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A study at the wildlife-livestock interface unveils the potential of feral swine as a reservoir for extended-spectrum β-lactamase-producing *Escherichia coli*

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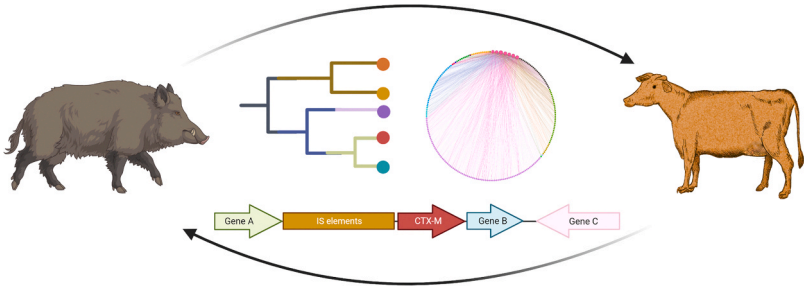
HIGHLIGHTS

- This study evaluated wildlife as a reservoir for antimicrobial resistance.
- This study showed a high prevalence of extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* in wildlife.
- This study unveiled the transmission mechanisms of antimicrobial resistant genes at the wildlife-livestock interface.
- This study highlights the potential hazard of feral swine as a reservoir of ESBL-producing *E. coli*.
- This study suggests the need for comprehensive strategies to mitigate wildlife associated AMR transmission.

GRAPHICAL ABSTRACT



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ABSTRACT

Wildlife is known to serve as carriers and sources of antimicrobial resistance (AMR). Due to their unrestricted movements and behaviors, they can spread antimicrobial resistant bacteria among livestock, humans, and the environment, thereby accelerating the dissemination of AMR. Extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* is one of major concerns threatening human and animal health, yet transmission mechanisms at the wildlife-livestock interface are not well understood. Here, we investigated the mechanisms of ESBL-producing bacteria spreading across various hosts, including cattle, feral swine, and coyotes in the same habitat range, as well as from environmental samples over a two-year period. We report a notable prevalence and

Abbreviations: AMR, antimicrobial resistance; ARB, antimicrobial resistant bacteria; SNPs, single nucleotide polymorphisms; ESBL, extended-spectrum β-lactamase; CRB, cefotaxime resistant bacteria; ARGs, antibiotic resistance genes; MLST, multi locus sequence type; CARD, comprehensive antibiotic resistance database; VF, virulence factor; HGT, horizontal gene transfer; IS, insertion sequences; MGEs, mobile genetic elements; MDR, multidrug resistance; MIC, minimum inhibitory concentration; AST, antibiotic susceptibility test; KEGG, Kyoto encyclopedia of genes and genomes; ST, sequence type.

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clonal dissemination of ESBL-producing *E. coli* in feral swine and coyotes, suggesting their persistence and adaptation within wildlife hosts. In addition, *in silico* studies showed that horizontal gene transfer, mediated by conjugative plasmids and insertion sequences elements, may play a key role in spreading the ESBL genes among these bacteria. Furthermore, the shared gut resistome of cattle and feral swine suggests the dissemination of antibiotic resistance genes at the wildlife-livestock interface. Taken together, our results suggest that feral swine may serve as a reservoir of ESBL-producing *E. coli*.

1. Introduction

The dissemination of antimicrobial resistant bacteria (ARB) continues to pose a threat to public health, contributing to increased mortality in both humans and animals [1,2]. Notably, the rapid spread of extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae*, with their rising prevalence in humans, animals, and the environment, underscores the urgent global need for solutions to mitigate the transmission of these pathogens [3–8]. ESBL is an enzyme that inactivates most third- and some fourth generation cephalosporins by hydrolyzing the β-lactam rings [9–11]. The resistance to the third-generation cephalosporin antibiotic cefotaxime has been attributed to the acquisition of the $bla_{\text{CTX-M}}$ gene, which stands as the most prevalent genetic element encoding ESBL [12]. Numerous variations of $bla_{\text{CTX-M}}$ genes are present on plasmids and in chromosomes of major human pathogens, such as pathogenic *Escherichia coli* and *Klebsiella pneumonia* [13,14]. Intriguingly, a prototype of the $bla_{\text{CTX-M}}$ gene has been traced to the environmental bacteria *Kluyvera* spp. [15]. Therefore, ESBL-producing *Enterobacteriaceae* diminish the effectiveness of medically important β-lactam antibiotics used to treat infectious diseases in humans and animals. Since first identified in human clinical settings, ESBL-producing pathogens have prevailed in diverse niches, including food-producing animals, wildlife, and the environment [15–20]. ESBL-producing pathogens are suggested to disseminate through complicated pathways, such as human-to-human, human-to-animal, and environmental routes [7,8].

Cow-calf operations are highlighted as a hotspot of ARB transmission because cattle graze on open pastures and rangelands, where direct or indirect interactions with wildlife are common due to shared resources like surface water, soil, and feed [21–23]. This dynamic interaction at the wildlife-livestock interface has gained importance for the study of zoonotic disease transmission, involving diseases like avian influenza, rabies, salmonellosis, and bovine tuberculosis [24]. Recently, Teng *et al.* isolated ESBL-producing *E. coli* in grazing beef cattle raised without antibiotic use, and these multi-drug resistant ESBL-producing *E. coli* encoded versatile virulence factors capable of causing severe diseases in animal and human hosts, suggesting that these bacteria transmit at the interface of wildlife-livestock [18]. Furthermore, one of the beef cattle isolates, JEONG5446 clustered with a variety of isolates originated from humans, animals, and environmental sources, suggesting ESBL-producing *E. coli* are widespread [18]. The two closest strains, PNUSAE004879 and 2015 C-3863, isolated from humans, had relatively small numbers of single nucleotide polymorphisms (SNPs) in their core genomes compared to the number in JEONG5446, indicating that these isolates are closely related. Similarly, Lee *et al.* [25] reported that feral swine may transmit microbiota and antibiotic resistance genes (ARGs) to cattle grazing in areas that overlap with feral swine habitats within a cow-calf operation. This study indicated that about 11 % of cattle microbiota in the gastrointestinal tract was sourced from feral swine. Based on these findings, we hypothesized that farm animals are important carriers and potential reservoirs of ESBL-producing bacteria, and the emergence of this pathogen in cattle raised without antibiotic use might have originated from wildlife.

In this study, we aimed to determine whether ESBL-producing *E. coli* are transmitted at the interface of wildlife-livestock. To achieve this, we attempted to isolate identical or clonal variants of these bacteria from wildlife and livestock coexisting in a shared habitat within a cow-calf operation. Subsequently, we conducted whole genome sequencing (WGS) and *in silico* analysis to trace their genomes. Our comprehensive isolation efforts targeted ESBL-producing *E. coli* from cattle and feral swine inhabiting the same environment in South Florida. To gain insight into bacterial colonization within hosts, we performed a thorough genetic profiling of the ESBL-producing *E. coli*. This analysis covered various aspects, including virulence factors, antibiotic resistance, and mobile genetic elements that facilitate the transfer of ARGs. Our findings highlight the potential of these isolates to establish colonization in wildlife, suggesting that feral swine may serve as a reservoir of ESBL-producing *E. coli*.

2. Materials and methods

2.1. Ethics statement

All the animals were processed and immobilized following the standard practices and released back into the wild environment after capture. All animal operations, including animal capture, animal care, and animal use, had prior approval by the University of Florida Institutional Animal Care and Use Committee (IACUC number, feral swine: 201408495 & 201808495, coyote: 201408477, and cattle: 201709994). All the animals were handled and immobilized following approved standard practices and either released back into the wild or ranch after sampled.

2.2. Sample collection and processing

We collected two batches of samples at Buck Island Ranch in South Florida, USA, in 2017 (n = 113) and 2018 (n = 364), respectively. The batch-2017 samples included animal feces from cattle (*Bos taurus*; n = 47), feral swine (*Sus scrofa*; n = 52) and coyotes (*Canis latrans*; n = 3), and environmental samples consisting of soil (n = 5) and water (n = 6). The batch-2018 samples included animal feces from cattle (*Bos taurus*; n = 100) and feral swine (*Sus scrofa*; n = 224) and environmental samples of soil (n = 20) and water (n = 20). Animal feces were collected with sterile cotton swabs from the recto-anal junction. The swab of animal feces was resuspended with 2 mL of Tryptic Soy Broth (TSB) and 2 mL of 30 % glycerol. Environmental samples were obtained within the cattle ranch. Soil samples were weighed at 2 g first, then resuspended with 2 mL of TSB and 2 mL of 30 % glycerol. In terms of water samples, bacterial cells were concentrated by centrifuging at 1500 x g for 10 min, and the pellet was resuspended with 1 mL of TSB and 1 mL of 30 % glycerol. Resuspended solutions were aliquoted into 2 mL sterile cryotubes and stored at − 80 °C until use. Frozen samples were transported to the Emerging Pathogens Institute at the University of Florida for further processing.

