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Antimicrobial use, antimicrobial resistance, and the microbiome in animals, volume II

Edited by
Moussa Sory Diarra, Xin Zhao and Patrick Rik Butaye

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Antimicrobial use, antimicrobial resistance, and the microbiome in animals, volume II

Topic editors

Moussa Sory Diarra — Agriculture and Agri-Food Canada (AAFC), Canada

Xin Zhao — McGill University, Canada

Patrick Rik Butaye — Ghent University, Belgium

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Michael Kogut,
Agricultural Research Service (USDA),
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*CORRESPONDENCE
Moussa S. Darria
✉ moussa.diarra@agr.gc.ca

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Editorial: Antimicrobial use, antimicrobial resistance, and the microbiome in animals, volume II

Moussa S. Darria[1]*, Xin Zhao[2] and Patrick Butaye[3]

[1]Guelph Research and Development Center, Agriculture and Agri-Food Canada, Ottawa, ON, Canada, [2]Department of Animal Science, McGill University, Montreal, QC, Canada, [3]Department of Pathobiology, Pharmacology and Zoological Medicine Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium

KEYWORDS

antimycobacterial, resistance, microbiota, animals, alternatives to antibiotic

Editorial on the Research Topic
Antimicrobial use, antimicrobial resistance, and the microbiome in animals, volume II

Introduction

As demonstrated by the COVID-19 pandemic, endemic diseases or epidemic outbreaks represent a significant financial risk to society. In veterinary medicine, risks also include the loss of animals and the reduction of productivity and market access. Antimicrobials contribute to the treatment and prevention of infectious diseases in both animals and humans while improving farm animals' productivity and welfare. However, antimicrobial resistance (AMR) is becoming an important and growing economic and social problem associated with annual costs estimated at US$1 trillion to US$3.4 trillion worldwide (1, 2), and is regarded as the silent pandemic. Even though AMR, a global threat to humans, animals, and the environment, is complex, the wide use of antibiotics has been linked to the emergence and spread of AMR in all ecosystems (One Health). This Research Topic presents 12 studies on antimicrobial use (AMU) and AMR, as well as on antimicrobial impacts on the microbiota and epidemiology, dissemination-transmission, and surveillance of AMR.

AMU and AMR

It is well known that antimicrobial resistant bacteria are selected mainly by antibiotics/antimicrobials use (AMU). In conventional production, antibiotics (although "antibiotics" and "antimicrobials" are sometimes used interchangeably, antibiotics are actually a subset of antimicrobials) have been used to prevent infectious diseases. However, this practice has been discontinued in more and more countries due to restrictions on antibiotic use. Antibiotics as feed additives to promote growth and eventually prevent diseases in healthy production animals were banned by the European Union in 2006. In 2018, the European Parliament approved new restrictions on the use of antimicrobials in healthy livestock. The Government of Canada (and also many other countries) has developed a Federal Framework and Federal Action Plan on AMR to initiate and take action

in the areas of surveillance, stewardship, and innovation https://www.canada.ca/en/health-canada/services/publications/drugs-health-products/tackling-antimicrobial-resistance-use-pan-canadian-framework-action.html. This responsible use in animal production is intended to preserve the effectiveness of antibiotics and minimize the development and spread of AMR.

In this Research Topic, Lagarde et al. evaluated the impact of regulation by comparing the AMR situation in dairy cattle in Québec before and after the introduction of the new regulation. They found that AMR was significantly decreased in generic *Escherichia coli* from dairy cattle, and this started 2 years after the initiation of the AMU regulation. Despite this positive effect on AMR, decreasing AMU should also be evaluated for other aspects, including health and productivity. Another study on the reduction of antimicrobial use by Khine et al. showed a decline in pathogenic *mcr*-positive *E. coli* following the withdrawal of colistin in pigs. The study by Furuya et al. reported significantly higher resistance rates in *E. coli* and *Enterococcus* spp. isolates from sick pet animals than in those from healthy ones and concluded that the use of antimicrobials could select resistant *E. coli* and *Enterococcus* spp. The optimization of therapeutic doses by better understanding of pharmacokinetics and pharmacodynamics (PKs and PDs) could reduce the burden of the therapeutic use of antimicrobials on AMR. This concept has been presented for danofloxacin in pigs by Zhou et al..

Microbiota and alternatives

The microbiota play critical roles in the gut and establish general health in the animal by maintaining/improving organ integrity and function and the provision and absorption of nutrients, as well as protecting against pathogens, including promoting immunity. A well-established microbiota plays a particularly important role in young animals. Feed additives, including alternatives to antibiotics, received attentions after the ban or restriction of in-feed antibiotics as growth promoters. Few studies have investigated the effects of antimicrobials on the metabolism, physiology, and immunity of animals. By contrast, several studies have reported their effects on microbiota and microbiomes. However, many other factors, such as genetics (line), physiological status, sex (male or female), health (clinical and subclinical), and housing/husbandry, influence the microbiota. Microbes respond to antimicrobials by developing and acquiring resistance mechanisms and changing gene expression patterns, which alter their metabolism and nutrient uptake and transport (4). The elimination or growth inhibition of bacteria by antibiotics results in changes in bacterial community structure and diversity.

In this Research Topic, the analysis of fecal microbiota in healthy, diarrheal, and treated weaned piglets showed differences between these three animal groups (Kong et al.). A comparison of the fecal microbiomes and antibiotic resistance genes (ARGs) in free-ranging and zoo-captive rhesus macaques by Jia et al., revealed that semi-captive wildlife might harbor a higher diversity of ARGs. Anemoside from *Pulsatillae Radix* has been found to potentially alleviate calf diarrhea, protect the integrity of the intestinal mucosa, and change the structure of intestinal microbiota (Lu et al.). Baicalin, another plant compound, this time from *Scutellaria baicalensis* (Baikal skullcap or Chinese skullcap), has been shown to potentially reverse azithromycin resistance in *Staphylococcus saprophyticus* (Wang et al.).

Transmission, epidemiology, and surveillance

It is important to intensify research to understand the circumstances leading to the emergence of pathogenic bacteria and antibiotic resistance. In animal production industries, better practices with respect to food and environmental safety, as well as public and animal health and welfare, still need to be developed. Microorganisms living in changing environmental conditions adapt and evolve. Bacterial resistance determinants can be spread through horizontal gene transfer (HGT), which could be related to temperature (3). Therefore, climate change and AMR are interlinked and both should be addressed to protect humans, animals, and the environment.

"One Health" approaches, using "omics" and well-structured government-controlled surveillance, are needed. Additionally, it is imperative to understand the impacts of environmental factors on the evolution of bacteria and the development of AMR. In this context, Günther et al. investigated environmental factors associated with the prevalence of extended-spectrum beta-lactamase (ESBL) and AmpC-producing *E. coli* in wild boar. The findings from this study improved our understanding of the distribution of AMR in humans and animals using "One Health" approaches. The plasmid pE165, a mobile genetic element (MGE) involved in the horizontal transfer of *erm(T)* in *Enterococcus faecalis*, and its intraspecies and interspecies transmission ability, was determined by Li X-Y et al.. Different types of multidrug-resistant *Klebsiella pneumoniae* harboring virulence and resistance genes on MGEs, including IncFIB-, IncFII-, IncR-, and IncX3-type plasmids, were shown to be present in diseased dogs and cats in China (Zhang et al.). Furthermore, Li A et al. showed the simultaneous occurrence of *tet(X4)*, $bla_{\mathrm{NDM-1}}$, and $bla_{\mathrm{OXA-58}}$ in a porcine *Acinetobacter towneri* isolate. The *tet(X4)* and the florfenicol *floR* resistance genes were flanked by IS91-like elements, emphasizing the importance of AMR surveillance in animals.

Perspectives

Antimicrobial resistance is a "One Health" issue because AMR genes can be spread across humans, animals, and the environment. Surveillance, whole-genome sequencing, microbiota/microbiome, and antibiotic stewardship research are needed to determine important AMR drivers. The identification of hot spots and the ability to predict phenotype and transmission pathways, along with the adoption of best AMU practices, will help mitigate AMR. New knowledge contributing to the improvement of animal health and production, as well as studies providing science-based evidence about AMR transmission through the food chain and the environment, are needed. Owing to the high load of ARGs in animal manure and their potential spread to the environment when manures are used as soil fertilizers, the effects of different treatments of raw manures, such as composting (thermophilic composting and vermicomposting) and anaerobic digestion, should be investigated.

Author contributions

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

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Characterization of the Gut Microbiome and Resistomes of Wild and Zoo-Captive Macaques

Ting Jia[1†], Wei-Shan Chang[2†], Vanessa R. Marcelino[2,3,4], Sufen Zhao[1], Xuefeng Liu[1], Yuyan You[1], Edward C. Holmes[2], Mang Shi[5*] and Chenglin Zhang[1*]*

[1] Beijing Key Laboratory of Captive Wildlife Technologies, Beijing Zoo, Beijing, China, [2] Sydney Institute for Infectious Diseases, School of Life and Environmental Sciences and School of Medical Sciences, The University of Sydney, Sydney, NSW, Australia, [3] Department of Molecular and Translational Sciences, Monash University, Clayton, VIC, Australia, [4] Centre for Innate Immunity and Infectious Diseases, Hudson Institute of Medical Research, Clayton, VIC, Australia, [5] School of Medicine, Sun Yat-sen University, Guangzhou, China

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Edited by:
Patrick Rik Butaye,
Ross University School of Veterinary Medicine, Saint Kitts and Nevis

Reviewed by:
Ava Michelle Trent,
University of Minnesota, United States
Meera Surendran Nair,
The Pennsylvania State University (PSU), United States

***Correspondence:**
Wei-Shan Chang
wei-shan.chang@sydney.edu.au
Mang Shi
shim23@mail.sysu.edu.cn
Chenglin Zhang
zhch6465@263.net

† These authors have contributed equally to this work and share first authorship

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Rhesus macaques (*Macaca mulatta*) are the most widely distributed species of Old World monkey and are frequently used as animal models to study human health and disease. Their gastrointestinal microbial community likely plays a major role in their physiology, ecology and evolution. Herein, we compared the fecal microbiome and antibiotic resistance genes in 15 free-ranging and 81 zoo-captive rhesus macaques sampled from two zoos in China, using both 16S amplicon sequencing and whole genome shotgun DNA sequencing approaches. Our data revealed similar levels of microbial diversity/richness among the three groups, although the composition of each group differed significantly and were particularly marked between the two zoo-captive and one wild groups. Zoo-captive animals also demonstrated a greater abundance and diversity of antibiotic genes. Through whole genome shotgun sequencing we also identified a mammalian (simian) associated adenovirus. Overall, this study provides a comprehensive analysis of resistomes and microbiomes in zoo-captive and free-ranging monkeys, revealing that semi-captive wildlife might harbor a higher diversity of antimicrobial resistant genes.

