-9	repUS15, rep1	0	18	M	0
2916	P12 C A28	*E. faecium*	28	SAL + BAC	CECC	Unknown ST	pAM-β-1, repUS15	0	12	L	0
2917	P12 CL A28	*E. faecium*	28	SAL + BAC	CLOA	ST-9	rep2, repUS15	0	18	M	0
2919	P13 CL A28	*E. faecium*	28	SAL + BAC	CLOA	ST-9	rep(pUB110), pAM-β-1, repUS15, rep2,	0	23	H	0
2951	P12 C A35	*E. faecium*	35	SAL + BAC	CECC	Unknown ST	pAM-β-1, rep2, repUS15	0	17	M	0
2952	P12 CL A35	*E. faecium*	35	SAL + BAC	CLOA	ST-9	rep2, repUS15	0	18	M	0
2980	LIT8 A36′	*E. faecalis*	36	SAL + BAC	LIT	ST-256	repA(pPD1)	0	13	L	1
2987	LIT12 A36	*E. faecalis*	36	SAL + BAC	LIT	ST-256	repA(pPD1)	0	12	L	0
2988	LIT12 A36′	*E. faecium*	36	SAL + BAC	LIT	ST-26	rep(pUB110), repUS15	0	12	L	0
2989	LIT13 A36	*E. faecalis*	36	SAL + BAC	LIT	ST-21	No plasmid replicons found	0	12	L	1
2990	LIT13 A37′	*E. faecalis*	36	SAL + BAC	LIT	ST-21	No plasmid replicons found	0	14	M	1

[$] *Sequenced additionally by Pacbio.*

[a] *CONTROL, non-antimicrobial supplemented basal diet; basal diet supplemented with: BAM, bambermycin; PEN, penicillin; SAL, salinomycin; BAC, bacitracin; SAL+BAC, salinomycin & bacitracin combination; CLOA, cloaca; CECC, ceaca; LIT, litter.*

[b] *β-Hem, Hemolytic.*

[c] *BS, bile salt hydrolysis, the number shows the size of precipitation zones.*

[d] *BS Class; L, Low; M, medium; H, High.*

[e] *GEL, Gelatinase.*

TABLE 2a | Distribution of MICs and resistance among *Enterococcus* spp. isolated from broiler chickens.

Class*	Antibiotic	Species**	MIC_{50}	MIC_{90}	Resistance (%)	No. of isolates with MIC (μg/ml)***													
						0.25	0.5	1	2	4	8	16	32	64	128	256	512	>1,024	>2,048
I	Ciprofloxacin	*E. faecium*	>1	4	32 (55.2)			5	21	32									
		E. faecalis	1	1	2 (8.3)			10	12	2									
		E. gallinarum	2	2	1 (20)		1	1	2	1									
		E. hirae	0.5	0.5	0		3	1											
		E. avium	1	>2	1 (50)			1		1									
	Daptomycin	*E. faecium*	4	4	13 (22.4)	1	1	4	3	36	13								
		E. faecalis	2	>2	0			2	17	5									
		E. gallinarum	4	>4	1 (20)					4	1								
		E. hirae	2	2	0				2	4									
		E. avium	0.5	>0.5	0		1			1									
	Linezolid	*E. faecium*	>2	>4	6 (10.3)		5	19	21	7	6								
		E. faecalis	2	2	3 (12.5)		3	10	6	2	3								
		E. gallinarum	>8	>8	3 (60)				1	1	3								
		E. hirae	4	>4	2 (50)			1		1	2								
		E. avium	1	1	0		1			1									
	Quinupristin/Dalfopristin	*E. faecium*	2	2	6 (10.3)		6	7	30	9	4		2						
		E. faecalis	>4	8	20(83.3)				2	1	16	4							
		E. gallinarum	>8	>8	3 (60)					1	2		1						
		E. hirae	2	2	0				1	3									
		E. avium	1	1	0			1		1									
	Vancomycin	*E. faecium*	2	2	0		15	36	6	1									
		E. faecalis	2	>2	0		2	15	6	1									
		E. gallinarum	>32	>32	5 (100)								5						
		E. hirae	1	1	0			4											
		E. avium	0.5	0.5	0		2												
II	Erythromycin	*E. faecium*	>8	>8	30 (51.7)	4	1	7	8	8	30								
		E. faecalis	>8	>8	20 (83.3)	4					20								
		E. gallinarum	0.5	8	2 (40)		3				2								
		E. hirae	>8	>8	4 (100)						4								
		E. avium	8	8	1 (50)	1					1								
	Gentamicin	*E. faecium*	>128	>128	0										58				
		E. faecalis	>128	>128	2 (8.3)										22			2	
		E. gallinarum	>128	>128	0										5				
		E. hirae	>128	>128	0										4				
		E. avium	>128	>128	0										2				
	Kanamycin	*E. faecium*	256	<512	26 (44.8)									2	12	14	4	26	
		E. faecalis	>1024	>1024	12 (50)										8	2	2	12	

(Continued)

TABLE 2a | Continued

Class*	Antibiotic	Species**	MIC_{50}	MIC_{90}	Resistance (%)	No. of isolates with MIC (μg/ml)***												
						0.25	0.5	1	2	4	8	16	32	64	128	256	512	>1,024	>2,048
		E. gallinarum	>512	>1024	2 (40)											1	2	2	
		E. hirae	128	128	0										3	1			
		E. avium	128	>128	1 (50)										1			1	
	Lincomycin	*E. faecium*	8	>8	56 (96.5)					2	54	2							
		E. faecalis	>8	>8	24 (100)						24								
		E. gallinarum	>8	>8	5 (100)						5								
		E. hirae	>8	>8	4 (100)						4								
		E. avium	>8	>8	2 (100)						2								
	Penicillin	*E. faecium*	>4	>8	22 (37.9)	1	1	3	7	20	4	22							
		E. faecalis	>2	>2	2 (8.3)				5	17		2							
		E. gallinarum	>4	>4	1 (20)			1	2	4		1							
		E. hirae	1	>2	1 (25)			2	1			1							
		E. avium	2	>4	1 (50)				1			1							
	Streptomycin	*E. faecium*	512	>512	16 (27.5)					2							40	6	10
		E. faecalis	512	512	7 (29.2)												17	3	4
		E. gallinarum	512	512	0												5		
		E. hirae	512	512	0												4		
		E. avium	512	512	0												2		
	Tylosin	*E. faecium*	<8	>16	20 (34.5)	3			4	10	13	8	20						
		E. faecalis	>32	>32	19 (79.2)				3	2			19						
		E. gallinarum	>4	>4	1 (20)				2	1		1	1						
		E. hirae	32	> 32	3 (75)							1	3						
		E. avium	1	16	1 (50)		1						1						
III	Bacitracin	*E. faecium*	>128	>128	47 (81)							1	1	9	47				
		E. faecalis	>128	>128	21 (87.5)								1	2	21				
		E. gallinarum	>64	>64	2 (40)									3	2				
		E. hirae	>128	>128	4 (100)										4				
		E. avium	>128	>128	2 (100)										2				
	Chloramphenicol	*E. faecium*	>8	>8	0				1	3	51	3							
		E. faecalis	> 8	> 8	0					1	20	3							
		E. gallinarum	4	8	0					2	3								
		E. hirae	8	8	0						4								
		E. avium	4	>4	0			1	1										
	Nitrofurantoin	*E. faecium*	>64	>64	0			1					1	56					
		E. faecalis	>16	>32	0						1	15	4	4					
		E. gallinarum	>16	>16	0						1	1	1	2					

(Continued)

TABLE 2a | Continued

Class*	Antibiotic	Species**	MIC_{50}	MIC_{90}	Resistance (%)	No. of isolates with MIC (μg/ml)***												
						0.25	0.5	1	2	4	8	16	32	64	128	256	512	>1,024	>2,048
		E. hirae	32	>32	0								2	2					
		E. avium	>64	>64	0									2					
	Tetracycline	*E. faecium*	>32	>32	40 (68.9)		17	1					40						
		E. faecalis	>32	>32	23 (95.8)			1					23						
		E. gallinarum	>32	>32	5 (100)								5						
		E. hirae	>32	>32	3 (75)			1					3						
		E. avium	>32	>32	2 (100)								2						
	Tigecycline	*E. faecium*	<0.25	<0.25	0	56					2								
		E. faecalis	<0.25	<0.25	0	24													
		E. gallinarum	0.25	0.25	0	5													
		E. hirae	0.25	0.25	0	4													
		E. avium	<0.25	<0.25	0	2													
IV	Flavomycin	*E. faecium*	>32	>32	52 (89.7)			1		2	2	1	52						
		E. faecalis	>8	>8	10 (41.7)			3	6	2	3		10						
		E. gallinarum	>32	>32	4 (80)						1		4						
		E. hirae	>32	>32	4 (100)								4						
		E. avium	32	32	1 (50)					1			1						

*The antibiotic class is based on relevance to human medicines.

**E. faecium (n = 58); E. faecalis (n = 24); E. gallinarum (n = 5); E. hirae (n = 4); E. avium (n = 2); One isolate of each E. durans and E. casseliflavus not included, details in the results section.

***Breakpoints adopted from CLSI (Clinical and Laboratory Standards Institute) recommended for enterococci.

The dotted and solid bars represents the intermediate and complete resistance to the respective antibiotic.

TABLE 2b | Distribution of resistance phenotypes among enterococci isolated from chickens fed antimicrobials[a].