2.3. Isolation and identification of cefotaxime resistant bacteria

To isolate cefotaxime resistant bacteria (CRB), 100 μL of aliquots from fecal and environmental samples were spread on MacConkey agar plates (BD, USA) containing 4 μg/mL of cefotaxime and incubated at 37 °C overnight. Up to 10 colonies with different morphologies were selected per sample for future studies, and purified isolates were stored at − 80 °C until use. In the case of CRB negative samples from direct plating, enriched culture samples at 37 °C overnight were also plated on

MacConkey agar plates with cefotaxime to prevent false-negative samples.

To specify isolated CRB, the 16 S rRNA gene was amplified with primers (F: 5'-CAGGCCTAACACATGCAAGTC-'3, R: 5'-GGGCGGWGTGTACAAGGC-'3) using a polymerase chain reaction (PCR) method [26]. The PCR products were purified with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and sent to Genewiz (NJ, USA) for Sanger sequencing. The 16 S rRNA sequences were blasted against the NCBI database to identify CRB isolates. The presence of bla_{CTX-M} genes was initially confirmed with all of the isolated CRB by PCR with the primer sets (CTX-M-F: 5' TTTGCGATGTGCAGTACCAGTAA-3', CTX-M-R: 5'-CGATATCGTTGGTGGTGCCATA-3') as previously described [27]. Amplified PCR products were visualized on an agarose gel stained with ethidium bromide by electrophoresis. The bla_{CTX-M} positive isolates, designated ESBL-producing bacteria, were used for further studies.

2.4. Whole genome sequencing of cefotaxime resistant bacteria

To further investigate the features of ESBL-producing bacteria, WGS was conducted using Illumina MiSeq with 250 bp paired-end sequencing with the selected isolates that harbor bla_{CTX-M} genes. A total of 78 bla_{CTX-M} positive *E. coli* isolates were subjected to WGS, with 13 isolated in 2017 and 65 isolated in 2018. Bacterial genomic DNA was extracted from each isolate with the DNeasy blood and tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The DNA libraries were constructed by using the Nextera XT sample preparation kit (Illumina, San Diego, CA) following the manufacturer's protocol. The sequencing results were trimmed for low-quality reads with the Sickle [28] and *de novo* assembled with SPAdes 3.0 [29]. After assembly, contigs of less than 200 bp were eliminated. Then, Parsnp v1.2 [30] was applied to generate the phylogenetic tree based on core-genome single nucleotide polymorphisms (SNPs). The final tree was visualized using FigTree [31]. The number of SNPs within the clades was calculated by NCBI Pathogen Detection [32], and the number of SNPs between clades was estimated by Parsnp.

2.5. Antibiotic susceptibility of cefotaxime resistant bacteria

Based on the genome architecture of phylogenetic tree, fifteen representative isolates were further selected, and the minimum inhibitory concentration was determined for cefotaxime using the microbroth dilution method [33]. An antimicrobial susceptibility test against 12 antibiotics was applied to identify the multidrug resistant character of the isolates using the disk diffusion susceptibility method on Mueller Hinton agar [34,35]. All antibiotic susceptibility tests were followed based on the Clinical and Laboratory Standard Institute (CLSI) guidelines (M100-S25) [36]. The twelve antibiotics used in this study were amikacin (30 μg), ampicillin (10 μg), amoxicillin/clavulanic acid (30 μg), sulfisoxazole (0.25 mg), ceftiofur (30 μg), chloramphenicol (30 μg), cephalothin (30 μg), gentamicin (10 μg), nalidixic acid (30 μg), streptomycin (10 μg), sulfamethoxazole/trimethoprim (23.75 μg/1.25 μg), and tetracycline (30 μg) (BD, USA). *E. coli* (ATCC 35401) strain was used for quality controls.

2.6. Genetic characterization of ESBL-producing bacteria

To determine the multi locus sequence type (MLST) and serotype information of these isolates, assembled genomes were submitted to the Center for Genomic Epidemiology (CGE) (https://www.genomicepidemiology.org/) [37]. Virulence genes were identified by using BLAST to align the whole genome sequences against the Virulence Factor Database (VFDB) through the Pathosystems Resource Integration Center [38,39]. Virulence factor genes with < 70 % subject coverage or < 70 % query coverage were eliminated. ARGs in each isolate were predicted with the Comprehensive Antibiotic Resistance Database (CARD) (version 2.0.0) [40]. Briefly, the whole genome sequence of each isolate was submitted to the Resistance Gene Identifier (RGI, version 4.0.3) in CARD with the default setting to predict resistant genes. Only "perfect" and "strict" hits with > 70 % gene coverage and > 70 % gene identity were selected. To identify whether bla_{CTX-M} genes were located in the plasmid or chromosome, a PLACNETw tool was applied to generate the contig networks that reflected the loci of the genes [41]. The genetic environment of the bla_{CTX-M} genes was analyzed with GenBank files of sequenced strains. Gene annotation and sequence similarity among isolates were visualized by EasyFig [42]. Mobile genetic elements (MGEs), including plasmid and insertion sequence (IS) elements, were identified through the BLASTn tool of PlasmidFinder and ISFinder [43,44].

2.7. Shotgun metagenomic sequencing and downstream analysis

To conduct shotgun metagenomic sequencing, we pooled cattle fecal samples into CRB-positive and CRB-negative samples. Concurrently, feral swine fecal samples were grouped according to the presence of CRB and ESBL-producing *E. coli* in fecal samples. The library for six pooled DNA samples was constructed using the Illumina TruSeq Nano DNA Library Prep kit (Illumina, Inc., USA) according to the manufacturer's protocols. Paired-end sequencing was performed on the Illumina HiSeq 2000 (Novogene, USA). The paired-end reads were filtered for quality control using Trimmomatic [45] as described in the previous study [46]. Briefly, Trimmomatic's 'ILLUMINACLIP' command was used for the TruSeq3 adapter sequences. A maximum of two mismatches were allowed in the initial seed, and adapter clipping occurred if a match score of 30 was reached. Both reads were retained upon clipping. Then a sliding window of four nucleotides was used to remove nucleotides from the 3' end once the Phred score within the window fell below 20.

To identify ARGs, bovine and pig DNA sequences were removed by aligning the trimmed sequences to *Bos taurus* (UMD_3.1) and *Sus scrofa* (Sscrofa_11.1) reference genomes, respectively, using the BWA version 0.7 [47]. The remaining sequences were aligned to MEGARes database version 2.0 using ResistomeAnalyzer [48]. Only ARGs that have greater than 80 % gene fraction and did not require SNP confirmation were retained for further downstream analysis.

To analyze the bacterial taxonomy, trimmed paired-end sequence reads were merged and analyzed using MG-RAST pipeline [49]. Briefly, artificial replicate sequences produced by sequencing artifacts, and host specific sequences (*Bos taurus*, UMD v3.0 and *Sus scrofa*, NCBI v10.2) were removed [50,51]. Sequences of length shorter than 50 bases and quality scores lower than 20 were removed for further analysis [52]. Sequence similarity was computed against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database with a maximum e-value of 1×10^{-5} and a minimum cutoff of identity and alignment length at 60 % and 15 bp, respectively.

2.8. Co-occurrence network analysis

To predict the interactions between ARGs and their bacteria hosts in the microbial community, co-occurrence patterns of ARGs and bacterial genera were evaluated in the network interface using pairwise Spearman's rank correlations (rs) [53]. The Spearman rank correlations were analyzed based on the bacterial relative abundance using the Hmisc program of the R software package. A significant rank correlation between two values ($rs > 0.67$ or $rs < -0.67$, P-value < 0.05) was considered to be a co-occurrence event. The network was visualized using the Circular algorithm in the interactive platform Gephi (http://gephi.org). Network nodes represented different ARGs or bacterial genera, and edges indicated correlations between nodes. The size of the nodes represented the degree of connection, and the thickness of the edges indicated the strength of the correlation.

To investigate the association between bla_{CTX-M} genes and MGEs, the co-occurrence networks of bla_{CTX-M} genes with conjugative plasmids and

IS elements were assessed. Pairwise Spearman's rank correlations (rs) [53] were evaluated based on the frequency of MGEs using the Hmisc program in R. Significant rank correlations between two values (rs > 0.67 or rs < −0.67, *P*-value < 0.05) were interpreted as co-occurrence events. The resulting network was visualized using the ForceAtlas 2 algorithm in Gephi (http://gephi.org). Depicted nodes represented $bla_{CTX\text{-}M}$ genes or various MGEs, and edges indicated correlations between nodes. Node size conveyed the degree of connection, and edge thickness reflected correlation strength.