Keywords: monkey, microbiome, antimicrobial resistance gene, adenoviruses, captive primates, metagenomic

INTRODUCTION

Rhesus macaques (*Macaca mulatta*) are a species of Old World Monkey with a wide geographic distribution. Because of their close phylogenetic relationship with humans, they are extensively used as biomedical models for understanding human disease. A handful of publications have demonstrated that the non-human primate (NHP) gut microbiome is shaped by diet, evolutionary features, age, sex, geographical habits (1–4), and notably captivity, indicating that human-mediated life styles and living locations could alter the gut-associated microbial communities of primates (5). Many previous studies have investigated the impact of captivity, diet and anthropogenic activity on microbiome composition. For example, Clayton et al. examined the gut microbiome in different species of NHPs such as douc and howler monkeys, showing that diversity of native gut microbial taxa was reduced among the captive groups (5). In black howler monkeys, the

environmental and dietary changes associated with captivity had a major impact on intestinal microbial methanogenesis (6). In contrast, similar bacterial compositions were observed in wild vs. captive chimpanzees (7).

Driven by advances in next-generation sequencing technologies, microbiome and resistome focused studies of are increasing importance, expanding our knowledge of microbial communities and their interactions with humans, animals and the environment. Amplicon sequencing is sufficient for family-level and genus-level bacterial classification, although the variation captured by 16S sequencing is insufficient for strain-specific identification (8). Additionally, metagenomic approaches provide a means to characterize non-bacterial microbes, including viruses and eukaryotic pathogens (9). Recent studies have also revealed that host-associated intestinal microbiota may impact viral susceptibility and the ensuing host immune responses (10, 11). The widespread use of antibiotic agents in veterinary and human medicine has revolutionized the therapeutic options of bacterial infection, although at the same time it has increased the selection pressure for the rapid emergence and evolution of antimicrobial resistance (12).

Herein, we used both 16S rRNA and whole genome shotgun DNA sequencing approaches to identify the differences of fecal microbial composition and resistome between zoo-captive and wild rhesus monkeys in China. Our results provide important insights on the impact of captivity on microbial diversity and antimicrobial resistance properties.

MATERIALS AND METHODS

Animal Ethics Statements

This study was approved by the Beijing Municipal Committee of Animal Management before sample collection. All experiments were performed in accordance with the approved guidelines and regulations under approval number #SYSU-IACUC-MED-2021-B0123.

Study Sites and Sampling Information

This study was conducted from July to August in 2014 at Shennongjia Forestry District natural reserves (SR), a zoo located in Beijing (BR) and a wildlife zoo located in inner Monglia (ER). All fecal specimens of rhesus monkeys (*Macaca mulatta*) were collected following defection at three sampling locations: one wild (SR), one semi-captive (ER), and a zoo-captive population (BR). Details of the sample collection sites, sample groups and food usage are presented in **Table 1**. DNA extraction of the fecal samples was performed using the TruSeq™ DNA Sample Prep Kit (Illumina) following the manufacturer's instructions.

TABLE 1 | Sample location and size of zoo-captive and wild rhesus monkeys.

Group name	Type	Location	Sample size	Latitude	Food source
BR	Zoo-captive	Beijing	24	39.94°N	Potatoes, fruits, vegetables, steamed corn bread
ER	Semi-zoo-captive	Inner Mongolia	57	39.8°N	Fruits, vegetables, steamed corn bread
SR	Free-ranging	Shennongjia Forestry District natural reserves	15	31.46°N	Wild plants

Comparisons of Bacterial Composition and Diversity Using 16S RRNA Sequencing

Fecal samples from each monkey were subject to 16S rRNA amplicon sequencing. The V3–V4 hypervariable regions of the bacterial 16S ribosomal RNA (rRNA) gene were amplified using barcoded primers, 341F- 5′-CCTACACGACGCTCTTCCGATCTN (barcode) CCTACGGGNGGCWGCAG-3′ and 805R-5′-GACTGGAGTTCCTTGGCACCCGAGAATTCCA (barcode) GACTACHVGGGTATCTAATCC-3′, according to the Illumina 16S Metagenomic Sequencing Library Guide. The amplicons generated were sequenced on an Illumina HiSeq platform in a 2 × 250 paired-end mode. All sequencing and library preparation procedures were performed by Sangon Biotech (Beijing, China).

The raw amplicons generated were screened, trimmed, filtered, denoised, and chimera-depleted using QIIME2 version 2018.2 (http://qiime.sourceforge.net). Short, ambiguous sequences and chimeras deriving from the PCR process were removed using DADA2 plugins. Sequences were clustered into Operational Taxonomic Units (OTUs) and then assigned to bacterial sequences with at least 99% similarity to representative sequences from the SILVA 132 database (http://www.arb-silva.de/). For statistical analysis, all the sequences were rarefied to 1,112 reads for the downstream analysis. For each sample, the relative abundance of each bacterium identified was expressed as the percentage of total reads. QIIME2 was applied to profile the taxonomy of microbial composition in each group and to calculate alpha diversity matrices (including ACE, Shannon diversity index and Simpson index) (13, 14). To evaluate the variation between different groups, beta-diversity distance matrices (including Bray-Curtis distances, weighted and unweighted UniFrac values) were performed using rarefied data sets, and subsequently principal coordinate analysis (PCoA) was conducted to visualize the dissimilarities in the fecal bacterial communities among different groups of rhesus monkeys.

Fecal Microbiome Characterization

All reads from the high-throughput DNA sequencing data were mapped to reference genomes of *Macaca mulatta* (NCBI txid:9544) using Bowtie2 (15) to remove genetic material of host origin after quality-trimming by Trimmomatic (16). To profile the bacterial results from microbial composition, CCMetagen (17) was used for taxonomic annotation against nt database. To screen for viruses, host-filtered reads from the metagenomic

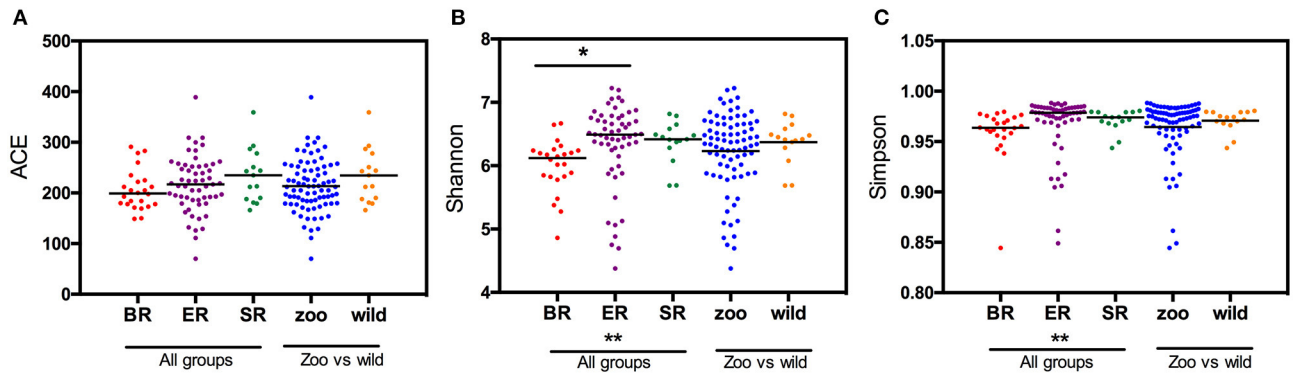

FIGURE 1 | Estimated OTUs richness and diversity index in different groups of monkeys. The index of OTUs richness in different groups was estimated using ACE **(A)** metrics. To estimate OTU diversity, Simpson's index **(B)** and Shannon's index **(C)** were performed. No significant statistical differences in ACE ($p = 0.23$) between the three groups was obtained using Kruskal-Wallis tests. Statistically significant differences were found between ER and other groups ($p < 0.05$) using the Simpson and Shannon metrics.

sequencing data sets were assembled using MEGAHIT (18) then compared against the entire nr database in GenBank using Diamond BlastX e value $< 10^5$) (19). Any viral reads and contigs identified by Blast were then extracted and reassembled using the assembler implemented in Geneious v.11. This process identified abundant adenovirus sequences that were then reassembled into an entire adenovirus genome. This genome was then translated into amino acid sequences for gene annotation and functional prediction using Conserved domain databases (CDD).

The assembled sequences were then aligned using the MAFFT version 7 with implemented E-INS-I algorithm (20). Conserved domains within the E1A and 100k protein of adenoviruses were used for subsequent phylogenetic analyses. After removing all ambiguously aligned regions using TrimAl (21), the final lengths of E1A and 100K protein alignments were 832 and 1,379 amino acid residues, respectively. Phylogenetic trees of these data were inferred using the maximum likelihood method (ML) implemented in PhyML version 3.0, employing a Subtree Pruning and Regrafting topology searching algorithm. Statistical support for specific groupings in the tree was assessed using the approximate likelihood-ratio test (aLRT) with a Shimodaira-Hasegawa like procedure with 1,000 replicate bootstrap. The phylogenetic trees were visualized using the FigTree program (http://tree.bio.ed.ac.uk/software/figtree).

Detection of Antimicrobial Resistance Genes

To determine the presence of putative antimicrobial resistance (AMR) genes in the data, we analyzed the shotgun sequencing data using the KMA program (22) combined with the ResFinder reference database (23). To reduce false-positive results, genes were only considered in downstream analyses when *p*-values for the conclave score were lower than 0.05, only two genes were excluded due to their $p > 0.05$ (22). We also excluded the blaTEM116 gene which has been previously identified as a common laboratory contaminant (24). AMR diversity and abundance was visualized in R with the package *ggplot2*.