Antibiotic class[b]	No. (%) of antibiotic resistant enterococci isolates relative to the total no. of isolates (*n*) tested per treatment											
	Control (*n* = 11)		BBM (*n* = 14)		PEN (*n* = 13)		SAL (*n* = 15)		BAC (*n* = 13)		SAL + BAC (*n* = 16)	
	E.FAEM (*n* = 7)	E.FAEL (*n* = 4)	E.FAEM (*n* = 10)	E.FAEL (*n* = 04)	E.FAEM (*n* = 11)	E.FAEL (*n* = 02)	E.FAEM (*n* = 13)	E.FAEL (*n* = 02)	E.FAEM (*n* = 11)	E.FAEL (*n* = 02)	E.FAEM (*n* = 06)	E.FAEL (*n* = 10)
CLASS I												
Ciprofloxacin	4 (57.1)	0 (0.0)	7 (70)*	0 (0.0)	6 (54.5)	0 (0.0)	5 (38.5)	0 (0.0)	8 (72.7)*	1 (50)	2 (33.3)	1 (10)
Daptomycin	3 (42.9)	0 (0.0)	2 (20)	0 (0.0)	1 (9.1)	0 (0.0)	3 (23.1)	0 (0.0)	2 (18.2)	0 (0.0)	2 (33.3)	0 (0.0)
Linezolid	2 (28.6)	1 (25)	1 (10)	0 (0.0)	0 (0.0)	0 (0.0)	2 (15.4)	0 (0.0)	1 (9.1)	0 (0.0)	0 (0.0)	2 (20)
Quinupristin/Dalfopristin	2 (28.6)	2 (50)	1 (10)	4 (100)	1 (9.1)	2 (100)	1 (7.7)	2 (100)	2 (18.2)	2 (100)	0 (0.0)	8 (80)
CLASS II												
Erythromycin	1 (14.3)	4 (100)	4 (40)*	4 (100)	7 (63.6)*	1 (50)	7 (53.8)*	1 (50)	7 (63.6)*	2 (100)	4 (66.7)*	8 (80)
Gentamicin	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (50)	0 (0.0)	1 (50)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Kanamycin	3 (42.9)	2 (50)	4 (40)	3 (75)	4 (36.4)	2 (100)	5 (38.5)	2 (100)	6 (54.5)	0 (0.0)	4 (66.7)	3 (30)
Lincomycin	7 (100)	4 (100)	10 (100)	4 (100)	11 (100)	2 (100)	13 (100)	2 (100)	9 (81.8)	2 (100)	6 (100)	10 (100)
Penicillin	4 (57.1)	0 (0.0)	5 (50)	0 (0.0)	7 (63.6)*	2 (100)	2 (15.4)	0 (0.0)	0 (0.0)	0 (0.0)	3 (50)	0 (0.0)
Streptomycin	2 (28.6)	2 (50)	3 (30)	1 (25)	6 (54.5)	1 (50)	2 (15.4)	0 (0.0)	1 (9.1)	1 (50)	2 (33.3)	2 (20)
Tylosin	1 (14.3)	4 (100)	2 (20)	4 (100)	4 (36.4)	1 (50)	4 (30.8)	1 (50)	6 (54.5)	1 (50)	3 (50)	8 (80)
CLASS III												
Bacitracin	5 (71.4)	4 (100)	9 (90)	4 (100)	8 (72.7)	1 (50)	12 (92.3)	1 (50)	8 (72.7)	2 (100)	5 (83.3)	9 (90)
Tetracycline	5 (71.4)	4 (100)	7 (70)	4 (100)	9 (81.8)*	2 (100)	7 (53.8)	2 (100)	8 (72.7)	2 (100)	4 (66.7)	9 (90)
CLASS IV												
Flavomycin	6 (85.7)	1 (25)	8 (80)	2 (50)	11 (100)	2 (100)	12 (92.3)	2 (100)	10 (90.9)	1 (50)	5 (83.3)	2 (20)

[a] *Bambermycin (BBM), penicillin (PEN), salinomycin (SAL), bacitracin (BAC), and a salinomycin-bacitracin combination (SAL + BAC).*

[b] *Based on their importance in human medicine, class I; very high; class II, High; class III Medium; class IV low.*

All isolates were susceptible to vancomycin, chloramphenicol, nitrofurantoin, and tigecycline.

* *Values are statistically different ($P < 0.05$).*

varying degrees of resistance to other antimicrobials. Among *E. faecalis,* the highest resistance to macrolides ($P < 0.05$) was observed in isolates from birds treated with BAM, BAC, and SAL + BAC, while levels of kanamycin and streptomycin resistance were higher in isolates from birds receiving BAC than the control or those receiving other treatments (**Table 2b**).

Other enterococci isolates, including *E. gallinarum* ($n = 5$), *E. hirae* ($n = 4$), *E. avium* ($n = 2$), *E. casseliflavus* ($n = 1$), and *E. durans* ($n = 1$), were susceptible to gentamicin and streptomycin (**Table 2a**). Among these isolates, a high frequency of resistance to lincomycin (100%), tetracycline (92.3%), flavomycin (84.6), bacitracin (76.9%), and erythromycin (53.8%) was observed, while resistance to other antimicrobials such as ciprofloxacin and daptomycin (15.4%), linezolid and tylosin (38.5%), quinupristin/dalfopristin and kanamycin (30.8%), and penicillin (23.1%) was less frequent. Only *E. gallinarum* and *E. casseliflavus* isolates were resistant to vancomycin. Differences between antimicrobial feeding were observed in the frequencies of resistance to erythromycin, kanamycin, and quinupristin/dalfopristin. For example, the highest resistance level to erythromycin was observed in isolates receiving BAC and SAL, while levels of kanamycin and quinupristin/dalfopristin resistance were higher in isolates from broilers receiving PEN as compared to the control or other treatments.

Antimicrobial Resistance Genes (ARGs)

The PacBio sequencing data from two isolates in this study provided high quality long reads, allowing accurate detection of plasmids or transposons associated with specific ARGs. A total of 40 ARGs were detected in the genome of all 95 sequenced *Enterococcus* spp. (**Table 3**). Among these isolates, the most frequently detected resistance genes included *ermA/B* (52.6%), *tetL* (54.7%), *msrC* (60%), *efmA* (62.1%), *bcr* (62.1%), *lsaA/E* (66.3%), *tetM* (68.4%), and *aac(6′)-Ii* (69.5%).

E. faecium

Overall the number of resistance genes was higher in *E. faecium* than in other *Enterococcus* species ($P < 0.05$). Among the 58 isolates, the number of detected genes in individual isolates ranged from 1 to 23, with 63.7% of isolates carrying at least 10 resistant genes. Nine aminoglycoside resistant genes were detected among *E. faecium*, with *aac(6′)-Ii* ($n = 58$), *ant(6)-Ia* ($n = 19$); *aph(3')-III* ($n = 19$), *aadE* ($n = 18$), and *ant(9)-Ia* ($n = 14$) being the most common genes detected. The *aac(6')-Ii* gene was the most prevalent in isolates from BAC-fed birds. Four macrolide resistance genes, *lsaA/E* ($n = 35$ isolates), *ermA/B* ($n = 24$ isolates), *mefA* ($n = 5$ isolates), and *mprF* ($n = 21$ isolates) were detected, with *ermA/B* being predominant in isolates from PEN-fed birds ($P < 0.05$). Thirty-one and 37 isolates carried the tetracycline resistance genes *tetL* and *tetM*, respectively, which were more prevalent in isolates from birds treated with PEN, BAC and SAL + BAC. The 35 isolates carrying the bacitracin resistance gene *bcrA* were prevalent in BAC and SAL + BAC treated birds compared to the control birds ($P < 0.05$). The macrolide-lincosamide-streptogramin B (MLS_B) genes such as *msrC* and *lnuB/*, as well as the gene cluster *PBP5* (∼36% amino acid similarity to the *mecA* specific to staphylococci; alignment shown in **Figure S1**), trimethoprim-sulfamethoxazole *dfrE* gene homolog and fluoroquinolone *mfd* resistance genes, were detected in 48, 28, 24, 24, and 24 isolates, respectively. Additionally, a multitude of antimicrobial efflux pump genes such as *efmA* were found in 52 isolates, along with *arlR, efrA/B, lmrB/C/D, pmrA/E,* and *taeA* in 24 additional isolates. In addition, the *fabI* gene, encoding enoyl reductase, which prevents the inhibition of fatty acid synthesis by triclosan, was found in 24 *E. faecium* isolates. Several *E. faecium* isolates were found to carry multiple resistance genes, with the most prevalent resistance combination being *bcrR-ermA/B-msrC-mefA-aac-aphA-tetL-tetM*. Moreover, 24 *E. faecium* genomes contained several mutations in the quinolone resistance-determining region (QRDR) of *parC*, *parE*, and *gyrA* genes, and six carried nucleotide substitutions (G2576T) in 23S rRNA (*E. coli*-K12 strain GenBank accession #HG738867 was used as reference) (data not shown). However, no vancomycin resistance gene was detected in any *E. faecium* isolate.

E. faecalis

Among all 24 studied isolates, 0 to 23 different resistant genes were detected from individual isolates, with 41.6% harboring at least 10 resistance genes. Consistent with their resistant phenotypes, eight aminoglycoside resistance genes, including *sat4* (9 isolates), *aph(3′)-III* (8 isolates), *aad(6)* (7 isolates), *ant(6)-Ia* (7 isolates), *aac(6′)-Ii* (2 isolates), *aac(6′)-aph(2″)* (2 isolates), *spc/str* (2 isolate), and *ant(9)-Ia* (1 isolate), were found among the 24 *E. faecalis* isolates. No significant effects of antimicrobial feeding on the distribution of these genes were observed. For macrolide resistance, all *E. faecalis* isolates (100%) carried the *lsaA/E* gene, while the *ermA/B* and *mprF* genes were detected in 20 and 7 of them, respectively. The trimethoprim-sulfamethoxazole resistance *dfrE* gene homolog was detected in 22 isolates. The tetracycline resistance *tetM* and *tetL* genes and the bacitracin resistance *bcr* gene were detected in 21, 19, and 17 bacitracin-resistant *E. faecalis* isolates across the birds with antimicrobials in their diet. The efflux pump genes *arlR, cdeA efmA, efrA/B, emeA, lmrB/C/D, pmrA/E,* and *taeA*, as well as the triclosan resistance *fabI* gene were found in seven *E. faecalis* isolates. Like *E. faecium,* multiple substitutions in the quinolone resistance-determining region (QRDR) of *parC, parE*, and *gyrA* were detected in two *E. faecalis* and three contained nucleotide G2576T substitutions in 23S rRNA. No vancomycin-resistant gene was detected in any of *E. faecalis* isolates.

The other *Enterococcus* species harbored a limited number of resistance genes. Among those, *aac(6')-Ii*, *bcrA*, *ermA/B, tetL,* and *tetM* were identified in some of the *Enterococcus* species included in this study. The *vanC* gene was exclusively detected in *E. gallinarum* and *E. casseliflavus* genomes.

Correlation Between AMR Phenotype and Genotype

For the majority of antimicrobials, there was a strong positive correlation between resistance phenotypes and genotypes (**Table 4**).

TABLE 3 | Distribution of resistance genes among *Enterococcus* spp. isolated from broiler chickens.