2.9. Statistical analysis

Statistical analyses were conducted using RStudio Version 1.1456 [54]. Differences in the relative abundance of ARGs in metagenomes and the number of ARG types in cattle and feral swine were compared using Student's *t*-tests. Associations between bacteria genera and ARGs, and the interactions between $bla_{CTX\text{-}M}$ genes and MGEs, were analyzed by Spearman rank correlations. Significance was considered for correlations > 0.67 or < −0.67. Statistical significance was defined as a *P*-value < 0.05.

3. Result

3.1. Prevalence and identification of ESBL-producing E. coli at the interface of wildlife-livestock

We used cefotaxime, a third-generation cephalosporin antibiotic, to select cefotaxime resistant bacteria (CRB) at the interface of wildlife-livestock. Over a span of two years, we consistently isolated CRB across all sample types (Fig. 1). In 2017, CRB were isolated from all environmental samples analyzed. Among animal samples, notably higher CRB prevalence was observed in feral swine (51.9 %, 27/52) and coyotes (66.7 %, 2/3) in comparison to cattle (8.5 %, 4/47) (Fig. 1A). Similarly, in 2018, CRB were prevalent in all sample categories, with environmental samples, specifically soil (40 %, 8/20) and water (50 %, 10/20), displaying higher CRB prevalence than animal feces. Within animal samples, the prevalence of CRB in feral swine (37.5 %, 84/224) exceeded that in cattle (14 %, 14/100) (Fig. 1B). Intriguingly, PCR genotyping of confirmed CRB revealed the exclusive presence of $bla_{CTX\text{-}M}$ gene carrying isolates, known as ESBL-producing bacteria, only in wildlife samples over the two-year period but absence in cattle. In 2017, the prevalence of ESBL-producing bacteria was 3.8 % (2/52) in feral swine and 33.3 % (1/3) in coyotes (Fig. 1A). However, this prevalence escalated to 25.9 % (58/224) in feral swine feces collected in 2018 (Fig. 1B). Taken together, a two-year screening demonstrates high prevalence of ESBL-producing bacteria in feral swine, but these bacteria were absent in grazing cattle.

3.2. Potency of wildlife as reservoirs for ESBL-producing E. coli

To investigate the inter- and intra-species transmission dynamics of ESBL-producing *E. coli*, we explored the genetic relatedness of isolates from animals coexisting in a shared habitat within a cow-calf operation. We sequenced the whole genomes of 78 isolates from feral swine and coyotes and conducted a core-genome-based SNPs phylogenetic analysis. These 78 genomes formed 14 distinct clades, with 72 isolates aggregating into eight multi-isolate clades, while the remaining six genomes were classified into six discrete single-isolate clades. The prevailing sequence type (ST) was ST155, encompassing ESBL-producing *E. coli* from both 2017 and 2018, indicating persistent colonization by ST155 within the feral swine population. Most notably, strains within the ST155 clade exhibited minimal genetic divergence, as evidenced by a limited number of SNPs (38–86 SNPs), underscoring a high degree of genetic homogeneity among these bacteria (Fig. 2). ESBL-producing *E. coli* isolated from a coyote belonged to ST398, and they showed only three SNPs, indicative of clonal variants. Similarly, five strains (KCJK7799, KCJK7800, KCJK7804, KCJK7805, and KCJK7806) isolated from single feral swine in 2017 were identified as clonal variants, although their specific STs remained unassigned. In contrast, ESBL-producing *E. coli* isolated from feral swine in 2018 exhibited greater genetic heterogeneity, manifesting across multiple clades. In particular, ESBL-producing *E. coli* from different feral swine (D-swine) coalesced within identical clades, encompassing ST1201, ST10, ST46, ST361, and ST155 (Fig. 2). This clustering indicates the potential for transmission and colonization of these bacteria among feral swine populations. Furthermore, our observations revealed that the same feral swine (S-swine) carried multiple STs, including ST155, ST685, ST10, ST1201, and ST8076. However, none of the feral swine and coyotes harboring these bacteria exhibited any discernible symptoms of animal diseases attributable to ESBL-producing *E. coli*. In summary, we provide evidence of the persistent presence and diversity of ESBL-producing *E. coli* within asymptomatic wildlife populations, suggesting the potential role of wildlife as reservoirs for ESBL-producing *E. coli*.

3.3. The profile of ARGs in ESBL-producing E. coli

To assess multidrug resistance (MDR) of the isolates, we conducted minimum inhibitory concentration (MIC) tests for cefotaxime and antibiotic susceptibility test (AST) against various classes of antibiotics. We selected 15 genetically distinct ESBL-producing *E. coli* strains, based on Fig. 2, as representatives of distinct clades for functional genomic analyses. All isolates exhibited an exceptionally high MIC for cefotaxime (≥128 μg/mL, Fig. 3A), which is 32 times higher than the breakpoint for cefotaxime resistance (≥4 μg/mL) [36]. Furthermore, all isolates were resistant to ampicillin, ceftiofur, cephalothin, chloramphenicol,

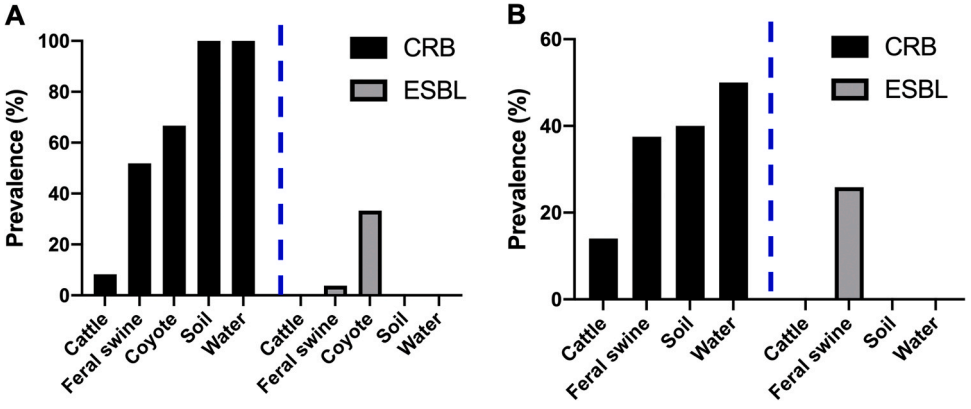


Fig. 1. Prevalence of antimicrobial resistant bacteria. The prevalence of cefotaxime resistant bacteria (CRB) and ESBL-producing bacteria are presented by sample types in 2017 (A) and 2018 (B). The prevalence of CRB is shown in black, and ESBL-producing bacteria is in dark grey.