TABLE 2 | Kruskal-Wallis tests of Alpha diversity in three groups of monkeys.

K-W test (all groups)	ACE	Shannon index	Simpson index
H value	2.97	14.87	14.31
P value	0.23	0.00059	0.00059

RESULTS

Overall Characterization of 16S and Shotgun DNA Sequencing Results

The 16S rRNA amplicon sequencing generated a total of 2,572,794 reads and 2,680 OTUs. The total number of raw reads across all groups from the high-throughput shotgun DNA sequencing data was 1,425,675,194. Rarefaction curves showed a similar trend in all three populations. Observed numbers of OTUs (Observed_OTUs), an indicator of alpha diversity, is a qualitative measure of community richness. By this metric, the population of SR (wild monkeys) harbored the highest numbers of OTUs among all three groups under the same sequencing depth (orange: SR; blue: BR; cyan: ER) (**Supplementary Figure 1A**). The Shannon-Wiener curves showed that the samples from all groups had plateaued (**Supplementary Figure 1B**). The rarefaction curves indicate that sequencing depth was sufficient to capture the bacterial diversity in all samples (**Supplementary Figure 1**).

Association Between Bacterial Richness and Diversity and Animal Captivity

Based on the OTU data, we examined the bacterial richness and diversity of captive (BR), semi-captive (ER), and wild (SR) groups using ACE, the Shannon index and the Simpson index (**Figure 1**). The number of OTUs identified in the samples depicted species richness, as estimated by ACE. A non-parametric Kruskal-Wallis test was performed in all groups. The richness indices

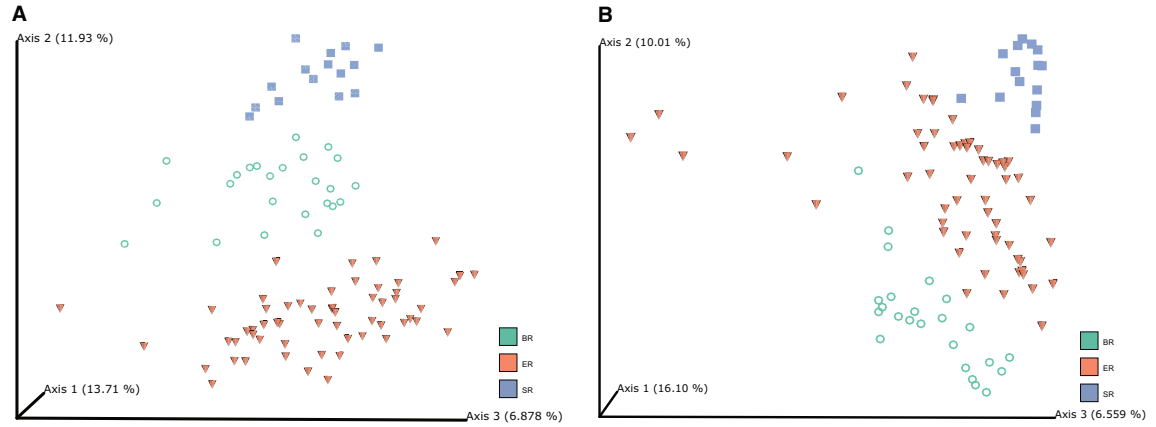

FIGURE 2 | Microbiome clustering of different groups of monkeys. Principal coordinate analysis (PCoA) 3D-plots display bacterial community structure based on unweighted UniFrac distance **(A)** and Bray-Curtis distance **(B)**. The numbers of Axis 1, Axis 2 and Axis 3 showed the percent variation explained by the PCoA plots, indicating three distinctive clusters of microbiome groups.

(ACE) revealed no significant difference ($p > 0.05$) between the wild and zoo-captive groups of macaques (**Table 2**). However, bacterial diversity was significantly different ($p < 0.05$) among all groups, as evaluated with the Shannon and Simpson indexes. Furthermore, Shannon indexes revealed significant differences between the captive (BR) and semi-captive (ER) groups, whereas no differences between wild (SR) and semi-captive groups (ER) were found by any of the methods.

Monkeys From Different Groups Have Distinct Microbiomes

Principal coordinate analysis (PCoA) was performed based on unweighted UniFrac (**Figure 2A**) and Bray-Curtis distances (**Figure 2B**) to visualize the dissimilarities in the bacterial communities among different groups of monkeys. The unweighted UniFrac analysis provided a much stronger clustering by population than either the weighted UniFrac or Bray Curtis distances, indicating that the clustering is likely driven by presence or absence of key taxa in different populations, rather than by shifts in the ratios of dominant members of the microbiota. In addition, PCoA plots based on Bray–Curtis distance matrices revealed that the samples from different locations formed distinct clusters, indicating that bacterial community composition conforms with the groups they were in, and hence that there were clear differences among wild, captive, and semi-captive monkeys. Analyses of distances based on relative abundance showed semi-captive groups overlapped more with captive group than with the wild group. We additionally performed Permutational Multivariate Analysis of Variance Using Distance Matrices (PERMANOVA) based on unweighted-uniFrac dissimilarity matrices (**Supplementary Figure 2**). Accordingly, the PERMANOVA results, indicated that ($p = 0.001$, number of permutations is 999) higher pseudo-F value in comparison of SR and ER groups with others (**Supplementary Table 1**).

Comparisons of Microbial Composition Results Between 16S and WGS Approaches

Based on 16S rRNA sequencing, the clustered operational taxonomic units identified in fecal samples were assigned to 32 bacterial phyla. Both 16S and WGS approaches identified Firmicutes, Bacteroides, Proteobacteria, and Actinobacteria as the most abundant phyla in all samples, although the proportion of Bacteroides and Proteobacteria differed substantially (**Figures 3A,C**). At the class level, the two approaches revealed different bacterial compositions (**Figures 3B,D**). The main differences lie in the Epsilonproteobacteria that only appeared at high abundance in the wild (SR) group from metagenomic sequencing, but not in the corresponding group from 16S sequencing. Furthermore, the proportion of the class Bacilli also varied greatly between the two approaches. In general, the 16S sequencing resulted in relatively consistent results across three groups, whereas WGS sequencing revealed relatively high levels of variation. Further comparisons were performed at the family level using 16S sequencing results for microbial composition between the three groups (i.e., wild, semi-captive and zoo-captive) (**Figure 4**).

Detection of a Novel Simian Adenovirus in Zoo-Captive Monkeys

To assess the adenoviral reads and contigs identified from group ER, a near complete genome was derived from reassembled reads sequences that were mapped to a reference adenovirus genome (a double-strand DNA virus). The total length was 34,291 nucleotides with a GC content of 56.9%. To further characterize the adenovirus, phylogenetic trees were estimated based on a sequence alignment of the conserved region of the E1A and the 100 K protein, and utilizing reference adenovirus sequences downloaded from NCBI/GenBank. The novel virus shared 70.5% (E1A) and

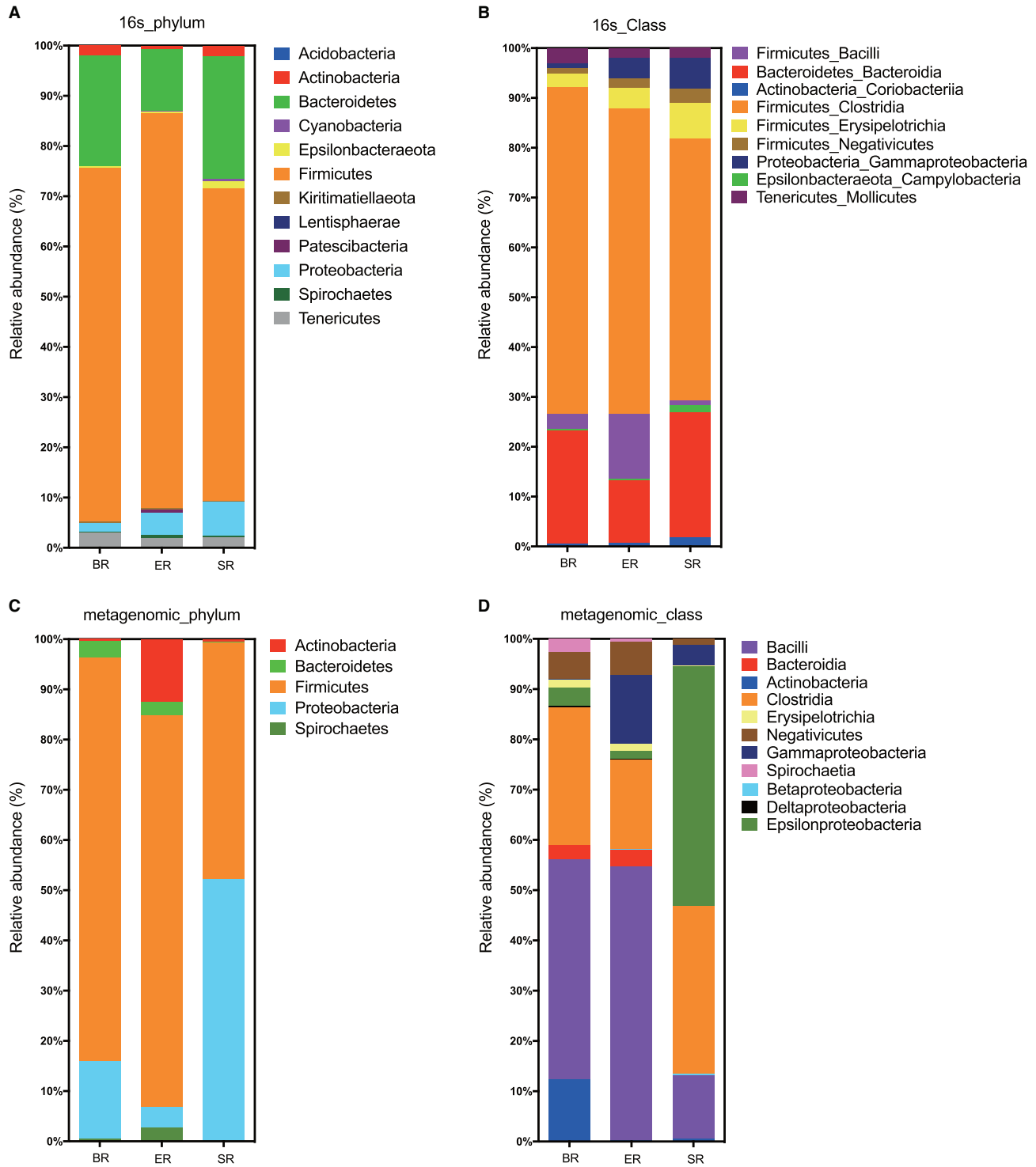

FIGURE 3 | Bacterial read profiling of 16S rRNA sequencing and metagenomic approaches at phylum level **(A,C)** and class level **(B,D)**. Stacked columns for the mean of each group of samples enrolled in this study, indicating the relative abundance as a percentage of the total bacterial sequences per group. All data with an abundance of at least 0.1% in at least one subject were included.