ARG Group	Gene name	No. (%) of isolates							
		E. faecium (*n* = 58)	*E. faecalis* (*n* = 24)	*E. gallinarum* (*n* = 5)	*E. hirae* (*n* = 4)	*E. avium* (*n* = 2)	E. durans (*n* = 1)	*E. casseliflavus* (*n* = 1)	Total (*n* = 95)
Aminoglycoside	*aac(6′)-Ii*	58 (100)	2 (8.3)	0 (0.0)	4 (100)	1 (50)	1 (100)	0 (0.0)	66 (69.5)
	aad(6)	6 (10.3)	7 (29.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	14 (14.7)
	aadE	18 (31)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	18 (18.9)
	ant(6)-Ia	19 (32.8)	7 (29.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	26 (27.4)
	ant(9)-Ia	14 (24.1)	1 (4.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	15 (15.8)
	aph(3′)-III	19 (32.8)	8 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	27 (28.4)
	aac(6′)-aph(2″)	2 (3.4)	2 (8.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (4.2)
	spc/str	3 (5.2)	2 (8.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (5.3)
	sat4	8 (13.8)	9 (37.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	17 (17.9)
β-lactam	*mecA homolog pbp5*	24 (41.4)	6 (25)	2 (40)	0 (0.0)	1 (50)	0 (0.0)	1 (100)	34 (35.8)
Fluoroquinolone[#]	*mfd*	24 (41.4)	6 (25)	2 (40)	0 (0.0)	1 (50)	0 (0.0)	1 (100)	34 (35.8)
StreptograminA/B	*msrC*	48 (82.6)	6 (25)	2 (40)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100)	57 (60)
Macrolide	*erm(A/B)*	24 (41.4)	20 (83.3)	2 (40)	4 (100)	0 (0.0)	0 (0.0)	0 (0.0)	50 (52.6)*
	lsaA/E	35 (60.3)	24 (100)	2 (40)	0 (0.0)	1 (50)	0 (0.0)	1 (100)	63 (66.3)
	mef(A)	5 (8.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (5.3)
	mprF	21 (36.2)	7 (29.2)	2 (40)	0 (0.0)	1 (50)	0 (0.0)	1 (100)	32 (33.7)
Lincosamide	*lnuB/G*	28 (48.3)	4 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	32 (33.7)
Tetracycline	*tet(L)*	31 (53.4)	19 (79.2)	0 (0.0)	2 (50)	0 (0.0)	0 (0.0)	0 (0.0)	52 (54.7)
	tet(M)	37 (63.8)	21 (87.5)	3 (60)	3 (75)	1 (50)	0 (0.0)	0 (0.0)	65 (68.4)
	tet(S)	2 (3.4)	0 (0.0)	4 (80)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	6 (6.3)
	tetO	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (50)	1 (100)	1 (100)	3 (3.2)
	tetA/B	0 (0.0)	0 (0.0)	1 (20)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100)	2 (2.1)
Fosfomycin	*murA*	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100)	1 (1)
Vancomycin	*VanC*	0 (0.0)	0 (0.0)	5 (100)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100)	6 (6.3)
Bacitracin	*bcr*	35 (60.3)	17 (70.8)	1 (20)	4 (100)	1 (50)	1 (100)	0 (0.0)	59 (62.1)*
Multi-drug efflux pump	*arlR*	24 (41.4)	7 (29.2)	2 (40)	0 (0.0)	1 (50)	0 (0.0)	1 (100)	35 (36.8)
	alaS	6 (10.3)	4 (16.7)	1 (20)	0 (0.0)	1 (50)	0 (0.0)	1 (100)	13 (13.7)
	cdeA	0 (0.0)	7 (29.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	7 (7.4)
	efmA	52 (89.7)	7 (29.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	59 (62.1)
	efrA/B	24 (41.4)	7 (29.2)	2 (40)	0 (0.0)	1 (50)	0 (0.0)	1 (100)	34 (35.8)
	emeA	0 (0.0)	7 (29.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	7 (7.4)
	lmrB/C/D	24 (41.4)	7 (29.2)	2 (40)	0 (0.0)	1 (50)	0 (0.0)	1 (100)	35 (36.8)
	pmrA/E	24 (41.4)	7 (29.2)	2 (40)	0 (0.0)	1 (50)	0 (0.0)	1 (100)	35 (36.8)
	TaeA	24 (41.4)	7 (29.2)	1 (20)	0 (0.0)	1 (50)	0 (0.0)	0 (0.0)	33 (34.7)
	patB	0 (0.0)	0 (0.0)	2 (40)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (2.1)
	salA	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100)	1 (1)
Triclosan	*fabI*	24 (41.4)	7 (29.2)	2 (40)	0 (0.0)	1 (50)	0 (0.0)	1 (100)	35 (36.8)
Trimethoprim-sulfamethoxazole	*dfrE*	24 (41.4)	22 (91.7)	2 (40)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100)	49 (51.6)
Mupirocin	*ileS/liaS*	7 (12)	4 (16.7)	2 (40)	0 (0.0)	1 (50)	0 (0.0)	1 (100)	15 (15.8)

#Several mutations in parC, parE, and gyr genes observed among ciprofloxacin resistant strains.

**Values are statistically different ($P < 0.05$).*

TABLE 4 | Resistance phenotype and genotype correlations among 95 enterococci isolated from broiler chickens.

Antibiotic	Antibiotics tested	Phenotypes (no. of isolates)[a]	Genotypes (no. of isolates)	Correlation (%)[b]
Tetracycline	TET	R, 75	*tet*(L) ($n = 52$), tet(M) ($n = 65$), tet(S) ($n = 6$), tet(O) ($n = 03$), tet(A/B) ($n = 02$)	100 (75/75)
		S, 20	None	100 (20/20)
Macrolides	ERY	R, 57	*erm*(A/B) ($n = 50$), mefA ($n = 05$)	91.2 (52/57)[c]
		S, 38	None	100 (38/38)
Lincosamide	LIN, Q/P	R, 93	*msrC* ($n = 57$), *lnu*(B/G) ($n = 32$)	95.7 (89/93)[d]
		S, 02	None	100 (02/02)
Fluoroquinolones	CIP	R, 36	*mfd* ($n = 34$), QRDR ($n = 10$)	100 (36/36)
		S, 59	None	100 (59/59)
Bacitracin	BCR	R, 78	*bcr* ($n = 59$)	75.6 (59/78)[e]
		S, 17	*bcr* ($n = 2$)	88.2 (15/17)[f]
Aminoglycosides	GEN, KAN, PEN, STR	R, 57	*aaC(6′)* ($n = 66$), aad(6) ($n = 13$), *aadE* ($n = 18$), *ant(6)-Ia* ($n = 26$), *ant(9)-Ia* ($n = 13$), *aph(3′)-III* ($n = 22$), *aac(6′)-aph(2″)* ($n = 02$), *spc/str* ($n = 02$), *sat4* ($n = 17$)	100 (36/36)
		S, 38	*aaC*(6′) ($n = 09$)	76.3 (29/38)[g]
Linezolid	LIN	R, 14	23S rRNA G2576T ($n = 14$)	100 (14/14)
		S, 81	None	100 (81/81)
Vancomycin	VAN	R, 5	*vanC* ($n = 5$)	100 (5/5)
		S, 90	None	100 (90/90)
Chloramphenicol	CHL	S, 95	None	100 (95/95)
Tigecycline	TGY	S, 95	None	100 (95/95)
Nitrofurantoin	NIT	S, 95	None	100 (95/95)
Daptomycin	DAP	R, 14	*liaS (n = 15)*	93.3 (14/15)[h]
		S, 81	None	100 (81/81)

[a] *R, resistant; S, susceptible.*
[b] *The values in parentheses represent the number of isolates with a resistance element(s)/number of isolates with the phenotype.*
[c] *Five isolates had an ERY^R phenotype, but did not contain the corresponding resistant gene.*
[d] *Four isolates had an LIN^R phenotype, but did not contain the corresponding resistant gene.*
[e] *Nineteen isolates had a bcr^R phenotype, but no bcr gene was detected.*
[f] *Two isolates had a bcrS phenotype, but bcrR gene was detected.*
[g] *Nine isolates had a sensitive phenotypes, but aaC(6′) gene was detected.*
[h] *One isolate was DAPS, but had liaS gene.*

Tetracycline

At least one tetracycline resistance gene was detected in all 75 isolates with phenotypic resistance to tetracycline. Of these, 65 contained the *tetM* gene while 52 carried both the *tetM* and *tetL* genes. The *tetS*, *tetO,* and *tetA/B* genes were found in six, three, and two tetracycline-resistant isolates, respectively. No tetracycline resistance gene was detected in any of the 20 tetracycline susceptible isolates.

Macrolide, Lincosamide Streptogramin B, and Quinupristin-Dalfopristin (MLS_B)

Fifty of the 57 isolates resistant to erythromycin (macrolide) harbored *ermB* or *ermA* alone, while three contained both genes. Five isolates harbored the *mefA* gene, of which three contained both *ermB* and *mefA* genes, while two had a combination of *ermA*, *ermB,* and *mefA*. No corresponding macrolide resistance genes were detected in five resistant isolates and none were detected in the 38 macrolide-susceptible isolates. *E. faecalis* is intrinsically resistant to lincomycin and streptogramin B and all 24 (100%) and 20 (83.3%) isolates of this species carried *lsaA/E* and *erm(A/B)* gene, respectively. The *msrC* gene, which is known to be intrinsic to *E. faecium* (Champagne et al., 2011) and confers resistance to macrolides, was found in 48 (82.6%) and 6 (25%) of the *E. faecium* and *E. faecalis* isolates, respectively.

Fluoroquinolone

Either the *mfd* gene or mutations in *parC*, *parE*, or *gyrA* were detected in all 36 resistant isolates associated with ciprofloxacin resistance. None of the susceptible isolates had the *mfd* gene or *parC*, *parE*, or *gyrA* mutations detected in their genomes.

Bacitracin

Among 78 bacitracin resistant isolates, 59 (75.6%) harbored the *bcr* gene, while this gene containing no mutation was also detected in 2 of the 17 phenotypically susceptible isolates.

Aminoglycoside

Various levels of resistance to aminoglycosides were observed, and their corresponding genes *aac(6′)li*, *aac(6′)li,* and *aac(6′)lih* were detected within the genomes. The two *E. faecalis* isolates with high levels of gentamicin resistance carried a corresponding bifunctional aminoglycoside-modifying enzyme,

TABLE 5 | Prevalence of virulence factors among poultry-associated *Enterococcus* spp.

Enterococcus spp.	Virulence factor	Function
*E. faecium** ($n = 58$)	*efaAfm*	*E. faecium* specific cell wall adhesins
	acm	Surface-exposed antigen, cell wall adhesion
*E. faecalis*** ($n = 24$)	*ace*	Adhesion to collagen and other extracellular proteins
	agg	Aggregation substance
	cad	Sex pheromone-associated
	camE	Sex pheromone-associated
	cCF10	Sex pheromone-associated
	cOB1	Sex pheromone-associated
	ebpA/B/C	Pilin subunit
	efaAfs	Adhesion protein, plays role in endocarditis
	ElrA	Enterococcal leucine-rich protein A
	fsrB	Biofilm formation
	gelE	Gelatinase
	hylA/B	Hyaluronidase
	SrtA	Anchor surface proteins to peptidoglycans
	tpx	Oxidative stress response
	cylA/B/L/M	Cytolysin, hemolysis

**efaAfm (57, 98.3%); acm (51, 87.9%).*
***ace, cad, cCF10, cOB1, fsrB, gelE, hylA/B, SrtA, tpx (24, 100%); agg (4, 16.7%) came, ElrA (22, 91.7%); ebpA/B/C, efaAfs (23, 95.8%); cylA/B/L/M (1, 4.2%).*

aac(6′)-aph(2″) [one isolate contained both *aac(6′)-Ie-aph(2″)-Ia* and *aph(2″)-Ic*], that conferred resistance to high concentrations of gentamicin.

Linezolid

All 14 enterococci that showed resistance to linezolid carried corresponding nucleotide substitutions in 23S rRNA.