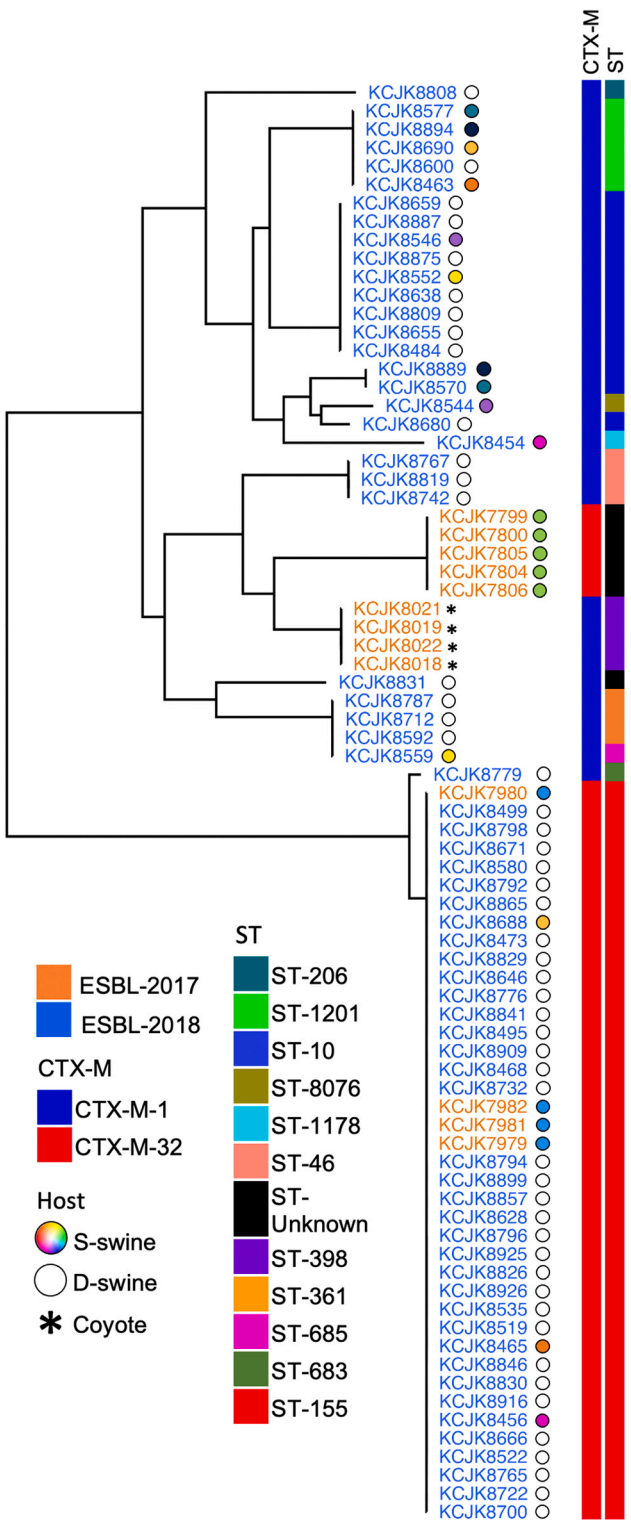


Fig. 2. Phylogenetic relatedness analysis of ESBL-producing bacteria. The maximum-likelihood phylogenetic tree was constructed based on the single-nucleotide polymorphisms identified in the core genomes of the 78 multidrug-resistant *E. coli* isolates by Parsnp. The sequencing types (ST) and CTX-M types of isolates were identified by using the MLST 2.0.4 software of the Center for Genomic Epidemiology. S-swine indicates that ESBL-producing bacteria, marked with the same color circle, were isolated from the same swine, while D-swine indicates that ESBL-producing bacteria, marked with an open circle, were isolated from different swine.

sulfisoxazole, and tetracycline. However, most isolates demonstrated intermediate or susceptible response to amoxicillin/clavulanic acid (15/15), gentamicin (13/15), nalidixic acid (14/15), and sulfamethoxazole/trimethoprim (12/15). Regarding amikacin and streptomycin, resistance was prevalent in 46.7 % (7/15) and 93.3 % (14/15) of isolates, respectively (Fig. 3A). Moreover, ESBL-producing *E. coli* identified in 2017 and 2018 displayed similar patterns of resistance susceptibility. All tested ESBL-producing *E. coli* were resistant against more than three classes of antibiotics, indicating a state of MDR.

In pursuit of identifying ARGs responsible for the observed MDR in our isolates, we subjected the whole genomes of representative isolates of each clade shown in Fig. 3A to CARD analysis (Fig. 3B). This investigation unveiled a total of 72 ARGs, spanning 14 distinct drug classes. A noteworthy finding was that the majority of these ARGs (51.4 %, 37 out of 72) were associated with MDR, consistent with our AST results. In addition, ESBL-producing *E. coli* isolates exhibited a shared repertoire of genes implicated in metal resistance. Regarding β-lactam resistance, all our representative isolates carried either $bla_{\text{CTX-M-1}}$ or $bla_{\text{CTX-M-32}}$ genes. In addition, several other β-lactamase genes, namely *ampC* and $bla_{\text{TEM-1}}$, were identified.

Furthermore, our investigation revealed the presence of several ARGs in ESBL-producing *E. coli* isolates from the year 2017. The *aadA* gene, responsible for aminoglycoside resistance, was harbored in strains isolated from both feral swine (KCJK7799) and coyote (KCJK8018). KCJK7799 harbored the *linG* gene, conferring lincosamide resistance, and the *SAT-2* gene, associated with nucleoside resistance. In ESBL-producing *E. coli* isolates from coyote (KCJK8018), we detected several other genes, including *qnrB19, mef(B), sul3, tet(B), tetR,* and *dfrA12*, contributing to resistance against quinolone antibiotics, macrolides, sulfonamides, tetracyclines, and trimethoprim, respectively (Fig. 3B). Moreover, we observed that ESBL-producing *E. coli* isolated in 2018 exhibited similar ARG profiles, with minor variations occurring in the β-lactamase genes.

3.4. Virulence factors in MDR ESBL-producing E. coli

Virulence factors, inherent to pathogens, are pivotal for assessing potential pathogenicity within hosts. In this study, we identified the virulence factor (VF) profiles within MDR ESBL-producing *E. coli* to elucidate their potential pathogenic attributes. Although these strains carried critical virulence genes that may cause severe disease in hosts, the VF profiles shows a range of potential virulence genes, numbering from 35 to 61, among the isolates (Fig. 4). All ESBL-producing *E. coli* strains consistently harbored the *ompA* gene, implicated in bacterial adhesion and invasion. Furthermore, the *ent* and *fep* gene families, essential for iron acquisition to support bacterial growth, were universally present as were genes from the *flg* family, crucial for flagellar formation and consequently, bacterial adhesion. Virulence genes, particularly those affiliated with the Type II secretion system (*gsp* gene families) were found in strains from feral swine (10 out of 14 isolates), contrasting with their absence in the coyote strain (KCJK8018). This observation suggests a diversity of virulence attributes among ESBL-producing *E. coli* found in feral swine (Fig. 4). Notably, ESBL-producing *E. coli* isolates collected over a two-year period exhibited a consistent VF profile.

3.5. Loci of the ESBL genes

The genetic localization of ESBL genes, either within plasmids or chromosomes, has long served as a classical strategy to differentiate various CTX-M enzyme variants [12]. This distinction has accumulated an increasingly significant role in understanding the evolutionary dynamics and dissemination of the ESBL genes. Therefore, we first ascertained the genomic location of ESBL genes. We conducted a PLACNET analysis, allowing us to pinpoint the specific contigs harboring $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-32}}$ genes within either chromosomal or plasmid contexts.

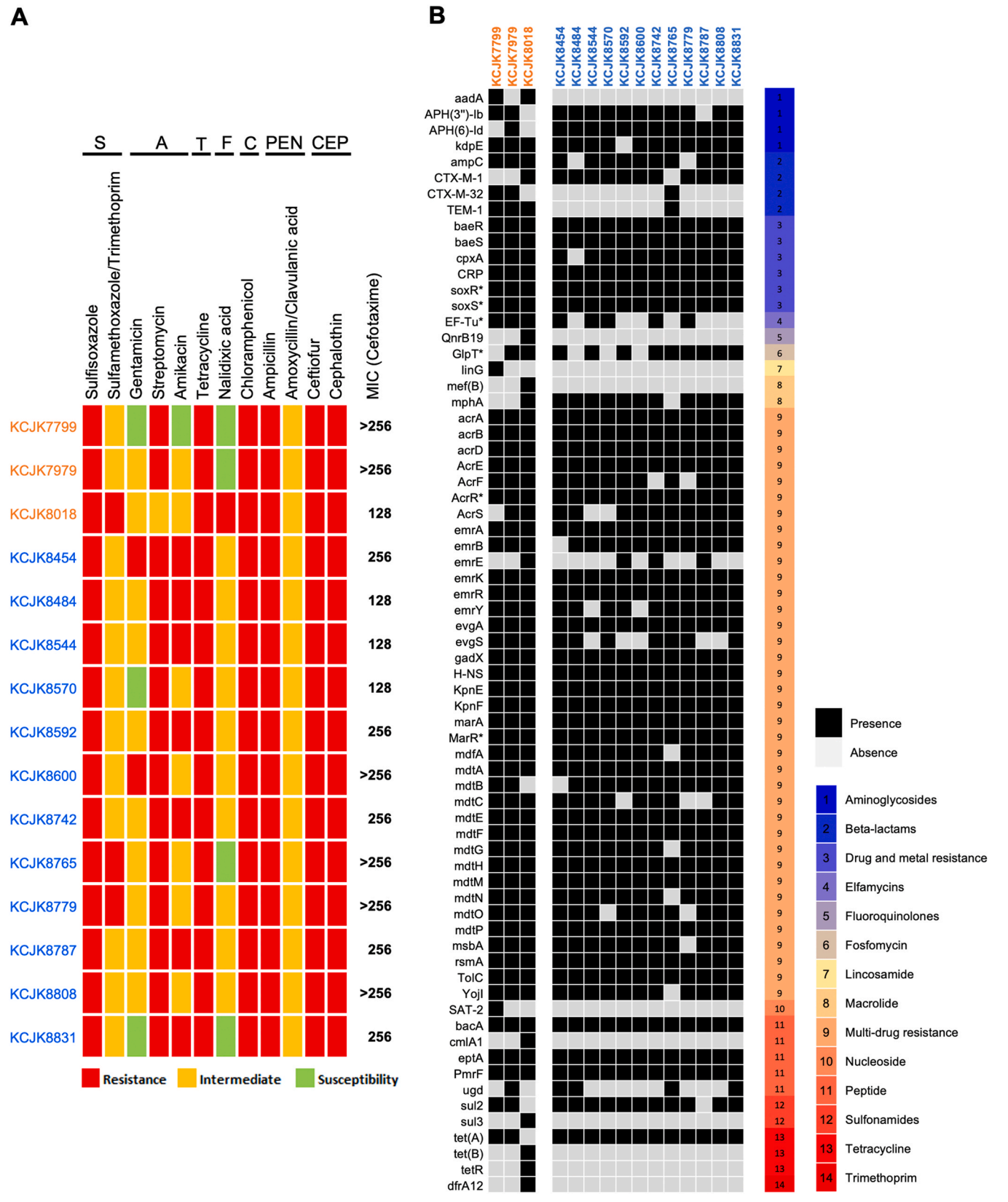
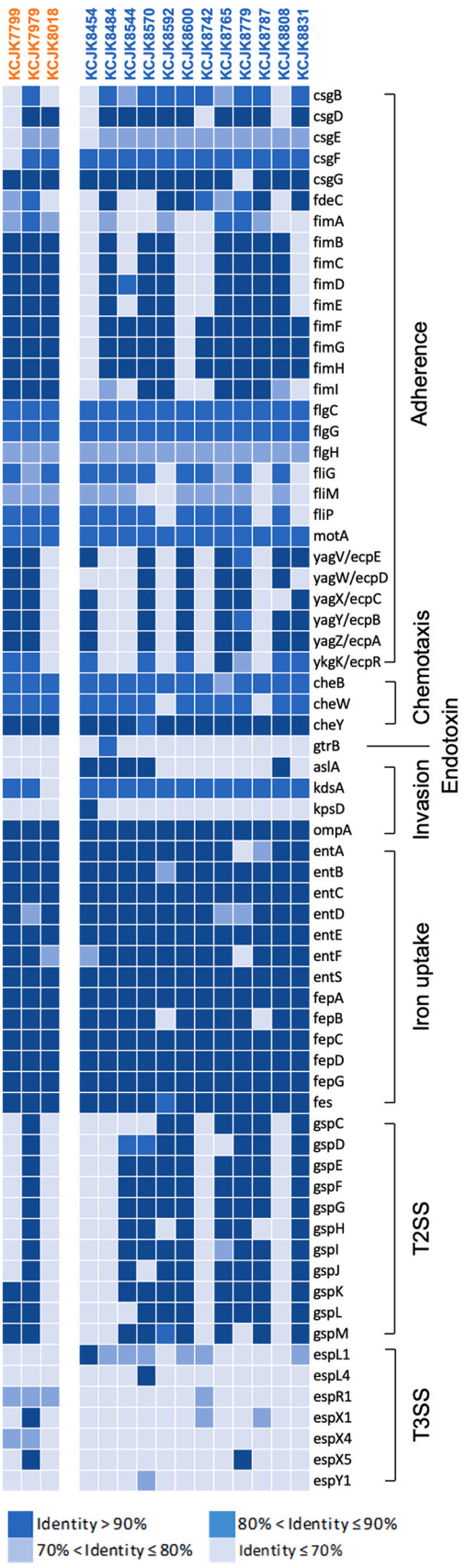