88.7% (100 K) sequence identity with the closest relative—Simian adenovirus 3—within the Simian adenovirus clade (**Figure 5**). Based on its level of sequence divergence, the newly discovered virus likely represents a new virus species that we tentatively termed "simian adenovirus ER" (GenBank accession number: MZ062897).

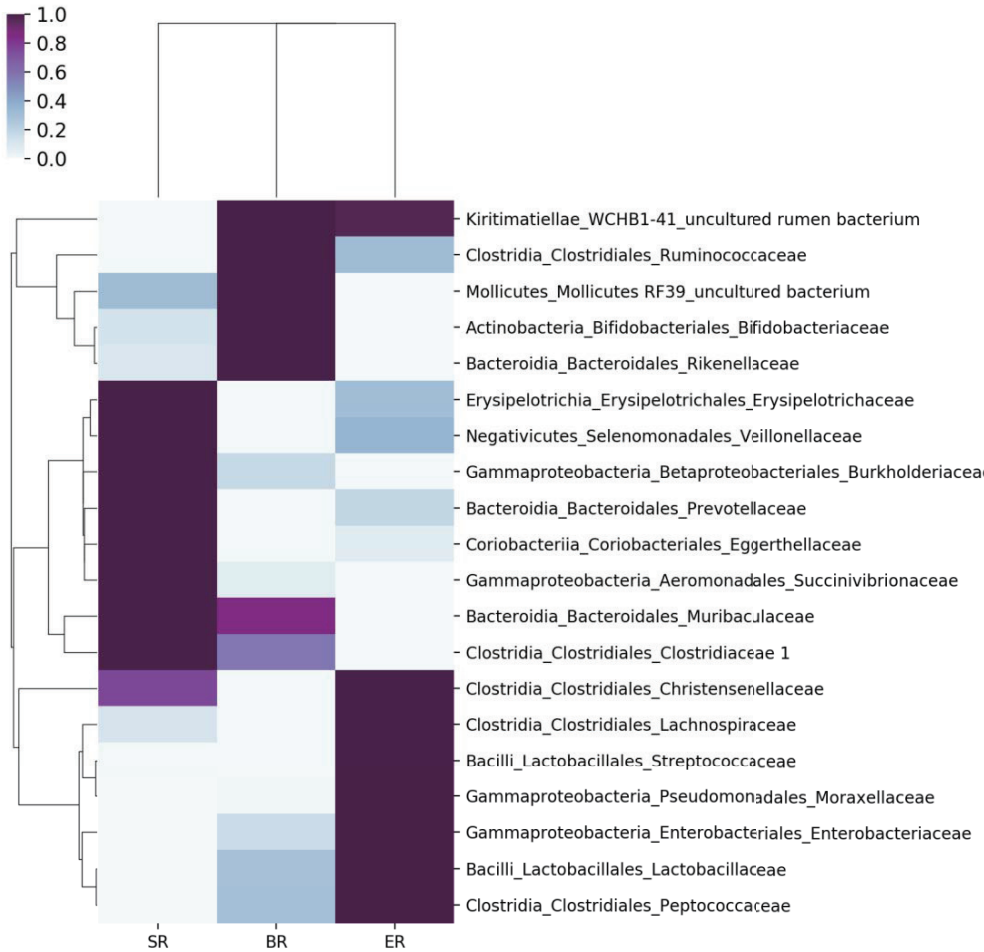

FIGURE 4 | Comparisons of microbial community at the family level in different groups of monkeys. A heatmap was used to visualize the microbial composition in three groups of monkeys by 16S rRNA sequencing.

WGS-Based Characterization of the Diversity and Abundance of AMR Genes

A total of 67 acquired AMR genes were detected in the DNA-seq data sets, representing resistance against nine classes of antibiotics (**Figure 6**). Genes providing resistance to aminoglycosides, beta-lactams, MLS (including macrolides, lincosamides, streptogramin) and tetracyclin were found across all locations tested (**Supplementary Table 2**). The semi-captive group (ER) showed highest variety and abundance of antibiotic genes, followed by captive group (BR). Diversity measures indicate the number of AMR genes detected against the ResFinder database in each class. Abundance was calculated as the sum of Reads Per Kilobase of each class of AMR maker per Metagenome (RPKM) in each library. Accordingly, the wild group(SR) had the lowest variety and abundance, while genes conferring resistance to sulphonamide, rifampicin, trimethoprim and phenicol were only detected in ER group (**Figure 6**). In both zoo-captive groups, genes conferring resistance against Tetracycline had the highest relative abundance [i.e., AMR genes abundance (RPKM)/Total AMR genes abundance (RPKM)] at 68% in ER and 88% in BR, while genes conferring resistance against Vancomycin were the most abundant in the SR group (97.5%).

DISCUSSION

We present a detailed fecal microbiome analysis of the zoo-captive and wild rhesus monkeys in China. Since non-human primates are the most relevant animal models for human research, a wide range of microbial composition studies have provided important information on the features that shape host-microbiome interactions (25). To date, however, only a few studies have investigated the fecal microbiome and resistome of wild and captive primates.

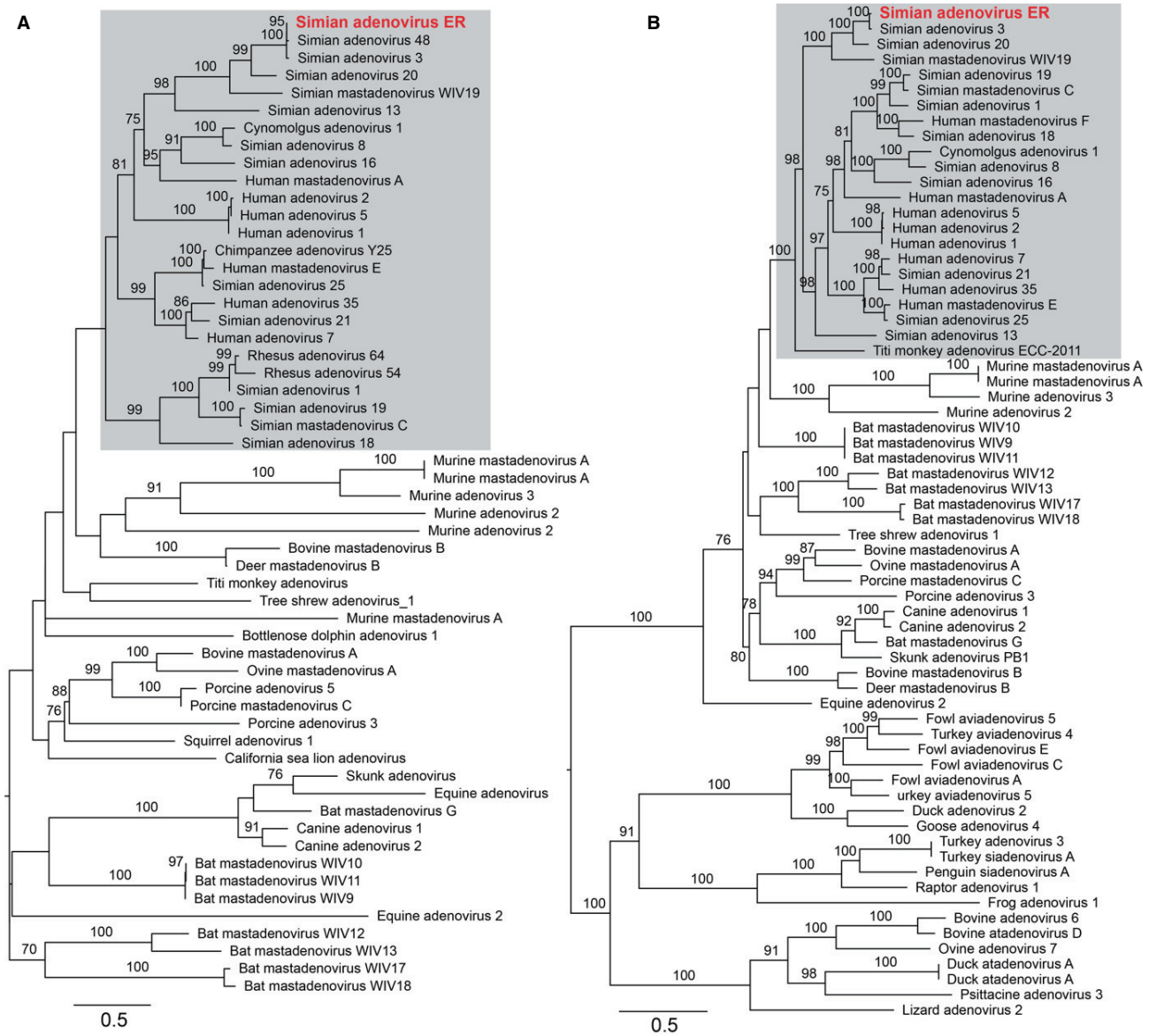

FIGURE 5 | Characterization of the newly identified simian adenovirus ER in zoo-captive monkeys. Phylogenetic analysis was performed based on the amino acid sequence of E1A **(A)** and 100 K protein **(B)**. The gray shading indicates the primate adenoviruses. Branch lengths are scaled according to the number of amino acid substitutions per site. The trees were mid-point rooted for clarity only.