Vancomycin

The resistance to vancomycin in *E. gallinarum* and *E. casseliflavus* was associated with the presence of *vanC* operon, comprising up to seven genes as detected in their respective genomes. Moreover, all vancomycin susceptible isolates lacked the corresponding *van* related genes in their genomes.

Chloramphenicol, Tigecycline, and Daptomycin

The isolates that were susceptible to chloramphenicol, tigecycline, and daptomycin consistently had no corresponding genetic determinant detected in their genomes.

Virulence Genes of *Enterococcus* spp.

Genes encoding virulence factors from the 95 assembled genomes of the *Enterococcus* isolates were identified using VirulenceFinder (**Table 5**). Overall, no significant differences in the distribution of virulence factors or their associated virulence phenotypes were observed among isolates of the same species collected from different antimicrobial feeding groups, source, or day of isolation (data not shown).

E. faecium

The majority of *E. faecium* genomes were positive for two well conserved virulence genes: *efaAfm* (57/58, 98.3%) and *acm* (51/58, 87.9%). Additionally, a putative virulence gene, *bsh* (978 bp), encoding a 326-amino acid bile hydrolysis enzyme (EC 3.5.1.24) was identified in the majority of *E. faecium* (54/58, 93.1%) genomes, and such isolates exhibited a positive bile salt hydrolyze (BSH) phenotype. Among the BSH-positive isolates, 20 (34.5%) had low, 25 (43.1%) medium, and 13 (22.4%) exhibited high BSH activity based on the size of precipitation zones (**Table 1**). Moreover, a 663-bp gene coding for a putative membrane protein hemolysin III homolog was detected in four hemolysin-positive *E. faecium* (6.8%). Interestingly, in contrast to human clinical cases, all *E. faecium* isolates of poultry origin tested in this study lacked the virulence gene *espfm*, encoding a putative surface protein precursor that plays a major role in biofilm formation and is associated with urinary tract infections (Semedo et al., 2003).

E. faecalis

A total of 15 well-defined virulence genes were detected among *E. faecalis* genomes. Nine of the 15 genes were identified in all isolates, while four *E. faecalis* genomes (IDs: 2807, 2810, 2891, and 2879) were positive for the *agg* gene, encoding an aggregation protein involved in adherence to host cells. The genes associated with activation, transportation and modifications of the cytolysin (*cylA*, *cylB*, *cylL, and cylM*) (Hallgren et al., 2009) were identified in a single *E. faecalis* genome (day 36, litter sample ID 2968) that clustered closely with *E. faecalis* of human origin. All *E. faecalis* were positive for a chromosomal-mediated gene *gelE*, encoding gelatinase, an extracellular zinc metallo-endopeptidase secreted by *E. faecalis* species that hydrolyze gelatin, collagen, hemoglobin and other bioactive peptides. Furthermore, the *gelE*-bearing isolates were found positive for the locus *fsrB*, encoding a processing enzyme that liberates gelatinase biosynthesis activating pheromone (GBAP) peptide, indicating the importance of *fsr* in virulence and disease (Hancock and Perego, 2004). Consistent with their *gelE* and *fsrB* genotypes, all *E. faecalis* isolates showed *in vitro* gelatinase phenotypes (a turbid halo zone around the colonies) (**Table 1**). The *bsh* gene was identified in all *E. faecalis* isolates, and these isolates also showed BSH activity *in vitro*. Our study also highlighted that all *E. faecalis* isolates carried a chromosomal *hylA/B* gene, which encodes the hydrolytic enzyme hyaluronidases (Golinska et al., 2013), and accordingly these isolates exhibited BSH phenotypes *in vitro* (**Table 1**). The hemolysin III homolog was detected in three *E. faecalis* (12.5%) which aligned with their hemolytic activity. Other virulence factors identified in *E. faecalis* in this study included sex pheromone-associated genes *camE*, *cad*, *cCF10,* and *cOB1* in 91.7–100% of isolates, respectively, and the cell wall adhesion expressed in the serum gene *efaAfs* (95.8%). Additionally, a novel enterococcal leucine-rich protein A gene, *elr* (91.7%), which facilitates bacterial escape from host immune defenses, and the thiol peroxidase gene *tpx* (100%), which protects pathogenic bacteria against oxidative stress (Fisher and Phillips, 2009), were detected. The endocarditis and biofilm-associated pili gene locus *ebpABC*, an associated sortase gene

srtA, and a cell wall-anchored adhesion gene *ace* were also detected in all *E. faecalis* isolates. The *espfs* surface protein found in human clinical isolates (Diarra et al., 2010) was not detected in any of the *E. faecalis* isolates originating from poultry.

Among other enterococci, including *E. gallinarum, E. casseliflavus, E. avium, E. hirae, and E. durans,* only three isolates contained the hemolysin III homolog and exhibited their corresponding hemolytic phenotypes while all carried the *bsh* gene, which was consistent with their BSH phenotype (**Table 1**). No other virulence genes were detected among these isolates. Additionally, all enterococci isolates were negative for *asa1* (aggregation substance), a virulence gene commonly found in human pathogenic isolates (Hallgren et al., 2009).

Mobile Genetic Elements

Plasmids

All three classes of plasmids, including the rolling circle replication (RCR), *Inc*18 and the pheromone-responsive plasmids, were detected among the sequenced genomes using the plasmid finder database. There was at least a minimum of one to several plasmids detected in a majority of the genomes sequenced in this study. No significant effect of antimicrobial feeding on the distribution of plasmids was observed. The plasmids identified among the sequenced genomes are listed in **Table 1**.

The pAMβ-1 family of non-conjugative broad host range plasmids was common among *E. faecium* genomes (Grohmann et al., 2003). Several ARGs, including *ermB, aad6*, *Aph(3′)-IIIa*, *tetM/L*, *Ant(9)-Ia*/*Ant(9)-Ia*, *lnuB, Sat4*, were present on the plasmid-bearing contigs in 6 of 58 (10.3%) *E. faecium* genomes sequenced in this study (**Table 6**). Interestingly, an aminoglycoside-streptothricin resistance gene cluster *aadE-sat4-aphA-3* of ∼3–3.5 kb in size was identified on plasmid-associated contigs in three *E. faecium* genomes (two from BAC treatment cloacal samples collected on day 14 and one from a BAM treatment litter sample collected on day 36). This gene cluster was located on a DNA fragment containing a Tn*5405*-like (putative) transposable element containing site-specific recombinase and other phage-related regions. Furthermore, all genomes that were positive for the *aadE-sat4-aphA-3* gene cluster were also positive for the *ermB* gene (**Figure 1**). No plasmid sequence was detected in four (6.9%) *E. faecium* genomes. The isolates from BAC and BAM treatment contained a relatively higher number of plasmids carrying ARGs as compared to the control or other treatment groups.

The pheromone-inducible plasmid pTEF2, structurally similar to pCF10 and pPD1, was the most prevalent type of plasmids detected in *E. faecalis*. Additionally, pTEF3, a non-conjugative plasmid harboring a pTEF2-like *prgZ* pheromone receptor adjacent to multiple insertion sequence (IS) elements (IS*1216* and IS*256*), was also identified in two *E. faecalis* genomes. Two other *E. faecalis* genomes contained a pUB110 plasmid. Several ARGs were detected on plasmid-containing contigs in 16 of 24 (66.7%) *E. faecalis* genomes. Like *E. faecium*, an *aadE-sat4-aphA-3* cluster linked with the *erm*B gene was detected on a plasmid-associated contig in one of *E. faecalis* genomes (**Table 6**). No plasmid sequence was detected in five (20.8%) *E. faecalis* genomes.

No plasmid was detected in the other sequenced *Enterococcus* species, including *E. gallinarum, E. casseliflavus, E. avium, E. hirae, and E. durans* (**Table 1**).

Transposons

The Tn*3*-like transposon Tn*917* (∼18-kb in size) was detected in several of the sequenced genomes. The Tn*917* transposon contained five open reading frames (ORFs); all oriented in the same direction on the transposon and were flanked by 38-bp inverted repeats. The presence of intact transposons and IS elements may lead to a variety of genetic rearrangements, including deletions, inversions, and translocations. All genomes that harbored the Tn*917* also harbored the macrolide erythromycin resistance gene *erm (B)* in close proximity. The other four ORFs identified were rRNA methylase, resolvase, transposase, and an ORF of unknown function. In 24 (41.3%) *E. faecium* and 20 (83.3%) *E. faecalis* genomes, the Tn*917* transposon was linked with the *ermB* gene. The Tn*917* transposon was completely absent in other species except in one *E. avium*.

Several *Enterococcus* genomes also contained a Tn*916* family of integrative conjugative elements (ICEs). Most studied enterococci also carried a tetracycline resistance gene, *tet*M, ∼11.4 kb upstream of Tn*916*. The Tn*916* elements were detected in 29 (50%) *E. faecium,* 21 *E. faecalis* (87.5%), 3 *E. gallinarum* (60%), and 2 *E. hirae* (50%). No sequence homology to Tn*916* was detected in other species.

The Tn*6000* (formerly EfcTn*1*), a novel Tn*916*-like conjugative transposon linked with the tetracycline resistance *tetS* gene, was detected in only one isolate of each *E. faecium* and *E. gallinarum*.

Two to three copies of putative Tn*552* transposons of the Tn*3* family were detected in 12 (20.7%) *E. faecium*. The mobility module of Tn*552* was comprised of genes such as *tnpA* (transposase), *tnpB (*ATP-binding protein), *bin3* (resolvase-integrase) as well as *arsR*, *tetR,* and *phoB* (family of transcriptional regulators).

Like *E. faecium,* the genomes of *E. hirae* and *E. durans* also harbored two to three copies of Tn*5252* conjugative transposons. No transposon was detected in the *E. casseliflavus* genomes.

Multi-Locus Sequence Typing (MLST) and Phylogenetic Analysis

Only the 58 *E. faecium* and the 24 *E. faecalis* genomes were included in MLST analysis due to the lack of an MLST scheme for other *Enterococcus* spp. (**Table 1**). The *E. faecium* isolates comprised 14 different MLST types, including 12 unknown sequence types (STs), including ST9 (9 isolates), ST26 (7 isolates), ST32 (9 isolates), ST-54 (5 isolates), and ST-329 (4 isolates). A total of 14 sequence types were found among *E. faecalis* isolates, with the most prevalent type being ST249 (7 isolates) and ST21 (3 isolates). No previously reported *E. faecalis* ST16 from poultry, swine and human urinary tract and endocarditis infections were detected among any of the studied isolates.

The initial phylogenetic tree was built using all 95 sequenced enterococci genomes based on the core genome single nucleotide polymorphism (SNPs). The *E. faecalis* V583 genome (GenBank

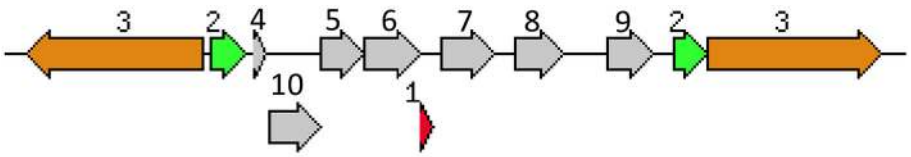
TABLE 6 | Plasmid associated Antibiotic Resistance Genes (ARGs).