Fig. 3. Profile of antimicrobial resistant genes (ARGs) in the ESBL-producing bacteria. (A) Fifteen isolates were tested for antibiotic susceptibility against 12 different antibiotics belonging to 8 classes (S: Sulfonamide, A: Aminoglycoside, T: Tetracycline, F: Fluoroquinolone, C: Chloramphenicol, PEN: Penicillin, and CEP: Cephalosporin) following the CLSI guidelines. Antibiotic resistance is indicated by different colors (red: resistant, yellow: intermediate resistant, and green: susceptible. (B) The antimicrobial resistant genes of 15 representative multidrug-resistant *E. coli* strains were identified by comparing their whole-genome sequences against the Comprehensive Antibiotic Resistance Database. Black blocks indicated ARGs with $\geq$ 70 % identity, and those with $<$ 70 % identity were shown in light gray blocks. ARGs were classified into different drug classes based on their functions, and the drug classes are listed next to the gene names. Asterisks mark genes with mutations conferring antibiotic resistance.



(caption on next column)

Fig. 4. Virulence factors in multi-drug resistant *E. coli*. The virulence factors of fifteen representative isolates from this study were identified by aligning protein sequences of the representative isolates (query sequences) against the reference sequences (subject sequences) in the Virulence Factor Database (VFDB) using BLASTp. The similarity of each virulence factor (query sequence) to the subject sequence in VFDB was calculated using the following formula: subject coverage (%) × query coverage (%) × identity of query and subject sequences (%). The high-to-low similarities were presented using different colors, ranging from dark blue to light blue.

These identified contigs are denoted in purple in Fig. 5. Among the 78 ESBL-producing *E. coli* strains isolated from wildlife, we observed the $bla_{\text{CTX-M-1}}$ gene predominantly within the plasmid contigs network of 33 ESBL-producing *E. coli* strains, spanning various STs (Fig. 5A). In contrast, the $bla_{\text{CTX-M-32}}$ gene was consistently located within the chromosomal network, predominantly associated with strains belonging to the ST155 lineage (Fig. 5B). These findings underscore the likelihood that the $bla_{\text{CTX-M-1}}$ gene undergoes dissemination among a diverse array of *E. coli* through plasmid transfer, contributing to the conversion into ESBL-producing *E. coli*. On the other hand, the $bla_{\text{CTX-M-32}}$ gene is more likely transferred through mobile genetic elements (MGEs).

3.6. *Dissemination of the ESBL genes mediated by horizontal gene transfer (HGT)*

As the dissemination of ARGs is frequently facilitated by MGEs that enhance horizontal gene transfer (HGT) and mobility of resistance genes [8,55], we further elucidate the role of MGEs in the transmission of ESBL genes. Initially, we conducted a comprehensive profiling of MGEs, encompassing conjugative plasmids and insertion sequences (IS) elements, within 15 representative ESBL-producing *E. coli* isolates. Our analysis revealed the presence of a total of 10 conjugative plasmids (Fig. 6A) among these ESBL-producing *E. coli*. Notably, IncR and IncN plasmids were prevalent across the population. IncR emerged as the predominant plasmid, harbored by the majority of isolates, while IncN was identified in 80 % of the isolates (12/15). Interestingly, KCJK8018, isolated from a coyote, did not carry either IncR or IncN plasmids. Instead, it harbored three other plasmids, Col440I, IncFIB(K), and IncFIA(HI1), indicating a distinct lineage compared to feral swine isolates. Occasional detection of other conjugative plasmids was noted in a few isolates. Furthermore, our correlation analysis delineated plasmids carrying the $bla_{\text{CTX-M-1}}$ gene, revealing a robust association between the $bla_{\text{CTX-M-1}}$ gene and the IncN and IncR plasmids (Fig. 6B). This suggests that these conjugative plasmids, particularly IncN and IncR, may serve as the primary drivers behind the dissemination of ESBL genes.

Within the ESBL-producing *E. coli* population, a diverse repertoire of IS elements was observed. IS*26* and IS*Vsa3* are consistently present in the majority of ESBL-producing *E. coli* population (14/15), with a few exceptions (Fig. 6C). IS*Kpn8* was exclusively found in the majority of strains isolated from feral swine in 2018, whereas Tn*2* and IS*Vsa5* were found exclusively in isolates from a coyote and feral swine in 2017. Strains from 2018 exhibited a higher abundance of IS elements compared to those from 2017. Several IS elements, including IS*6*, IS*Vsa3*, IS*26*, IS*Kpn8*, and IS*903*, displayed strong associations with the $bla_{\text{CTX-M-1}}$ gene, whereas IS*5*, IS*26*, IS*903* and IS*Ec38* were associated with the $bla_{\text{CTX-M-32}}$ gene (Fig. 6D). These findings indicate that ISs may play a crucial role in facilitating the widespread of both $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-32}}$ genes across a diverse ESBL-producing *E. coli*.

To gain insight into the dissemination mechanisms of ESBL genes among bacterial isolates, we further investigated the genetic environment of the $bla_{\text{CTX-M}}$ genes. ESBL-producing *E. coli* from different years and animal hosts were examined to explore the potential evolution and host diversity of ESBL gene transmission. $bla_{\text{CTX-M-1}}$ gene loci exhibited identical insertion sites in both KCJK8018, isolated from a coyote in 2017, and KCJK8742, isolated from feral swine in 2018 and were associated with the same IS elements, IS*6*/IS*26* and IS*256* (Fig. 7A).