Several previous studies have demonstrated that human activities such as captivity, confinement, diet and anthropogenic activity, may change the diversity and complexity of the primate gut microbiome (5). Although these studies provide evidence that captivity was associated with a reduction in diversity/richness in the gut microbiome compared to wild primates, our study revealed no such reduction, consistent with some other work (26). With respect to microbial composition, we found similarity at the phylum and class level among the three groups, but striking differences at the OTU level. The cause of such differences is still unclear. While captivity may be an important contributing factor, we are unable to exclude other factors such as geographic locations, diet and human interactions.

Notably, we used two sequencing strategies, 16S amplicon sequencing and WGS, to investigate the fecal microbiome. Generally, a similar trend of microbial composition was obtained from both approaches. However, some differences at different taxonomic levels were evident. For example, certain bacterial phyla (i.e., Tenericutes) were strongly underrepresented in shotgun WGS in comparison to 16S rRNA sequencing.

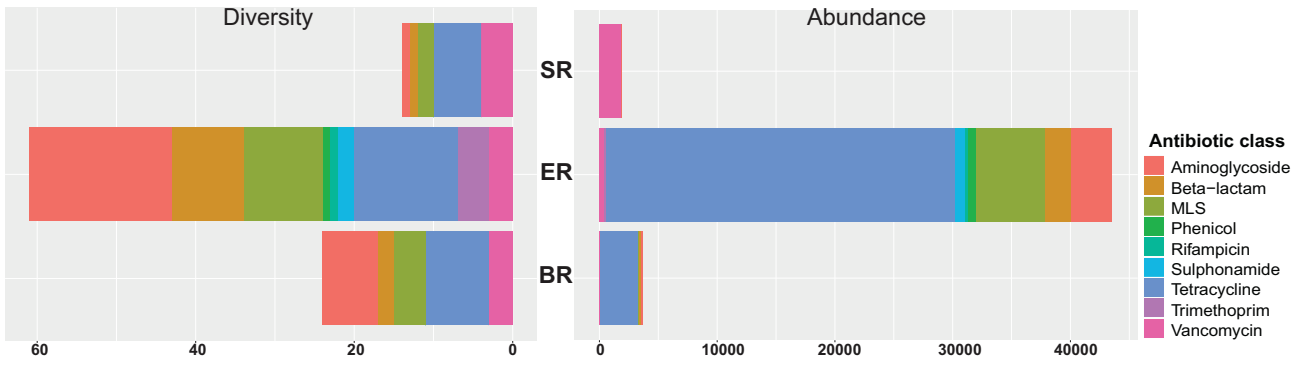

FIGURE 6 | Resistome profiling of wild and zoo-captive monkeys. The diversity measures indicate the number of AMR genes detected from the ResFinder database in each class. Abundance was calculated based on the sum RPK of each class of AMR makers per metagenome.

Conversely, at the class level, WGS identified more diverse bacterial classes (i.e., Epsilonproteobacteria and Bacilli) than 16S sequencing, which may reflect a lack of consistent marker genes.

Due to climate change and increasing anthropogenic activities, the habitat of many wildlife species has been threatened. As such, enclosed environments like zoos provide an opportunity for intermingling of human and monkey populations (27). Previous studies have detected several zoonotic pathogens were detected in free-ranging or zoo-captive monkeys in China, such as *Escherichia coli* O98 (28), *Mycobacterium tuberculosis* (29), *Bartonella quintana* infection in captive or wild rhesus macaques (30). In addition, canine distemper virus (31), novel noroviruses, enteroviruses and enteric parasites such as *Enterocytozoon bieneusi*, *Cryptosporidium* spp. and *Giardia duodenalis* (32, 33) have been identified from monkeys, raising public concerns about the risk of disease transmission from zoo animals to humans. In our study, a single vertebrate-associated virus—an adenovirus—was identified in one of the zoo-captive group, ER. This virus was relatively abundant and related to the previously identified Simian adenovirus type 3. Adenoviruses have a broad host spectrum including humans and cross-species transmission have been reported in non-human primates (27, 34, 35). Furthermore, Simian adenoviruses can result in infectious respiratory and diarrheal diseases in humans, but are asymptomatic in rhesus macaques (27), indicating that they are of public health concern.

Our analysis revealed a great diversity and abundance of AMR genes in zoo-captive groups. Although AMR genes exist in nature and are transmitted among wildlife animals, habitats that are more closely linked to anthropogenic activities tend to show significantly higher levels of antimicrobial resistance (36). Common sources of AMR genes for zoo-captive groups are through contact with humans (i.e., keepers, caretakers or tourists), diet, or through receiving veterinary medication. Interestingly, the highest level of antimicrobial resistance was observed in semi-captive monkeys (ER) rather than captive animals (BR), despite the fact that the latter are more subject to human interventions. However, since the study is limited in sampling size and locations, this needs to be examined with more data in the future studies.

We identified the AMR genes *Van*G, *Van*T-G, and *Van*XY-G genes in all groups of monkeys. These confer Vancomycin resistance in gram-positive cocci such as *Enterococcus faecalis* (37). Since the first vancomycin-resistant enterococci (VRE) cases were reported in the 1980s (38), VRE-associated infections and persistent colonization in humans have raised serious public health awareness and caused huge economic impacts (39). The emergence of VREs in food-animal production systems has been largely attributed to the heavily use of avoparcin as a growth promoter (40). Even though the use of growth-promoting antibiotics in farm animals has been banned since 1997, high rates of VRE carriage have been reported globally in economic animals, as well as in companion and laboratory animals (41) as well as wildlife (42), and which might act as reservoir populations (43–54). Accordingly, the continuous long-term monitoring of a broader range of microbiome and resistomes between captive and free-ranging wildlife for enterococcal species as well as other vancomycin-resistant genes dispersal is clearly required.

In comparison to wild populations, the captive populations studied here had much higher levels of tetracycline associated resistant genes. These genes are frequently found in human isolates of the two types of bacteria that were a substantial part of the normal microbiota of primates (Firmicutes and Bacteroidetes). It was previously observed that *Enterococcus* species showed high resistance in captive black capuchin monkeys in Brazil, characterized by a higher frequency of *msr*C (95%) and *tet*(L) (57%) genes when compared to wild monkeys (55). Although we did not find *msr*C in all groups, *tet*(M) and *tet*(L) resistance genes were found at high abundance in the semi-captive group (ER); nevertheless, these AMRs genes which have also been found overlapping with existing known human gut resistomes, suggesting potential transmission via human contact with wildlife. However, because our sample size was limited future studies are needed to clarify the essential reservoirs, carriers, and vectors on the transmission chain, and to identify the factors promoting and models assessing AMR gene exchange.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this article. All raw sequence reads were submitted to the Sequence Read Archive (SRA-NCBI) under BioProject PRJNA726842. The simian adenovirus ER was submitted to GenBank (accession number: MZ062897).

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee, Sun Yat-sen University.

AUTHOR CONTRIBUTIONS

TJ and CZ: conceived and designed the experiments. TJ and XL: collect the samples. TJ, SZ, XL, and YY: performed the experiments. W-SC, VM, TJ, SZ, and YY: analyzed the data. W-SC, MS, TJ, and CZ: wrote the paper. SZ, W-SC, VM, and EH: revised the paper. EH, MS, and CZ: supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.778556/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Comparison of PK/PD Targets and Cutoff Values for Danofloxacin Against *Pasteurella multocida* and *Haemophilus parasuis* in Piglets

Yu-Feng Zhou [1,2,3], Zhen Sun [1,3], Rui-Ling Wang [1,3], Jian-Guo Li [1,3], Chao-Yan Niu [1,3], Xian-An Li [1,3], Yun-Yun Feng [1,3], Jian Sun [1,2,3], Ya-Hong Liu [1,2,3] and Xiao-Ping Liao [1,2,3]**

[1] National Risk Assessment Laboratory for Antimicrobial Resistance of Animal Original Bacteria, College of Veterinary Medicine, South China Agricultural University, Guangzhou, China, [2] Guangdong Laboratory for Lingnan Modern Agriculture, Guangzhou, China, [3] Guangdong Provincial Key Laboratory of Veterinary Pharmaceutics Development and Safety Evaluation, South China Agricultural University, Guangzhou, China

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***Correspondence:**
Xiao-Ping Liao
xpliao@scau.edu.cn
Yu-Feng Zhou
zyf@scau.edu.cn

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Danofloxacin is a synthetic fluoroquinolone with broad-spectrum activity developed for use in veterinary medicine. The aim of this study was to evaluate the pharmacokinetic/pharmacodynamic (PK/PD) targets, PK/PD cutoff values and the optimum doses of danofloxacin against *P. multocida* and *H. parasuis* in piglets. Single dose serum pharmacokinetics was determined in piglets after intravenous and intramuscular administration of 2.5 mg/kg. Danofloxacin was well absorbed and fully bioavailable (95.2%) after intramuscular administration of 2.5 mg/kg. The epidemiological cutoff (ECOFF) values of danofloxacin from 931 *P. multocida* isolates and 263 *H. parasuis* isolates were 0.03 and 4 mg/L, respectively. Danofloxacin MICs determined in porcine serum were markedly lower than those measured in artificial broth, with a broth/serum ratio of 4.33 for *H. parasuis*. Compared to *P. multocida*, danofloxacin exhibited significantly longer post-antibiotic effects (3.18–6.60 h) and post-antibiotic sub-MIC effects (7.02–9.94 h) against *H. parasuis*. The mean area under the concentration-time curve/MIC (AUC_{24h}/MIC) targets of danofloxacin in serum associated with the static and bactericidal effects were 32 and 49.8, respectively, for *P. multocida*, whereas they were 14.6 and 37.8, respectively, for *H. parasuis*. Danofloxacin AUC_{24h}/MIC targets for the same endpoints for *P. multocida* were higher than those for *H. parasuis*. At the current dose of 2.5 mg/kg, the PK/PD cutoff (CO_{PD}) values of danofloxacin against *P. multocida* and *H. parasuis* were calculated to be 0.125 and 0.5 mg/L, respectively, based on Monte Carlo simulations. The predicted optimum doses of danofloxacin for a probability of target attainment (PTA) of > 90% to cover the overall MIC population distributions of *P. multocida* and *H. parasuis* in this study were 2.38 and 13.36 mg/kg, respectively. These PK/PD-based results have potential relevance for the clinical dose optimization and evaluation of susceptibility breakpoints for danofloxacin in the treatment of swine respiratory tract infections involving these pathogens.