ID	Species	Contig	Start	Stop	Orientation	Gene
2807	*E. faecalis*	36	7331	8239	+	*aad(6)*
		36	11401	12138	+	*ermB*
2810	*E. faecalis*	13	15678	16472	–	*Aph(3')-IIIa*
		13	18090	18998	–	*aad(6)*
		13	14191	14928	–	*ermB*
2815	*E. faecalis*	8	32992	33729	+	*ermB*
		8	19796	21715	+	*tetM*
2831	*E. faecalis*	9	32992	33729	+	*ermB*
		9	19796	21715	+	*tetM*
2877	*E. faecalis*	10	477	1271	+	*Aph(3')-IIIa*
		10	2021	2758	+	*ermB*
		10	15343	16719	–	*tet(L)*
		10	16913	18832	–	*tetM*
2879	*E. faecalis*	14	10235	10972	–	*ermB*
		14	11722	12516	–	*Aph(3')-IIIa*
		14	14134	15042	–	*aad(6)*
2881	*E. faecium*	72	4627	6003	+	*tet(L)*
		72	2514	4433	+	*tetM*
2893	*E. faecalis*	14	52900	54186	–	*tet(L)*
		14	42456	43193	–	*ermB*
		14	54470	56404	–	*tetM*
2898	*E. faecium*	32	1759	2496	+	*ermB*
		32	215	1009	+	*Aph(3')-IIIa*
2899	*E. faecalis*	37	477	1271	+	*Aph(3')-IIIa*
		37	2021	2758	+	*ermB*
2904	*E. faecalis*	21	12578	13954	+	*tet(L)*
		21	10465	12384	+	*tetM*
2905	*E. faecium*	26	25651	26193	+	*Sat4*
		97	326	1702	+	*tet(L)*
		26	16021	16830	+	*Ant(9)-Ia*
		26	27830	28567	+	*ermB*
		26	24746	25654	+	*aad(6)*
		26	6952	8169	+	*mefA*
		26	14455	15318	+	*Ant(6)-Ia*
		26	26286	27080	+	*Aph(3')-IIIa*
		26	20435	21238	+	*lnuB*
2939	*E. faecalis*	11	10465	12384	+	*tetM*
2973	*E. faecalis*	6	32831	33568	+	*ermB*
		6	801	2720	–	*tetM*
2978	*E. faecium*	10	11465	13384	+	*tetM*
2980	*E. faecalis*	10	5880	6422	–	*Sat4*
		10	6419	7327	–	*aad(6)*
		10	4993	5787	–	*Aph(3')-IIIa*
		10	3506	4243	–	*ermB*
		9	18006	19925	–	*tetM*
2981	*E. faecalis*	9	32992	33729	+	*ermB*
		9	19796	21715	+	*tetM*
2987	*E. faecalis*	8	18006	19925	–	*tetM*
		9	3506	4243	–	*ermB*
2993	*E. faecalis*	10	19796	21715	+	*tetM*

(Continued)

TABLE 6 | Continued

ID	Species	Contig	Start	Stop	Orientation	Gene
		10	32992	33729	+	*ermB*
2994	*E. faecalis*	7	19796	21715	+	*tetM*
		7	32992	33729	+	*ermB*
2999	*E. faecium*	28	16016	16825	+	*Ant(9)-Ia*
		28	14450	15313	+	*Ant(6)-Ia*
		28	20430	21233	+	*lnuB*
		28	26281	27075	+	*Aph(3')-IIIa*
		28	25646	26188	+	*Sat4*
		28	24741	25649	+	*aad(6)*
		28	27825	28562	+	*ermB*
3000	*E. faecium*	28	12261	13064	−	*lnuB*
		28	7306	7848	−	*Sat4*
		28	4932	5669	−	*ermB*
		28	18181	19044	−	*Ant(6)-Ia*
		28	16669	17478	−	*Ant(9)-Ia*
		28	6419	7213	−	*Aph(3')-IIIa*
		28	7845	8753	−	*aad(6)*



1. Streptothricin acetyltransferase (***sat-4***)
2. Site-specific recombinase, DNA invertase Pin related protein
3. TransposonsTn*5405* containing repeat elements
4. helix-turn-helix domain protein
5. Ubiquinone/menaquinone biosynthesis methyltransferase UBIE
6. aminoglycoside 6-adenylyltansferase (***aadE***)
7. Mobile element protein (hypothetical phage protein)
8. Aminoglycoside phosphotransferase (***aphA-3***)
9. RRNA (Adenine-N(6)-) -methyltransferase
10. Hypothetical protein

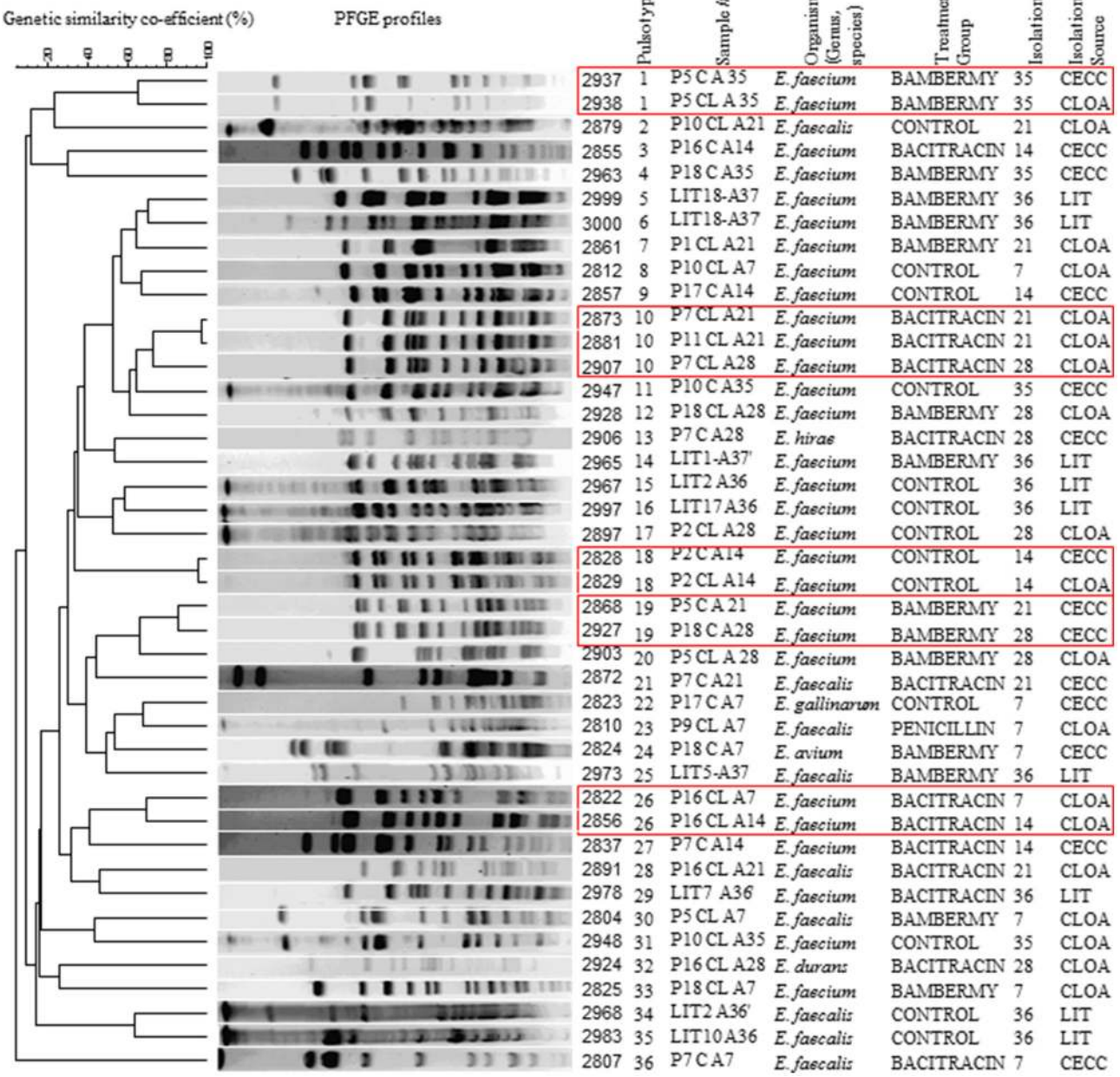
FIGURE 1 | Schematic of gene cluster (not scaled) containing *aadE-sat4-aphA-3* on a Tn*5405*-like (putative) transposable element linked with *ermB* gene found in three studied enterococci (not shown). The encoded proteins are labeled 1–10 on the gene cluster and their descriptions are provided at the bottom.

accession #NC_004668) was used as reference. Each genome was placed into a correct species-specific cluster, further validating the accurate identity of each species by WGS based on the rMLST scheme used in this study (**Figure S2**). The overall topology of the tree showed substantial inter-species similarities, although some inter-species variations were also noticed with the PFGE profiles (**Figure 2**) of selected isolates from the control group as well as from BAM- and BAC-fed birds, which showed the overall highest AMR prevalence.

Because of the limited numbers of *E. hirae*, *E. gallinarum*, and *E. casseliflavus* isolates in this study, only genomes of *E. faecalis* ($n = 24$), and *E. faecium* ($n = 58$) were comparatively analyzed, with their respective human clinical reference genomes obtained from the NCBI database.