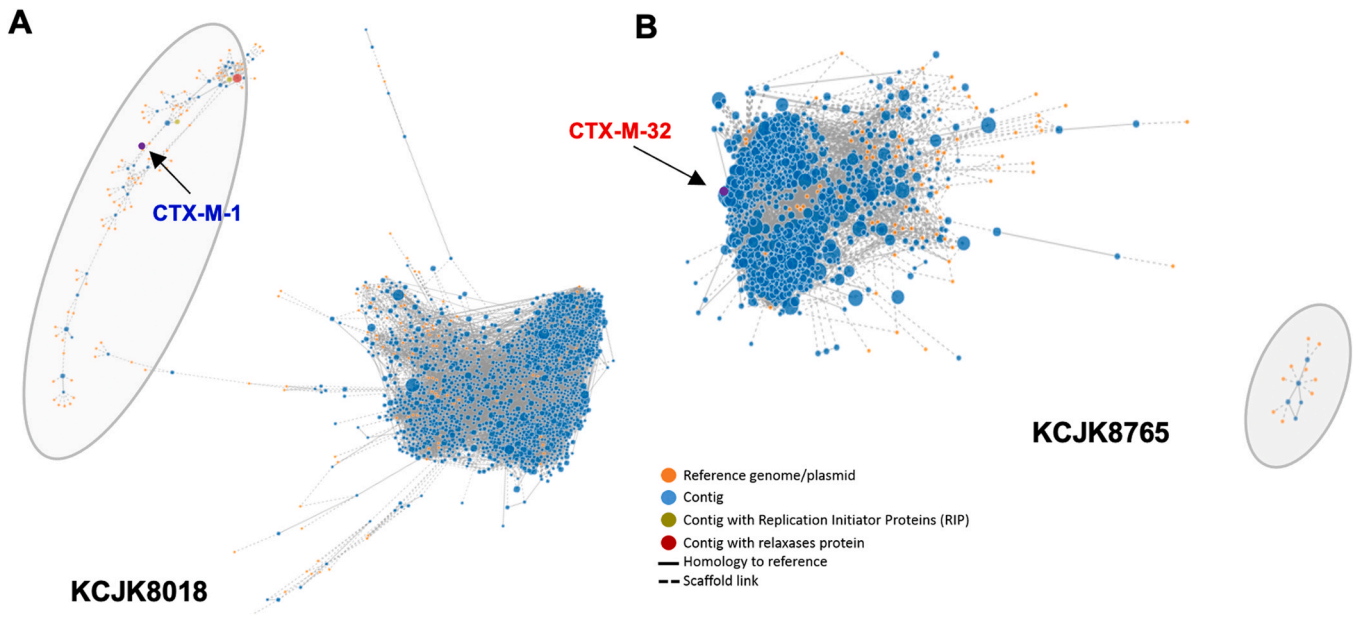


Fig. 5. Comparison of genetic location and surrounding environments of *bla*$_{CTX-M}$ genes. The genetic location of *bla*$_{CTX-M}$ genes in KCJK8018 (A) and KCJK8765 (B). The contigs (blue, green, red, and purple nodes) of the isolates were assigned to either the bacterial chromosome (white background) or a plasmid (grey background) based on their homology to reference genomes/plasmids (orange nodes) using PLACNETw and manual trimming. Purple nodes represent contigs containing a *bla*$_{CTX-M}$ gene.

Likewise, the *bla*$_{CTX-M-32}$ gene loci were located at consistent insertion sites in the chromosomes of KCJK7979, isolated in feral swine in 2017, and KCJK8765, isolated in 2018, featuring the same IS elements, IS*5* (Fig. 7B). These findings suggest an association between certain IS elements and the *bla*$_{CTX-M}$ gene loci, regardless of the animal source, suggesting ESBL genes may disseminate among diverse wildlife through horizontal gene transfer, such as conjugation and MGEs.

3.7. Potential ARGs transmission at the wildlife-livestock interface

In our attempt to provide direct evidence for transmission of ESBL-producing *E. coli*, the isolation of these bacteria from cattle was unsuccessful. Consequently, we investigated the gut resistome in both cattle and feral swine to identify ESBL genes in the cattle microbiome. Shotgun metagenomics analysis aimed to access the prevalence and transfer of ARGs, including *bla*$_{CTX-M}$ genes. To enhance sequencing coverage, we pooled cattle fecal samples into CRB-positive and CRB-negative samples. Feral fecal swine samples were pooled based on the presence of CRB and ESBL-producing *E. coli*. Six pooled samples were analyzed by shotgun metagenomic sequencing (Table 1), yielding an average of 13,621,043 reads after filtering low-quality and host-specific sequences. A total of 419,409 ARG counts were identified (Table 1), comprising 76 distinct ARGs in 11 resistance classes. Intriguingly, feral swine exhibited over six times more diverse ARGs than cattle, and the relative abundance of ARGs in feral swine (0.051 % of total filtered reads) was approximately three times higher than that in cattle sample (0.019 %) (Table 1 and Fig. 8B), supporting that feral swine as a reservoir of ARGs.

To elucidate the spectrum of ARGs in cattle and feral swine, we investigated the presence and the relative abundance of specific ARGs in each pooled sample using shotgun metagenomics sequencing. Consistent with the absence of ESBL-producing *E. coli* in cattle fecal samples, *bla*$_{CTX-M}$ genes were not detected in cattle while it was detected in CRB and ESBL positive samples in feral swine, suggesting that a lack of transmission of *bla*$_{CTX-M}$ genes between feral swine and cattle. Moreover, a total of 68 ARGs were exclusively detected in feral swine samples while only one ARG, *mefA* (MFS efflux pumps), was distinctively present in CRB positive cattle, suggesting the majority of ARGs are host specific.

However, seven ARGs, including tetracycline resistance genes (*tet32*, *tet40*, *tet44*, *tetO*, *tetQ*, and *tetW),* and genes resistant to macrolide, lincosamide, and streptogramin (MLS, *lnuC)* were detected in both cattle and feral swine (Fig. 8C). Therefore, we identified bacteria that carried these ARGs found commonly in two animal hosts by the Spearman rank correlation analysis. This analysis revealed 726 significant positive associations (P value < 0.05) between the relative abundance of 206 bacterial genera and the proportion of 7 ARGs shared by cattle and feral swine samples (Fig. 8D). Eighty-six bacteria belonging to Firmicutes, thirty-one bacteria belonging to Bacteroidetes, and twenty-seven bacteria belonging to Proteobacteria were the major genera positively associated with the ARGs belonging to tetracycline and MLS resistance classes. Especially, ARGs related to tetracycline resistance had positive associations with bacterial genera of Bacteroidetes, Firmicutes, and Proteobacteria. Taken together, although ARGs between hosts showed distinct profiling, a group of ARGs showed shared presence, suggesting the existence of common bacteria carrying the same ARGs in the gastrointestinal tract.

4. Discussion

Given the escalating spread of antibiotic resistance in recent years, comprehending the environment's pivotal role as a natural reservoir and carrier of antibiotic resistance has become imperative to preventing the global dissemination of AMR. Despite extensive investigations into antibiotic resistance in food-producing animals, our understanding of the transmission of ARGs and MDR ESBL-producing *E. coli* at the wildlife-livestock interface remains in a nascent stage. In this two-year study, we conducted a thorough investigation, revealing a high prevalence of ARGs and ESBL-producing *E. coli* in feral swine, while such isolations and responsible genes were notably absent in grazing cattle. These findings suggest that feral swine may serve as a potential reservoir for ESBL-producing *E. coli.*

Wildlife, once contaminated with AMR, can serve as vectors for the transmission of AMR across various habitats. Understanding AMR transmission at the wildlife-livestock and wildlife-human interface is crucial [7,8]. Due to the close proximity of feral swine to cattle, sharing resources like forage, livestock supplements, and water, there is a potential acceleration of ESBL-producing *E. coli* transmission [56]. In this