Keywords: PK/PD, cutoff, danofloxacin, *P. multocida*, *H. parasuis*

INTRODUCTION

P. multocida and *H. parasuis* play important roles in many outbreaks of swine respiratory disease (SRD) and act together to increase the severity and duration of lung damage caused by other symbiotic viruses and bacteria such as porcine circovirus type 2 and *Streptococcus suis* (1–3). Furthermore, studies indicated that *P. multocida* type A can act as the primary pathogen of porcine pneumonia and septicemia with a rising prevalence rate reported from 8% to 15.6% in China, Korea and United States (1, 4, 5). Due to the high prevalence of mixed infections with multiple bacterial species, the treatment of SRD generally includes the use of broad-spectrum antibiotics (6, 7). Fluoroquinolones, such as danofloxacin, possess excellent PK characteristics that may contribute to clinical success of treating SRD. Such advantages include high peak concentrations in plasma, extensive distribution to most tissues in animal body and deep penetration into lung fluids (8, 9). Despite these findings from previous studies, the precise pharmacokinetic/pharmacodynamic (PK/PD) targets and cutoff values of danofloxacin in pigs for SRD pathogens, especially for *P. multocida* have not been fully elucidated.

This study sought to determine and compare the PK/PD relationships of danofloxacin between *P. multocida* and *H. parasuis* with the goal to provide a framework for further study and optimization of danofloxacin dosing strategies for the treatment of bacterial respiratory mixed infections in piglets caused by SRD pathogens. By evaluating the drug kinetics, PK/PD targets, post-antibiotic effect (PAE) and postantibiotic sub-MIC effect (PA-SME), the optimum doses and PK/PD cutoffs (CO_{PD}) of danofloxacin were estimated for *P. multocida* and *H. parasuis*. A comparison of these results provides insights into the accurate antibiotic treatment for SRD.

MATERIALS AND METHODS

Bacterial Strains and Susceptibility Testing

The MIC distribution of 931 swine-origin *P. multocida* isolates (**Supplementary Table 1**) was obtained by merging data from our own laboratory and some previous studies (10, 11). A total of 263 isolates of *H. parasuis* were gathered during 5-year surveillance study in different provinces of China from 2015 to 2020 (**Supplementary Table 1**). All the isolates were collected from diseased pigs suffering polyserositis, pneumonia or arthritis, and cultured with Haemophilus test medium (HTM) broth and agar containing 20 mg/L β-NAD and 5% lysed horse blood. Bacterial species was identified using the Axima Assurance MALDI-TOF mass spectrometer (Shimadzu Corp., Kyoto, Japan), as previously described (12). The MICs of danofloxacin for these isolates were determined using the broth microdilution in accordance with CLSI guidelines (13). To determine if there is a potentiation effect of serum on susceptibility, the danofloxacin MICs against *P. multocida* and *H. parasuis* were further determined in both broth and porcine serum.

Epidemiological Cutoff Values Determination

The ECOFF defines the upper end of the wild-type MIC distribution devoid of phenotypically detectable acquired resistance mechanisms (14). The isolates carrying plasmid-mediated quinolone resistance (PMQR) genes [*qnrA-D*, *qepA*, *oqxAB* and *aac(6')-Ib-cr*] were consequently removed (15). The $\log_2$-transformed MIC distribution of danofloxacin for *P. multocida* and *H. parasuis* was subjected to the statistical goodness-of-fit and non-linear least-squares regression tests to obtain optimum normal distribution (16). The final ECOFF value was calculated as the MIC that captured at least 95% of the optimum MIC distribution using the ECOFFinder program (16, 17).

PAEs and PA-SMEs Determination

Two *P. multocida* and three *H. parasuis* strains were selected to expose to danofloxacin at 1× and 4× MICs for 1 h. After removal of drug by centrifugation at 3,000 g for 10 min, bacterial cells were resuspended in drug-free broth (PAE) and broth containing 0.1 to 0.3 × MICs of danofloxacin (PA-SME) for continuous measurement of the absorbance at 600 nm. Optical density was converted into bacterial counts using a standard curve, as our previously reported (17). The PAEs and PA-SMEs of danofloxacin against *P. multocida* and *H. parasuis* were calculated as follow: PAE/PA-SME = T/T_{PA}-C, where C is the time for 1-$\log_{10}$ control growth and T/T_{PA} is the time for 1-$\log_{10}$ growth after drug removal (T) or in the sub-MIC treated phase (T_{PA}) (18).

Pharmacokinetics of Danofloxacin in Piglets

Twelve healthy crossbred piglets (Duroc × Landrace × Yorkshire, 9.3 ± 1.9 kg from Jiahe Agricultural Stockbreeding Co., Qingyuan, China) were used for a two-period crossover study. Animal experimental protocols were approved by the Animal Ethics Committee of South China Agricultural University (approval no. 2018014). Each piglet received danofloxacin (Injectable solution; lot no. 190201; Hainan Yuqi Pharmaceutical Co., Dingan, China) at a dose of 2.5 mg/kg b.w. by intravenous (IV) and intramuscular (IM) injections. Feed and water were provided *ad libitum*. The dose of danofloxacin was chosen based on previous PK studies in pigs and the manufacturer's instruction (19–22). The serums samples for danofloxacin concentration determination were collected from the jugular veins into vacutainers without anticoagulant prior to dosing (0 h) and at 0.08, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 12, 24, 36, and 48 h after administrations of danofloxacin.

Danofloxacin concentrations in serum samples were measured by a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method (details are given in **Supplementary Data Sheet 1**). All PK parameters were calculated using the compartmental models in WinNonlin software (version 5.2; Pharsight, St. Louis, MO, USA). The Akaike information criterion (AIC) was used to guide the selection of the best PK model to describe the observed time-concentration data. Danofloxacin average bioavailability (F%)

after intramuscular injection was calculated by dividing each AUC_{IM} value by their respective AUC_{IV} value for each individual piglet according to the following standard equation (23): $F\% = AUC_{infinity(IM)} / AUC_{infinity(IV)} \times 100\%$.

Ex vivo Time-Kill and PK/PD Index Target for Efficacy

The abilities of danofloxacin to kill *P. multocida* and *H. parasuis* were assessed *ex vivo* as previously described (17). Serum samples collected from piglets at different time points were filtered to avoid bacterial contamination. Bacterial cells were subcultured and inoculated to each serum sample, giving an initial inoculum of $\sim 10^6$cfu/mL. The mixtures were serially diluted and plated using a drop-plate technique to enumerate bacterial CFUs after 3, 6, 9 and 24 h of incubation. The limit of detection (LOD) was 40 cfu/mL.

The correlation between antibacterial efficacy and the PK/PD parameter AUC_{24h}/MIC was determined by the non-linear WinNonlin regression program (version 5.2; Pharsight, St. Louis, MO, USA). The AUC/MIC ratio was chosen as the predictive PK/PD parameter as previous studies have demonstrated this index to be predictive for fluoroquinolones (24, 25). The sigmoid E_{max} model used was derived from the Hill equation: $E = E_0 + E_{max} \times C^N / (EC_{50}{}^N + C^N)$, where E_0 is the $\log_{10}$ change of bacterial count in the absence of danofloxacin, E_{max} is the maximum effect, C is the PK/PD index (AUC_{24h}/MIC), EC_{50} is the AUC_{24h}/MIC required to achieve 50% of the E_{max} and N is the slope of the dose-response curve. The coefficient of determination (R^2) was used to estimate the variance due to regression with the PK/PD parameter AUC/MIC. The AUC_{24h}/MIC targets in serum required to produce bacteriostatic ($E = 0$), bactericidal ($E = -3$) and eradication ($E = -4$) effects were calculated for each drug-organism combination.

PK/PD Cutoff Determination and Dose Assessment

Based on PK parameters and calculated PK/PD targets (AUC_{24h}/MIC) for bactericidal effect, a 10,000-subject Monte Carlo simulation was conducted to obtain the danofloxacin PK/PD cutoffs (CO_{PD}) for *P. multocida* and *H. parasuis* using Crystal Ball software (version 11.1.2, Oracle Corporation) (26). The AUC_{24h}/MIC was calculated with the following formula: $AUC_{24h}/MIC = Dose / (Cl \times MIC)$. Clearance (Cl) was assumed to be normally distributed in the form of mean ± SD (**Table 1**). Scenarios were simulated separately at each possible MIC. The CO_{PD} was defined as the highest MIC at which the PTA was ≥90% (27).

In order to ascertain the optimum dose regimens of danofloxacin to cover the overall MIC population distributions in this study (931 *P. multocida* strains and 263 *H. parasuis* isolates), the two population distributions of danofloxacin doses were predicted by a 10,000-subject Monte Carlo simulation. The dose was calculated by the equation as follow (17, 28): $Dose = (Cl \times AUC/MIC \times MIC_{distribution}) / (fu \times F)$, where Cl is the body clearance; AUC/MIC is the PK/PD target required for a bactericidal effect, in this case, the AUC_{24h}/MIC of 49.8 and 37.8 for *P. multocida* and *H. parasuis*, respectively; a scaling factor of 4.33 was used to bridge the MIC differences between HTM and serum when dose distribution was predicted for *H. parasuis*; *fu* is free drug fraction using protein binding rate of 44% in porcine serum (29); F is the bioavailability of IM administration.

TABLE 1 | The PK parameters of danofloxacin in porcine serum following single dose intravenous (IV) and intramuscular (IM) administrations at 2.5 mg/kg.