E. faecium

The genetic relatedness between the 58 studied *E. faecium* genome sequences and the 73 *E. faecium* reference strains from human clinical cases was determined (**Figure 3**). Seven distinct clades were identified, with most genomes of strains from human clinical cases constituting a major (clade III, $n = 48$) and a minor clade (clade V, $n = 23$). All 58 *E. faecium* clustered into five distinct clades, containing six isolates in clade I, five in clade II, 11 in clade IV, 28 in clade VI, and eight in clade VII. Interestingly, eight isolates in clade VII clustered together with the human clinical reference *E. faecium* strain 1070_EFCM (tree id, JWEB01_1, GenBank accession # JWEB01000000), obtained from a tertiary care hospital's intensive care unit (ICUs). This strain exhibited the highest



	Pulsotype	Sample #	Organism (Genus, species)	Treatment Group	Isolation day	Isolation Source
2937	1	P5 C A 35	*E. faecium*	BAMBERMY	35	CECC
2938	1	P5 CL A 35	*E. faecium*	BAMBERMY	35	CLOA
2879	2	P10 CL A21	*E. faecalis*	CONTROL	21	CLOA
2855	3	P16 C A14	*E. faecium*	BACITRACIN	14	CECC
2963	4	P18 C A35	*E. faecium*	BAMBERMY	35	CECC
2999	5	LIT18-A37	*E. faecium*	BAMBERMY	36	LIT
3000	6	LIT18-A37	*E. faecium*	BAMBERMY	36	LIT
2861	7	P1 CL A21	*E. faecium*	BAMBERMY	21	CLOA
2812	8	P10 CL A7	*E. faecium*	CONTROL	7	CLOA
2857	9	P17 C A14	*E. faecium*	CONTROL	14	CECC
2873	10	P7 CL A21	*E. faecium*	BACITRACIN	21	CLOA
2881	10	P11 CL A21	*E. faecium*	BACITRACIN	21	CLOA
2907	10	P7 CL A28	*E. faecium*	BACITRACIN	28	CLOA
2947	11	P10 C A35	*E. faecium*	CONTROL	35	CECC
2928	12	P18 CL A28	*E. faecium*	BAMBERMY	28	CLOA
2906	13	P7 C A28	*E. hiras*	BACITRACIN	28	CECC
2965	14	LIT1-A37'	*E. faecium*	BAMBERMY	36	LIT
2967	15	LIT2 A36	*E. faecium*	CONTROL	36	LIT
2997	16	LIT17 A36	*E. faecium*	CONTROL	36	LIT
2897	17	P2 CL A28	*E. faecium*	CONTROL	28	CLOA
2828	18	P2 C A14	*E. faecium*	CONTROL	14	CECC
2829	18	P2 CL A14	*E. faecium*	CONTROL	14	CLOA
2868	19	P5 C A 21	*E. faecium*	BAMBERMY	21	CECC
2927	19	P18 C A28	*E. faecium*	BAMBERMY	28	CECC
2903	20	P5 CL A 28	*E. faecium*	BAMBERMY	28	CLOA
2872	21	P7 C A21	*E. faecalis*	BACITRACIN	21	CECC
2823	22	P17 C A7	*E. gallinarum*	CONTROL	7	CECC
2810	23	P9 CL A7	*E. faecalis*	PENICILLIN	7	CLOA
2824	24	P18 C A7	*E. avium*	BAMBERMY	7	CECC
2973	25	LIT5-A37	*E. faecalis*	BAMBERMY	36	LIT
2822	26	P16 CL A7	*E. faecium*	BACITRACIN	7	CLOA
2856	26	P16 CL A14	*E. faecium*	BACITRACIN	14	CLOA
2837	27	P7 C A14	*E. faecium*	BACITRACIN	14	CECC
2891	28	P16 CL A21	*E. faecalis*	BACITRACIN	21	CLOA
2978	29	LIT7 A36	*E. faecium*	BACITRACIN	36	LIT
2804	30	P5 CL A7	*E. faecalis*	BAMBERMY	7	CLOA
2948	31	P10 CL A35	*E. faecium*	CONTROL	35	CLOA
2924	32	P16 CL A28	*E. durans*	BACITRACIN	28	CLOA
2825	33	P18 CL A7	*E. faecium*	BAMBERMY	7	CLOA
2968	34	LIT2 A36'	*E. faecalis*	CONTROL	36	LIT
2983	35	LIT10 A36	*E. faecalis*	CONTROL	36	LIT
2807	36	P7 C A7	*E. faecalis*	BACITRACIN	7	CECC

FIGURE 2 | Dendrograms of pulsed-field gel electrophoresis (PFGE) profiles of *smaI*-digested genomic DNA showing relationship between 42 enterococci isolated at day 7 to 35–36 from cecal, cloacal, and litter samples of broiler fed control, bambermycin (BAM), and bacitracin (BAC). Due to their significant effects on the overall AMR, subsets of isolates were selected from BAM and BAC treatments to assess their diversity. The dendrogram on the left represents the genetic similarity co-efficient (%). The gel image in the middle separated by white space represents the banding pattern of individual isolate labeled on the right. Pulsotype refers to subtypes labeled as 1, 2, 3, and so forth. Day of isolation refers to broiler age. Two distinct clusters were observed, cluster I (pulsotype 1–4) and cluster II (pulsotype 5–36). The genetically homogeneous isolates presenting similar pulsotype are shown in red boxes.

degree of genetic relatedness (>99%) with a minimum pairwise SNP difference in the range of seven to 15, with isolate ID 2997 from the cloacal samples of the 36 days BAM-fed broiler being the most closely related, with only seven SNP differences (**Figures 3**, **4**).

E. faecalis

Phylogenetic inferences of 24 genomes from this study and 82 references revealed that the poultry isolates interspersed throughout the tree (**Figure 5**). Genetic relationships were found between several genomes of studied isolates and those

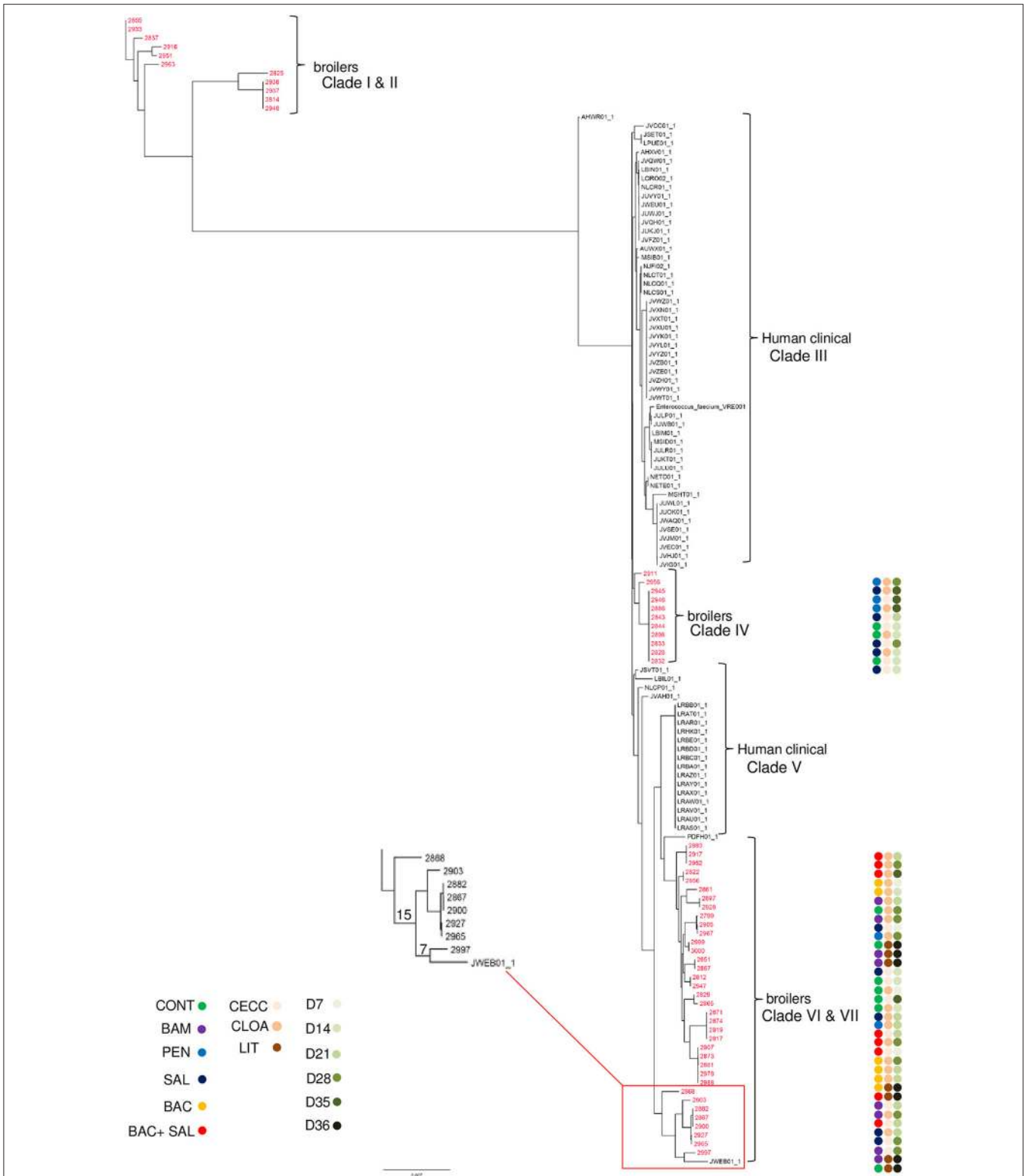


FIGURE 3 | Phylogeny of *E. faecium* based on core genome (>99%) single-nucleotide polymorphisms (SNPs) analysis using SNVPhyl. *E. faecium* strain Aus0004 used as reference in this analysis. A maximum likelihood tree of 58 *E. faecium* multi-resistant isolates obtained from broiler chickens in the present study (labeled) and 73 reference isolates from human clinical sources (labeled). Isolates in each clade are labeled as I- VII. Studied isolates showing some close similarity with isolates of human clinical origin are presented in the red box. The enlarged view of closely related isolates from broiler chickens and an isolate associated with clinical infection in humans. The number on the tree nodes represents SNP differences between the genomes.