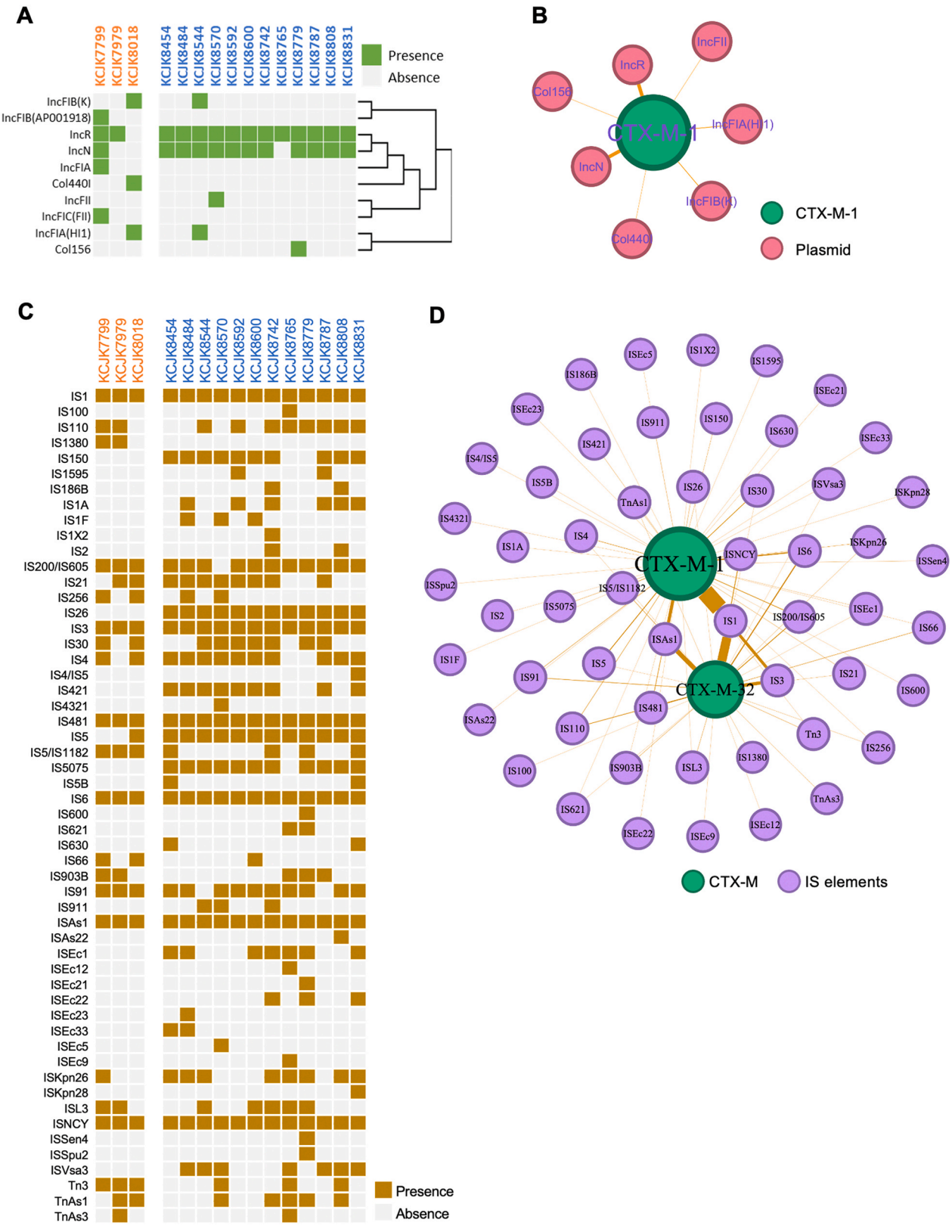


Fig. 6. Mobile genetic elements (MGEs) in ESBL-producing *E. coli*. The heatmaps showed the collection of conjugative plasmids (A) and insertion sequences (IS) element (C) identified in fifteen representative isolates through the BLASTn tool of PladmidFinder and MGEfinder with default settings, respectively. The presence of MGEs with $\geq$ 90 % identity was indicated in green (conjugative plasmids) and dark orange (IS). The MGEs (Y axis) were hierarchically clustered based on the similarity of their sequences. The maximum-likelihood phylogenetic tree was constructed using IQ-TREE and visualized using FigTree. The network displayed the correlation between the $bla_{\text{CTX-M}}$ genes with identified plasmid (B) and IS element (D), respectively. Network nodes represented the $bla_{\text{CTX-M}}$ genes in green, conjugative plasmid in pink (B), and IS elements in purple (D). Network edges indicated correlations between nodes, and the thickness of the edges denoted the strength of the correlation. The correlation network was visualized by Gephi.

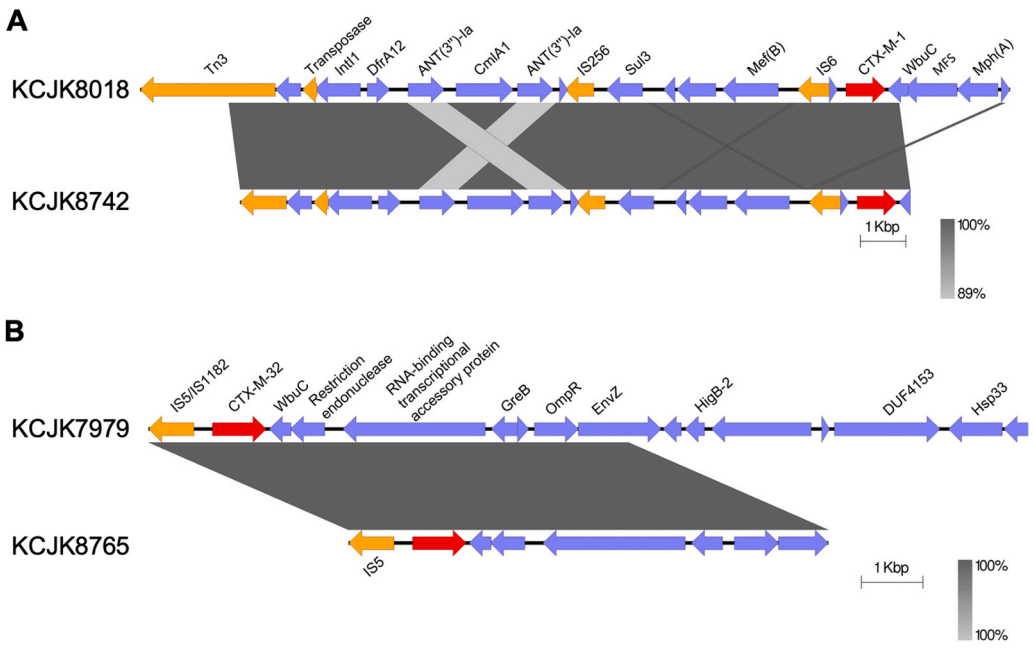


Fig. 7. Genetic environments of *bla*$_{CTX-M}$ genes. Comparison of genetic environments surrounding *bla*$_{CTX-M-1}$ (A) and *bla*$_{CTX-M-32}$ (B) genes in representative isolates. The genes surrounding each *bla*$_{CTX-M}$ (red arrows) gene contain insertion sequences (yellow arrows) and other genes as indicated (green arrows).

Table 1
The summary of shotgun metagenomic sequencing.

Sample Pooling Group	Total reads	Total ARG reads	ARG Reads > 80 % _without SNP confirmation	Relative ARG abundance %
Cattle_CRB_Positive (n = 14)	15803864	71064	3056	0.019342627
Cattle_CRB_Negative (n = 86)	11229563	50868	2036	0.017992041
Swine_CRB_Positive (n = 84)	20565813	112058	13287	0.06460722
Swine_CRB_Negative (n = 140)	6928003	37761	2368	0.034180124
Swine_ESBL_Positive (n = 58)	19299519	105083	13382	0.069338516
Swine_ESBL_Negative (n = 166)	7899494	42576	2749	0.034799697

study, aiming to provide direct supporting evidence of ESBL-producing *E. coli* transmission at the livestock-wildlife interface, we attempted to isolate identical strains at the genomic level from coexisting livestock and wildlife within a shared habitat. Contrary to expectations, our two-year comprehensive isolation effort unexpectedly revealed successful isolation of these bacteria only from feral swine, not from cattle. This contradicts our previous report indicating the natural occurrence of ESBL-producing *E. coli* in beef cattle raised without antibiotic use [18,19]. The absence of ESBL-producing *E. coli* in Buck Island may result from farm-specific management practices or other unknown factors, such as bacterial enrichment method, seasonal variation, or the farm environment. Therefore, further investigation is necessary to identify potential mitigation strategies against ESBL-producing bacteria in farm animals on this farm.

Thorough genetic profiling of the ESBL-producing *E. coli* using WGS and *in silico* analysis provided insights into their colonization within feral swine, including virulence factors, AMR, and MGEs that facilitate the transfer of ARGs. For MDR assessment, all evaluated isolates displayed resistance to various antibiotics, indicating multi-drug resistance, and a high MIC for cefotaxime. Whole genome analysis identified 72 ARGs across 14 drug classes, associated with multi-drug resistance, drug and metal resistance, and peptide resistance. A subset of genes involved in peptide resistance was identified and shared among these ESBL-producing *E. coli*, specially, *bacA*, *eptA*, and *pmrF*, which may suggest the potential resistance to bacitracin or colistin/polymyxins [57–59]. ESBL genes (*bla*$_{CTX-M-1}$ or *bla*$_{CTX-M-32}$) were prevalent in all isolates, along with other β-lactamases genes (*ampC* and *bla*$_{TEM-1}$). Interestingly, exclusive ARGs were found in 2017 isolates, with *aadA*, *linG*, and SAT-2 genes indicating aminoglycoside, lincosamide, and nucleoside resistance, suggesting the presence of specific bacteria are dynamic. In addition, we distinctively detected *qnrB19, mef(B), sul3, tet (B), tetR,* and *dfrA12*, in coyote isolate (KCJK8018), suggesting strains colonized in coyote may differ from the feral swine lineage as shown in Fig. 2. Across isolates, virulence factor profiles revealed 35 to 61 virulence genes, with consistent presence of *ompA*, *ent*, and *fep* genes involved in adhesion, invasion, iron acquisition, and bacterial growth. The *flg* gene family crucial for flagellar formation was also universally present. ESBL-producing *E. coli* from feral swine encode Type II secretion system-related genes *(gsp* families), contrasting with the absence of these genes in the coyote strain (KCJK8018). This suggests distinct virulence factor profiles among ESBL-producing *E. coli* in feral swine. Interestingly *E. coli* KCJK8484 in 2018 raised concerns due to harboring the *gtrB* gene, indicating potential pathogenicity through endotoxin production and survival mechanisms against antibiotics. This underscores the need for further exploration into the pathogenic potential of specific ESBL-producing *E. coli* strains. Our findings highlight the potential of these isolates to establish colonization in feral swine.