PK parameters[a]	Unit	IV route	IM route
K_a	1/h		4.99 ± 5.08
K_{el}	1/h		0.21 ± 0.12
A	mg/L	1.12 ± 0.68	–
α	1/h	4.44 ± 2.24	–
B	mg/L	1.67 ± 0.89	–
β	1/h	0.20 ± 0.05	–
$T_{1/2Kel}$	h		4.18 ± 1.81
$T_{1/2Ka}$	h		0.29 ± 0.20
$T_{1/2\alpha}$	h	0.25 ± 0.24	–
$T_{1/2\beta}$	h	3.76 ± 1.00	–
V_{ss}	L/kg	1.90 ± 1.22	–
Cl	L/kg/h	0.39 ± 0.27	–
$AUC_{infinity}$	mg·h/L	9.39 ± 5.79	8.17 ± 3.51
T_{max}	h		1.04 ± 0.52
C_{max}	mg/L		1.19 ± 0.55
F	%	–	95.2 ± 21.9

[a] *K_a, constant of absorption rate; K_{el}, constant of elimination rate; A, intercept for the distribution phase; α, distribution rate constant; B, intercept for the elimination phase; β, elimination rate constant; $T_{1/2Kel}$, elimination half-life in the one-compartment model; $T_{1/2Ka}$, absorption half-life; $T_{1/2\alpha}$, distribution half-life; $T_{1/2\beta}$, elimination half-life in the two-compartment model; V_{ss}, volume of distribution at steady state; Cl, systemic clearance; $AUC_{infinity}$, the area under the concentration-time curve from zero to infinity; T_{max}, time to reach the peak concentration (C_{max}); F, average bioavailability.*

RESULTS

MICs and ECOFF Determination

Of 931 *P. multocida* isolates, the fitted MIC distribution [Log_2 mean (−6.06) ± SD (0.27)] contained > 95% that possessed danofloxacin MICs ≤ 0.03 mg/L, and the ECOFF was consequently calculated to be 0.03 mg/L for *P. multocida* (**Figure 1A**). No difference in MIC was observed for *P. multocida* between broth and serum. However, of the 14 *H. parasuis* isolates tested, geometric mean of danofloxacin MIC in serum was significantly lower than that in HTM, with a HTM/serum ratio of 4.33 ($P < 0.05$; **Supplementary Figure 1**). The MICs of danofloxacin against our 263 clinical *H. parasuis* isolates ranged from 0.004 to 128 mg/L in HTM, with the MIC_{50} and MIC_{90} of 0.25 and 4 mg/L, respectively (**Supplementary Table 1**). In order to obtain a unimodal distribution, the 10 isolates with MICs of ≥64 mg/L were therefore removed. More than 95% of the best fitting normal distribution [Log_2 mean (−2.21) ± SD (2.49)] was in the range of 0.004 to 4 mg/L, thus the ECOFF value was determined to be 4 mg/L for *H. parasuis* (**Figure 1B**).

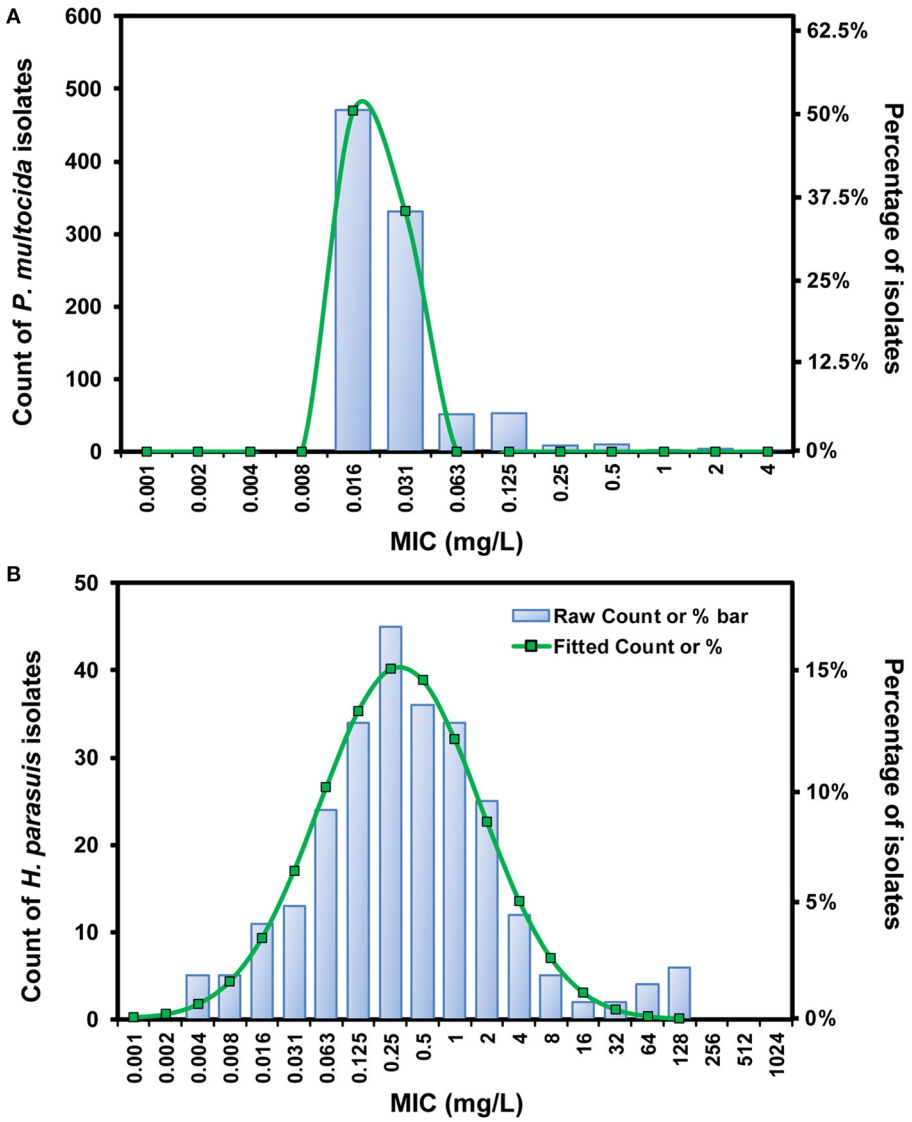

FIGURE 1 | The log_2-transformed MIC distribution of danofloxacin against swine-origin *P. multocida* **(A)** and *H. parasuis* **(B)**. The MIC distribution of *P. multocida* isolates ($n = 931$) was created by merging data from our laboratory and some previous studies (10, 11). *H. parasuis* isolates ($n = 263$) and MIC data were obtained in our own laboratory from 2015 to 2020. The number of isolates and the observed frequency corresponding to each MIC value are shown along the y-axes. The lines represent predicted frequency based on the best fitting log_2-normal distribution [log_2 mean (−6.06) ± SD (0.27) for *P. multocida* and log_2 mean (−2.21) ± SD (2.49) for *H. parasuis*, respectively].

PAEs and PA-SMEs

PAEs were calculated after removal of bacterial cells from danofloxacin exposures at 1× and 4× MICs. Persistent regrowth inhibition was observed in a concentration-dependent manner, resulting in PAE values of 0.96–4.46 h for *P. multocida* and 2.42–6.92 h for *H. parasuis*, respectively (**Figures 2A,B**). The addition of sub-MIC danofloxacin during the post-antibiotic phase substantially delayed bacterial regrowth, producing PA-SMEs of 4.30–6.86 h for *P. multocida* and 7.02–9.94 h for *H. parasuis*, respectively (**Figure 2C**). Despite this fact, the mean bacterial densities in the presence of sub-MIC danofloxacin remained at a lower level than their respective growth controls until at least 12 h (**Figure 2**; **Supplementary Figure 2**). Of note, the time suppression of regrowth (PAE and PA-SME) for *H. parasuis* was significantly longer relative to *P. multocida* ($P < 0.05$; two-tailed unpaired Students *t-test*; **Figure 2C**).

Danofloxacin PKs in Piglets

A two-compartmental model fit was shown for time-concentration profile of danofloxacin after IV injection (**Figure 3**), which was consistent with previous results observed in both healthy and infected pigs (29). Notably, the decline in serum danofloxacin concentrations was bi-exponential with half-lives of ($T_{1/2\alpha}$) 0.25 h and ($T_{1/2\beta}$) 3.76 h for distribution

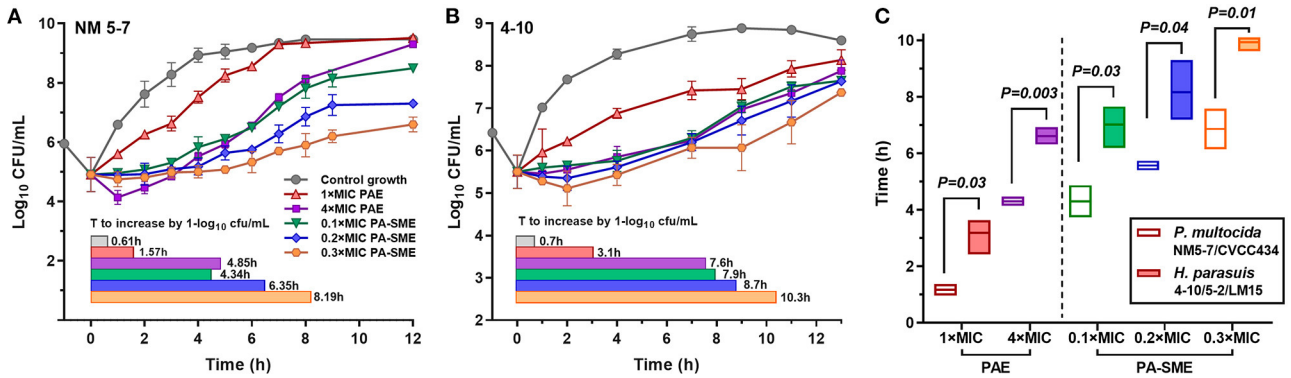

FIGURE 2 | The postantibiotic effects (PAEs) and post-antibiotic sub-MIC effects (PA-SMEs) of danofloxacin. **(A,B)** The PAEs were measured after initial exposure to danofloxacin at 1× and 4× MICs against *P. multocida* (A; strain NM5-7) and *H. parasuis* (B; strain 4–10). The PA-SMEs were measured after initial exposure to danofloxacin at 4 × MICs. The color horizontal bars represent the time that required bacterial counts to increase by 1.0-$\log_{10}$cfu/mL after drug removal (PAE) or at the sub-MIC phase (PA-SME). **(C)** Comparison of danofloxacin PAE and PA-SME between *P. multocida* and *H. parasuis*. Two *P. multocida* isolates and three *H. parasuis* isolates were tested and included. Statistical significance was determined using the two-tailed unpaired Student *t-test* ($P < 0.05$).