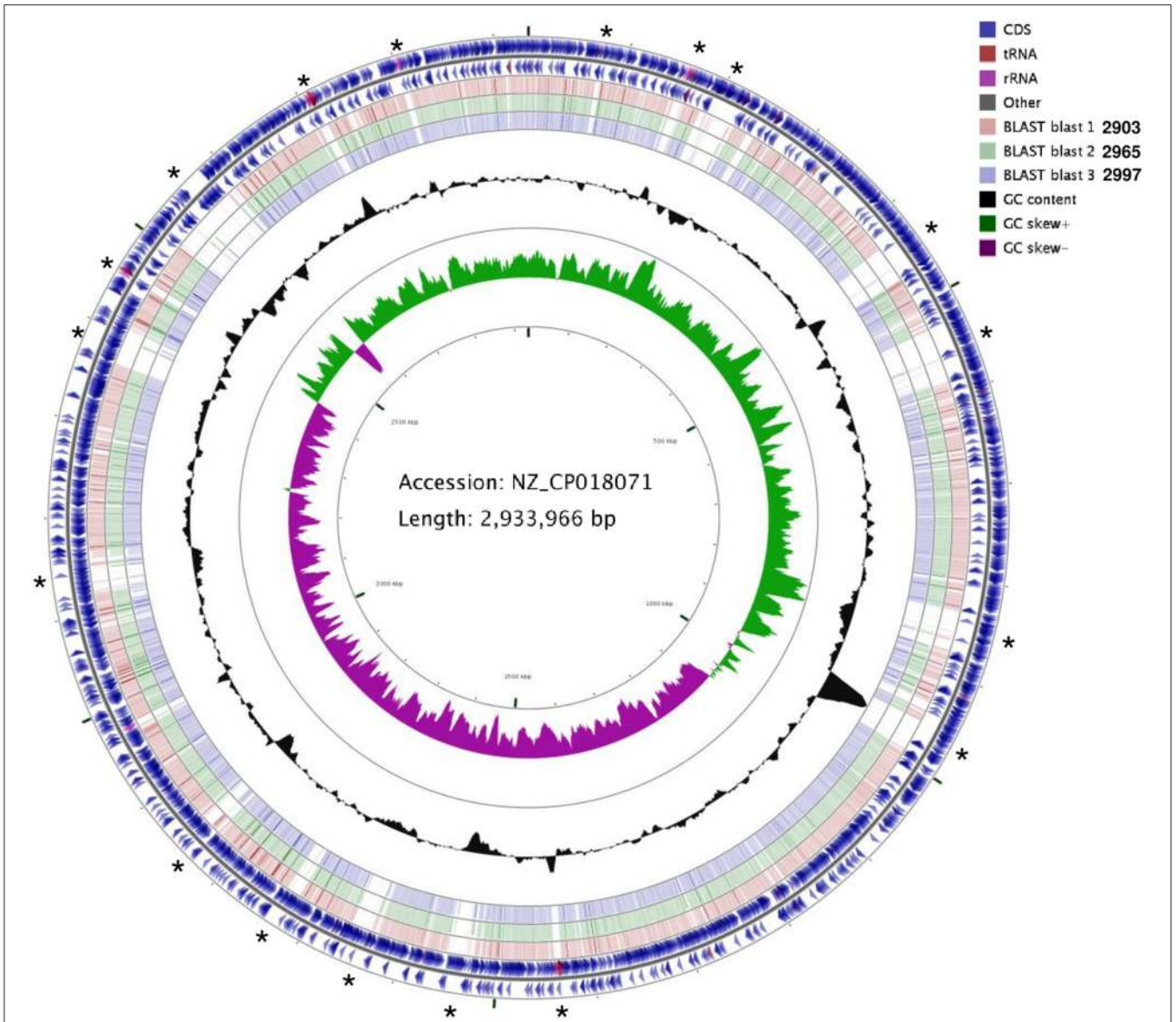


FIGURE 4 | CGView: comparison of a bacterial genome from isolates of broiler origin with isolate of human clinical origin (accession # NZ_CP018071) using blastn. The contents of the feature rings (starting with the outermost ring) are as follows. Ring 1, 2: forward and reverse strand features read from the primary sequence GenBank file. Rings 3, 4, 5: Genomes 2903, 2965, 2997 in *fasta* format. Rings 7, 8: GC content and GC skew. The gaps in the alignment represent the regions missing in the query genomes in comparison to the reference genome.

from environmental sources (aquatic, isolated from agricultural feeding sites). For example, four sets of isolates from broilers administered different antimicrobials, and at different sampling days, clustered with aquatic isolates. Furthermore, isolates from six different treatment groups were evenly distributed across the branches of the phylogenetic tree, except for a few isolates from litter that clustered together, suggesting their clonal nature. Overall, the genetic relatedness was higher among *E. faecalis* isolates that originated from similar habitats. The litter isolate (ID 2968 of ST82) from control group that harbored cytolysin genes showed some relationship with human isolates (**Figure 6**).

DISCUSSION

Enterococcal species are opportunistic pathogens in both humans and animals (Jett et al., 1994) and their ability to acquire antibiotic resistance presents a challenge for infection control. The ecology of AMR in enterococci in relation with antimicrobial usage in poultry is not well-characterized. In this study, the effect of in-feed antimicrobials on the diversity and promotion of AMR as well as relationship between resistance phenotypes and genotypes among enterococci isolated from broiler chickens were investigated.

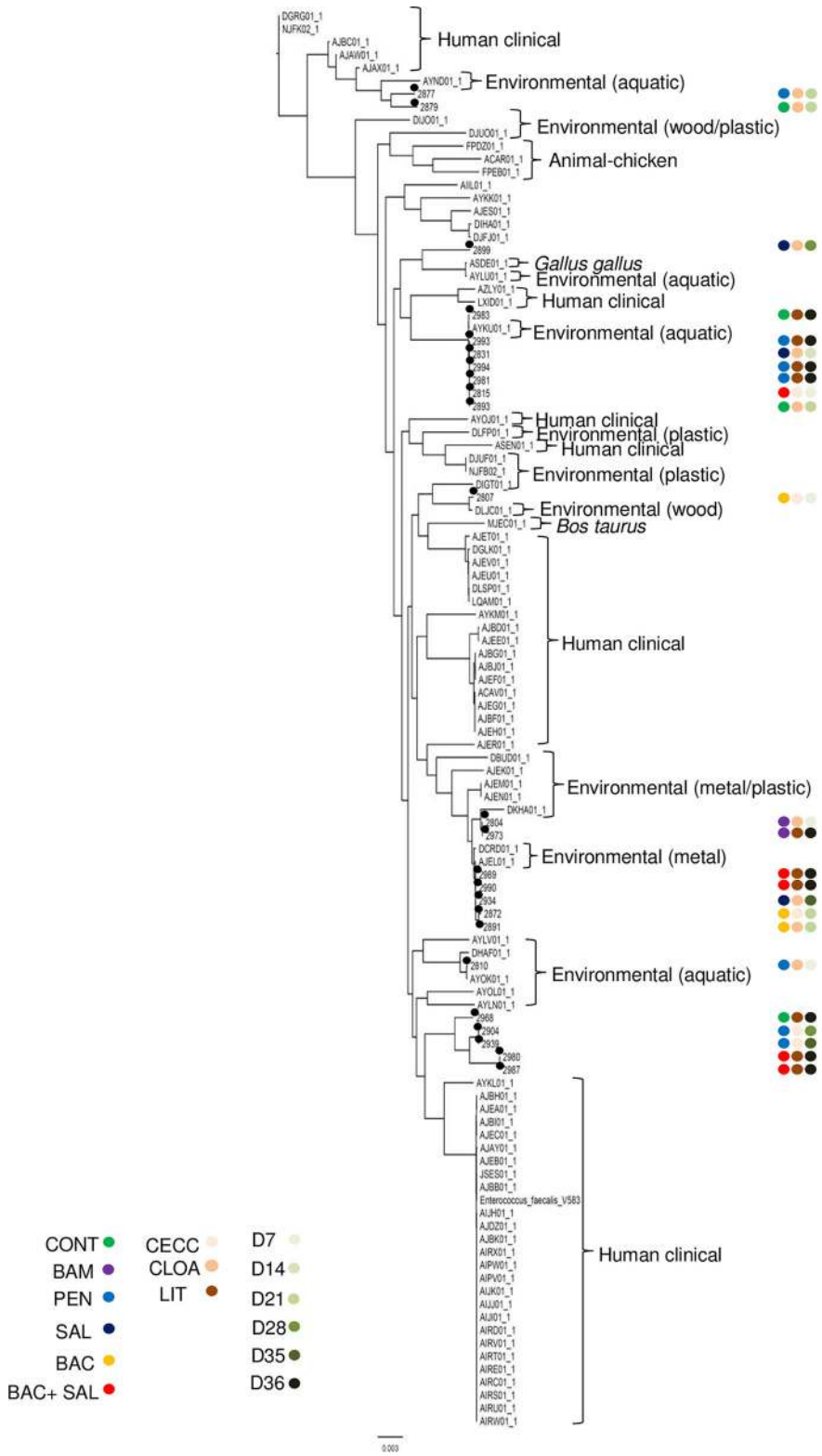


FIGURE 5 | Phylogeny of *E. faecalis* based on core genome (>99%) single-nucleotide polymorphisms (SNPs) analysis using SNVPhyl. *E. faecalis* strain V583 was used as reference in the analysis. A Maximum-likelihood tree of 24 *E. faecalis* multi-resistant isolates obtained from broiler chickens in the present study (dark dots) and 81 isolates from human clinical (from patients with bloodstream and urinary infections), environment (aquatic, metal, plastic, wood) and animal sources (labeled). The *E. faecalis* strain V583 served as a common reference.

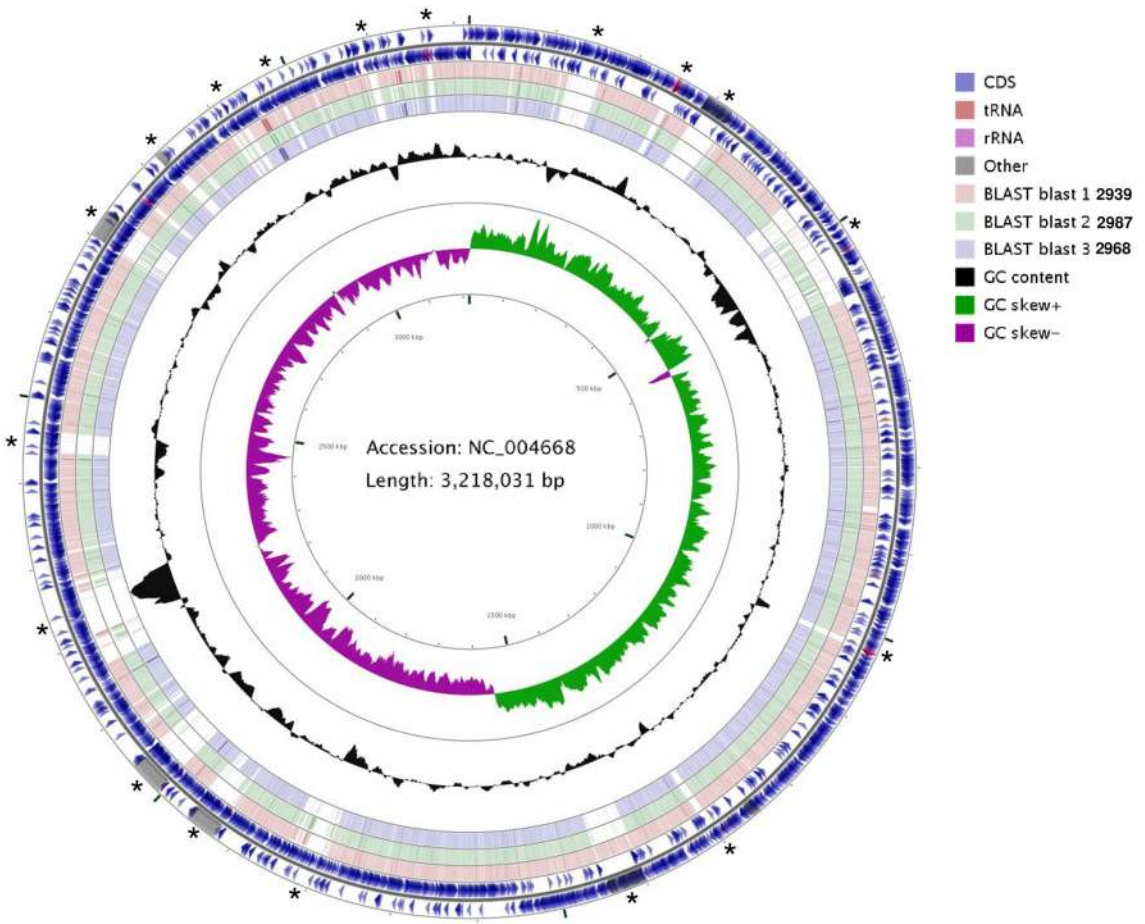


FIGURE 6 | CGView: Comparison of genome from study isolates with an isolate of human clinical origin (accession # NZ_CP018071) using blastn. The contents of the feature rings (starting with the outermost ring) are as follows. Ring 1, 2: forward and reverse strand features read from the primary sequence GenBank file. Rings 3, 4, 5: Query genomes 2939, 2987, 2968 in fasta format. Rings 7, 8: GC content and GC skew. The gaps in the alignment represent the regions missing in the query genomes in comparison to reference genome.