In addition to clonal transmission, ESBL genes can be transferred to other bacteria through HGT by conjugative plasmids and IS elements [60]. The shared presence of IncR and IncN plasmids among ESBL-producing *E. coli* suggests their involvement in ESBL gene spreading. IncN, a broad-host range plasmid, identified frequently in ESBL-producing *E. coli* from hospitalized patients, has been associated with *bla*$_{CTX-M-1}$ gene transmission [46,61,62]. Indeed, a previous study has reported within-farm transmission of IncN plasmid between pigs and farm workers [63], suggesting a potential link in spreading ESBL genes between wildlife and clinical settings. The IncR plasmid has been increasingly reported in ESBL-producing *E. coli* from animals and the environment as well [64–66]. IS elements, particularly IS*6* for *bla*$_{CTX-M-1}$ and IS*5* family for *bla*$_{CTX-M-32}$, may play a significant role in ESBL gene

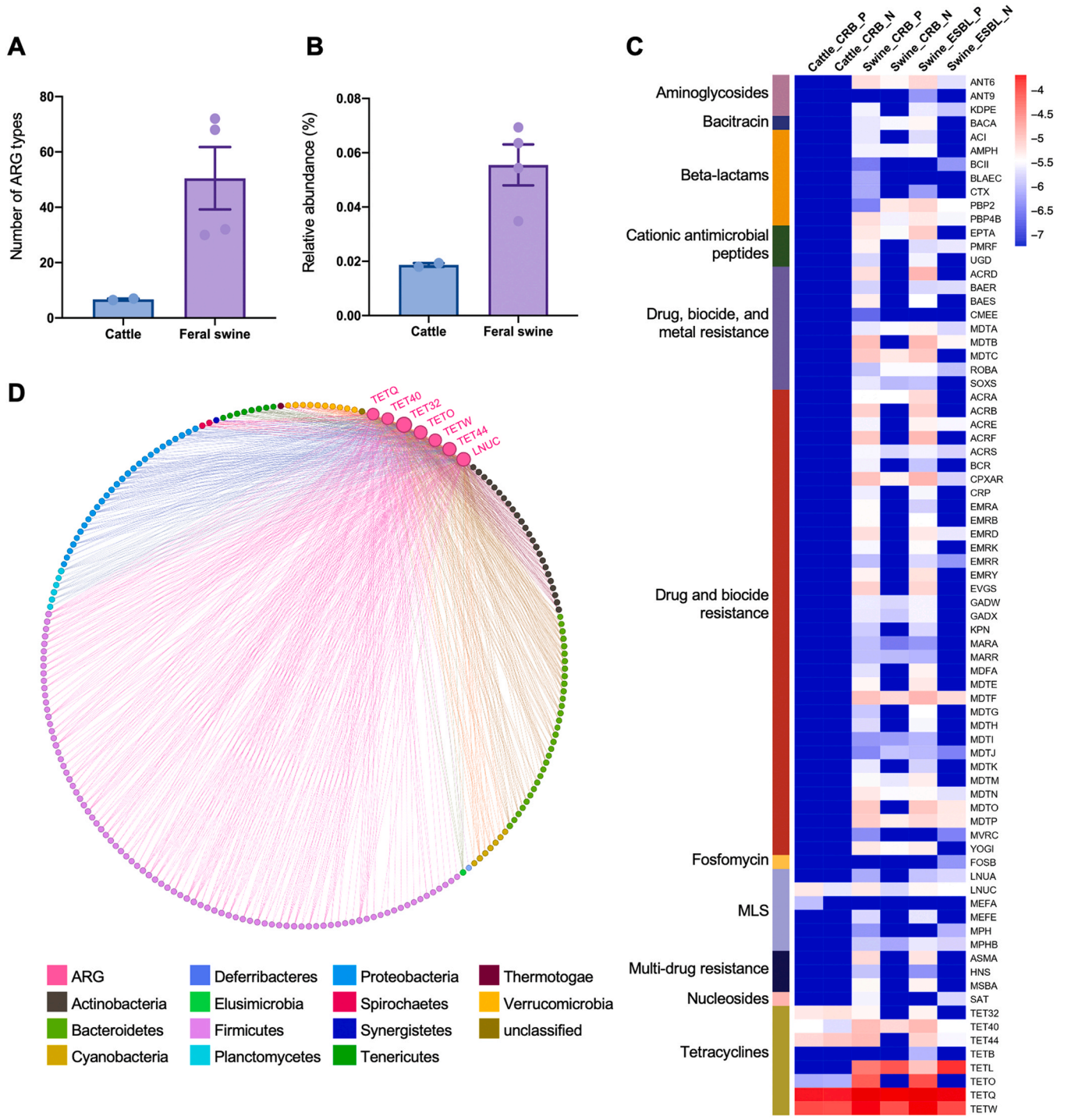


Fig. 8. The transmission of gut microbiota and ARGs at the wildlife-livestock interface. The number of ARG types (A) and relative abundance of ARGs (B) among filtered metagenomic sequences of cattle and feral swine samples. The differences between groups were compared using the student's *t*-test. (C) The heatmap showed the relative abundance of ARGs in different pools. (D) The positive association between shared bacteria and shared ARGs at the wildlife-livestock interface. Network nodes represented different ARGs or bacterial genera, and edges indicated correlations between nodes. The size of the nodes represented the degree of connection, and the thickness of the edges indicated the strength of the correlation.

dissemination (Fig. 7). IS*6* family elements, especially IS*26*, are known for their role in transmitting various resistant determinants, including $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{NDM-1}}$, and $bla_{\text{NDM-9}}$, in Gram-negative bacteria [67–70]. $bla_{\text{CTX-M-32}}$ transmission is associated with IS*5* in clinical and environmental isolates [71,72]. As shown in Fig. 7, IS elements are located upstream of $bla_{\text{CTX-M}}$ genes and it has been shown that IS elements located upstream of $bla_{\text{CTX-M}}$ genes can increase the mobilization frequency under some stress situations, such as elevated temperature or antibiotic selection pressure [12,73]. Therefore, the exceptional increase and spread of $bla_{\text{CTX-M}}$ genes in animals may be influenced by global warming, high temperatures in animal intestinal tract, and increased antibiotic use [74,75]. These findings collectively suggest the crucial role of IS elements in facilitating HGT, thereby promoting the acquisition and dissemination of ESBL genes.

5. Conclusions

In conclusion, our investigation underscores the significant role of wildlife as a central reservoir for ESBL-producing *E. coli*. Our findings revealed a notable prevalence of these bacteria in feral swine and coyotes, emphasizing their persistence and adaptation within wildlife hosts. A clonal dissemination of ESBL-producing *E. coli* isolates was observed between wildlife, but it was not directly observed due to the lack of isolation of these bacteria in cattle. Notably, the absence of the $bla_{\text{CTX-M}}$ gene in cattle samples, as determined by shotgun metagenomic sequencing, aligns with the lack of ESBL-producing bacteria in cattle feces, suggesting potential host specificity within the gastrointestinal tract of feral swine. Furthermore, our study identified transmission events of certain ARGs such as tetracycline and MLS resistance classes at the wildlife-livestock interface. Furthermore, our research highlights the role of horizontal gene transfer, including conjugative plasmids and IS elements, in supporting and mediating the occurrence of ESBL gene transmission. Focusing particularly on feral swine, our study suggests their critical role as a reservoir for ESBL-producing bacteria.

Environmental implication

This study underscores the environmental significance of wildlife as a reservoir for extended-spectrum β-lactamase-producing Escherichia coli, providing crucial insights into antimicrobial resistance (AMR) dissemination. Unrestricted wildlife movements across diverse habitats pose a substantial risk for transmitting multi-drug resistant pathogens to livestock, humans, and the environment. Our findings highlight the potential hazard of feral swine serving as reservoirs and carriers of multi-drug resistant pathogens, impacting animal and human health. Understanding transmission mechanisms at the wildlife-livestock interface is essential for addressing environmental challenges posed by AMR, emphasizing the need for comprehensive strategies to mitigate the environmental impact of wildlife-associated AMR transmission.

CRediT authorship contribution statement

KwangCheol Jeong: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Christina Boucher:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **Raoul Boughton:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Peixin Fan:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Miju Kim:** Writing – review & editing, Writing – original draft, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Shinyoung Lee:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ting Liu:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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