Of the 95 identified colonies *E. faecium* was the most predominant species across different treatment groups, sources (cloacae, ceca, or litter) and sampling days. These findings were consistent with previous studies (Yoshimura et al., 2000; Diarra et al., 2010). In contrast, other studies (Kaukas et al., 1986; Aarestrup et al., 2000a) have found that *E. faecalis* was the most predominant species from poultry. Furthermore, *E. cecorum* has been reported to be the most abundant species in the intestines of 3–5 week old broilers (Devriese et al., 1991; Gong et al., 2002), but this species was not identified in our study. The difference in the occurrence of species between studies may be due to differences in rearing conditions, source of chicks, sampling strategies, isolation and identification procedures, geographic disparities, or the use of medicated diets (Hayes et al., 2004). Susceptibility data of the 95 enterococci in this study showed a high proportion of resistant isolates from birds fed diets containing BAC. In agreement with an earlier study (Diarra et al., 2010), multiresistance was frequently detected among *E. faecium* and *E. faecalis* isolates that accounted for 61 and 25.3% of total enterococci population, respectively.

Bacitracin has been commonly used in poultry production to control infections caused by *Clostridium perfringens* (Diarra and Malouin, 2014). Our data showed that of the 59 isolates carrying the *bcr* gene, 93.3 and 93.4% were from birds fed diets containing BAC and SAL + BAC, confirming the relationship between bacitracin usage and the selection of isolates harboring the corresponding resistant determinant. The resistance to flavomycin has been described as an intrinsic property of enterococci (Aslam et al., 2012; Barros et al., 2012; Diarra and Malouin, 2014). The comparative genomics analysis of resistance and susceptible population in this study suggests the role of cell surface proteins as a possible resistance mechanism to this antimicrobial. Bambermycin (flavomycin) in feed was also found to promote flavomycin resistance in enterococci.

In the present study, ∼60% of total isolates were found to be resistant to erythromycin; however, a significantly higher frequency of resistance to erythromycin (83%) and

tylosin (79%) were observed in *E. faecalis*. Studies have reported resistance to MLS_B in enterococci involving 23S rRNA methylation as well as active efflux and inactivating enzymes (Hollenbeck and Rice, 2012; Jaglic et al., 2012). In our study, the co-resistance was also frequently observed in broilers receiving BAC. Approximately 22% of enterococci were simultaneously resistant to erythromycin, lincomycin, and quinupristin-dalfopristin, suggesting an acquired MLS_B related co-resistance. The streptogramin A resistance genes *vatD* or *vatE* were not detected in this study as in a previous study (Weisblum, 1985). Interestingly, the macrolide resistance *erm* gene was found to be associated with the *tet* gene, which was located on a transposable element in the majority of *E. faecalis* and *E. faecium* isolates. These transposable elements encode a full complement of machinery for conjugation as well as regulatory systems to control excision from the chromosome, suggesting that conjugative transfer of ARGs to a new bacterial host happen in the broiler gut (Roberts and Mullany, 2009; Wozniak and Waldor, 2010).

Overall, only 28.4% of the 95 enterococci were resistant to penicillin. However, a significant difference was found between *E. faecium* (38%) and *E. faecalis* (8%) isolated from poultry in the frequency of resistance to penicillin. It is well-known that penicillin resistance in *E. faecium* can arise due to low affinity or binding (due to point mutations in the C-terminal of *pbp5* gene) to penicillin-binding protein 5 (PBP5), as well as expression of β-lactamase (Hollenbeck and Rice, 2012). Other PBPs such as $PBP3^r$, which shares similarities with PBP2, which in turn confers resistance to methicillin in staphylococci, have also been reported to confer resistance to penicillin in other enterococci species (Rice et al., 2001). In the present study, analysis of *pbp5* gene of the isolates resistant to penicillin, revealed various point mutations in the C-terminal when compared to penicillin-susceptible isolates, which is consistent with previous reports (Ligozzi et al., 1996; Klibi et al., 2008). In spite of no β-lactamase genes being detected, a gene cluster homolog to the adaptive region of *mecA*, responsible for resistance to antibiotics such as methicillin, penicillin and other penicillin-like antibiotics (Stapleton and Taylor, 2002), was found in 24 (41.4%) and 6 (25%) of the *E. faecium* and *E. faecalis* isolates, respectively. However, no association was found between this gene cluster and resistance to PEN, as only 7 (25.9%) of the 35 isolates resistant to penicillin were positive for this *mecA*-like cluster. The characterization of this gene cluster may elucidate its role in β-lactam resistance in enterococci.

Resistance to linezolid has been documented among enterococci from poultry (Diarra and Malouin, 2014). Different mechanisms for linezolid resistance have been described, with the most common being mutations in genes encoding the 23S rRNA (Eliopoulos et al., 2004; Miller et al., 2014). We observed a G2576T mutation in 23S rRNA among the resistance isolates (position 2576 refers to the nucleotide position relative to 23S rRNA in *E. coli*). This particular mutation impedes the linezolid binding site and confers resistance. The importance of mannose phosphotransferase (ManPTS) in linezolid resistance in *E. faecium* has been recently reported (Geldart and Kaznessis, 2017), however no such observation was noted in this study.

Resistance to glycopeptide antibiotics like vancomycin has generated considerable research interest during the last few decades, as vancomycin is among the drugs of last resort to treat infections in humans caused by enterococci. As expected, all *E. gallinarum* and one *E. casseliflavus* isolated in this study carried the *vanC* operon, which is an intrinsic property of these enterococci species (Reid et al., 2001). The absence of vancomycin resistance and its associated genes in *E. faecium* and *E. faecalis* in the present study was expected and in agreement with an earlier study in the USA (Butaye et al., 2001). In contrast, resistance to vancomycin in enterococci was linked to the use of avoparcin in Europe (Aarestrup et al., 2000b).

Regardless of feed groups, 44 and 24% of the isolates were resistant to kanamycin. The prevalence of resistance to streptomycin (MIC > 2048) was relatively higher than resistance to other aminoglycosides, as only 14 isolates (14.8%) showed high level (HL) of resistance to this antibiotic, in agreement with others studies (Aarestrup et al., 2000a; Hayes et al., 2004; Tremblay et al., 2011). The aminoglycoside-modifying *aac (6′)-Ii* gene, encoding a 6′-*N*-aminoglycoside acetyltransferase found in almost all (98.5%) our aminoglycoside resistant isolates, has been reported to be chromosomally located in *E. faecium* and to confer resistance to synergism between cell-wall acting antibiotics and aminoglycosides (Eliopoulos et al., 2004). All 58 *E. faecium* and all four *E. gallinarum* harbored the *aac(6′)-Ii* gene. This gene was predominantly detected in isolates from birds fed BAC (86.7%) and those fed BAC and SAL (81.3%).

In the present study, the *tetM* gene was co-located with *ermB* on a conjugative transposon related to the Tn*916*-like family, thus the use of MLS_B antimicrobials could co-select for tetracycline resistance in broilers as concluded in a previous study (Cauwerts et al., 2007). Moreover, the Tn*552* transposons were found to be associated with a tellurite resistance gene, and the Czc efflux system which mediates resistance to heavy metals such as cobalt, zinc, and cadmium (Dressler et al., 1991). The genetic map of Tn*5252* was found to be somewhat similar to a streptococcal conjugative transposon carrying the β-lactamase class C family (100% sequence similarity to serine hydrolase). Several open reading frames (ORFs) also showed sequence homologies to DNA processing genes such as *trsE*-like transmembrane ATPase, integrases, excisionases, and transcriptional regulators responsible for conjugal transfer of these elements (Alarcon-Chaidez et al., 1997). The detected transposons and plasmids in the studied enterococci suggest their potential to disseminate ARGs associated with them, which could contribute to the mobilization of AMR.

A total of 17 virulence genes were examined in all 95 enterococcal genomes, including factors involved in bacterial replication, host-colonization and tissue damage and modulation of the host inflammatory system (Jett et al., 1994; Ike, 2017; Pillay et al., 2018). *E. faecalis* harbored a greater number of virulence genes (15 of 17 detected genes) than *E. faecium,* in agreement with a previous study (Champagne et al., 2011). The two virulence genes, *esp* and *asa1*, which are generally associated with human infections (Hallgren et al., 2009), were detected in none of the enterococci studied.

E. faecalis has been reported to be associated with pulmonary hypertension syndrome and endocarditis in broilers of all ages (Tankson et al., 2001, 2002). Except *E. faecium* and a few *E. faecalis*, the studied isolates were phylogenetically unrelated to human clinical isolates, suggesting that studies on their virulence potential in humans and even in poultry are warranted.

This longitudinal study provided useful information on the distribution of antimicrobial-resistant and virulent genotypes of enterococci from broiler chickens fed with different antimicrobial agents compared to control birds. In-depth generated genomic data could be used in AMR risk analysis and modeling to further improve understanding of AMR emergency and transmission, which could help to design production practices to mitigate antimicrobial resistance in broilers.

CONCLUSIONS

Multiple drug resistant enterococci continue to create issues for human health, and their presence in animals raised for food may threaten the sustainability and safety of food production. Data from this study showed that the use of BAC and BAM in feed significantly promoted the resistance phenotype and genotype of enterococci that persisted in broilers. The persistence of AMR enterococci specifically in litter could have an impact on the environment and subsequently on food safety when resistance genes are transferred to pathogenic bacteria. It is imperative to understand the molecular ecology of AMR enterococci in order to control their dissemination in poultry production. Despite the limited number of studied isolates, a correlation was found between WGS data and phenotype. Although a few discrepancies were noted for drugs for which enterococci show intrinsic resistance, our study is overall a critical step in advancing the use of WGS in resistance phenotype prediction among Gram-positive bacteria.

AUTHOR CONTRIBUTIONS

MD and ET conceptualized the study design. Whole genome sequencing was performed by NG and KA. Bioinformatics analyses and AST was performed by MR, JP, and MD. XY, JP, RZ, and TM contributed to experimental design. MD applied the statistical tests. MR and MD wrote the manuscript with input from all coauthors. MD was the principle investigator, who provided overall guidance, mentorship, and resources throughout the scope of this project.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2018.00083/full#supplementary-material

Table S1 | List of *E. faecalis* reference genomes.

Table S2 | List of *E. faecium* reference genomes.

Figure S1 | Amino acid sequence alignment of enterococci pbp5 and *Staphylococcus* mecA proteins.

Figure S2 | Species specific clustering of each species by WGS based SNV analysis.

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