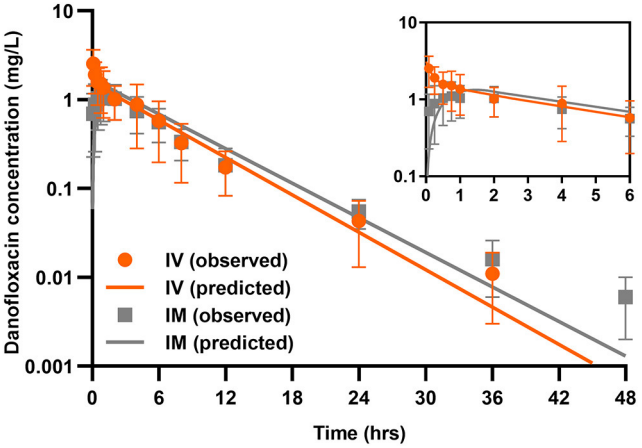

FIGURE 3 | Danofloxacin pharmacokinetics in piglets. Serum concentrations of danofloxacin following single dose intravenous (IV) and intramuscular (IM) administrations at 2.5 mg/kg in piglets ($n = 12$ per time point). Upper right panel shows details from 0 to 6 h. The lines drawn through the time points indicated the best-fitting lines based on the compartment models.

and elimination phases, respectively (**Table 1**). After IM dosing of danofloxacin, the mean peak concentration (C_{max}; 1.19 mg/L) was reached in serum within 1.04 h. While the prolonged terminal half-life ($T_{1/2Kel}$; 4.18 h) was observed in serum following IM dosing, $AUC_{infinity}$ values were comparable regardless of administration routes, indicating a high bioavailability of 95.2% after IM administration (**Table 1**).

Ex vivo Antimicrobial Activities and PK/PD Targets

Rapid activity against *P. multocida* strain NM5-7 (MIC_{serum} = 0.13 mg/L) was demonstrated with porcine serum collected up to 12 h at concentrations of 0.22 to 1.12 mg/L (**Figure 4A**). Notably, a concentration-dependent trend toward a greater level of *P. multocida* killing was observed with increasing danofloxacin concentrations in serums. Bacterial densities of *P. multocida* were driven below detectable limits by serums collected up to 6 h after 9 h of incubation (**Figure 4A**). Concentration-dependent killing activity was similarly observed for all *H. parasuis* strains tested (**Figure 4B**; **Supplementary Figure 3**). Accordingly, *ex vivo* activity was negligible for serums at 36 and 48 h, while sustained bactericidal activity was attained within 9 h of exposure to serums containing danofloxacin > 0.5 mg/L (**Supplementary Figure 3**). For *H. parasuis* 4–10 (MIC_{serum} = 0.06 mg/L), complete bactericidal activities reaching undetectable limits of eradication were noted within 24 h in response to serums collected up to 12 h (**Figure 4B**).

PK/PD analyses of the *ex vivo* time-kill data were performed to determine AUC/MIC targets of danofloxacin associated with the optimal activity. PK/PD relationships between AUC/MIC and *ex vivo* activity were strong, with an R^2 of > 0.93 (**Figures 4C,D**). For *P. multocida*, the mean AUC/MIC targets in serum for bacteriostatic, bactericidal and eradication effects were 32, 49.8 and 66.9, respectively. Of note, the PK/PD targets for *H. parasuis* were much lower than those for *P. multocida* ($P < 0.05$, two-tailed unpaired Student's *t*-test). Serum AUC/MIC targets for the same endpoints were 14.6, 37.8 and 62.9 (**Table 2**).

PK/PD Cutoff Determination and Dose Prediction

The probabilities of the current dose regimen (2.5 mg/kg) achieving typical AUC/MIC targets at each possible MIC were determined by a 10,000-iteration Monte Carlo simulation, from which PTAs were estimated (**Figure 5**). With a target AUC/MIC ratio of 49.8 (i.e., bactericidal action for *P. multocida*), the PTA was still 87.3% at a MIC of 0.125 mg/L. The CO_{PD} value of danofloxacin for *P. multocida* was consequently determined to be 0.125 mg/L (**Figure 5A**). In view of the significant potentiation

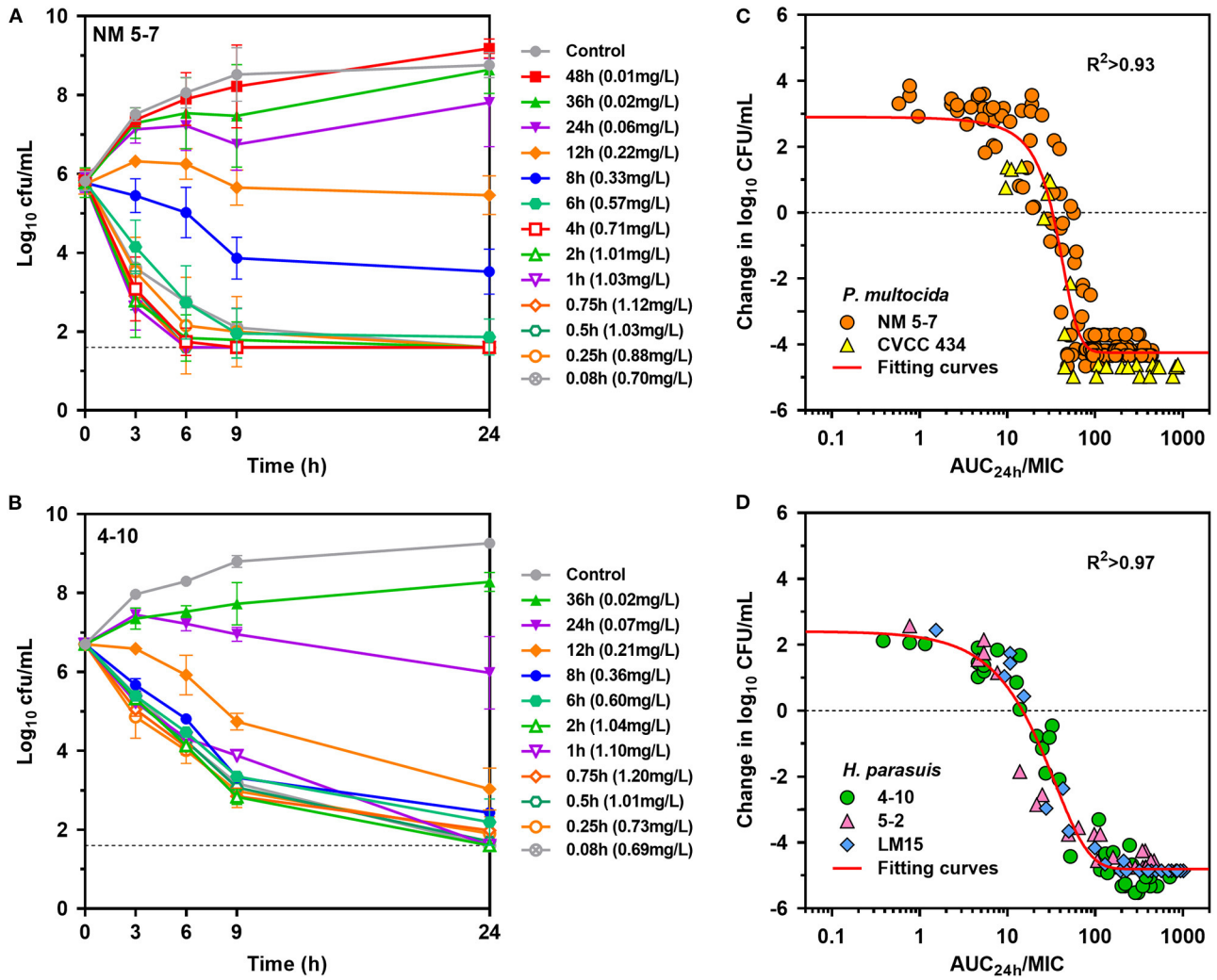

FIGURE 4 | *Ex vivo* activity and PK/PD relationships of danofloxacin. **(A,B)** *Ex vivo* time-kill curves of danofloxacin against *P. multocida* [**(A)** strain NM5-7; MIC_{serum} = 0.13 mg/L] and *H. parasuis* [**(B)** strain 4-10; MIC_{serum} = 0.06 mg/L] in serums of piglets receiving intramuscular injection of danofloxacin (2.5 mg/kg b.w.). Numerical values on right brackets represent the mean concentrations of danofloxacin in serums collected from different time points post-dosing. **(C,D)** Correlation plots between *ex vivo* activity and AUC_{24h}/MIC of danofloxacin using the sigmoid E_{max} equation. The fitting curves represent predicted values (two *P. multocida* and three *H. parasuis* included), and the points represent values of individual serum samples collected from 0 to 48 h.

effect of serum on activity of danofloxacin for *H. parasuis* (**Supplementary Figure 1**), a scaling factor of 4.33 was created to bridge the MIC variation between HTM and serum when calculating the CO_{PD} for *H. parasuis*. The PTA for AUC/MIC ratio of 37.8 was only 26.1% at a MIC of 1.0 mg/L and reached 99.7% when the MIC was 0.5 mg/L. The CO_{PD} of danofloxacin against *H. parasuis* was therefore defined as a MIC of 0.5 mg/L (**Figure 5B**).

Based on the results of the current PK parameters, PK/PD targets and the MIC distribution, if danofloxacin was given once daily intramuscularly in piglets, the predicted dosages for a PTA of 90% to cover the overall MIC population distributions in this study were 2.38 and 13.36 mg/kg (**Figure 6**), which were estimated to be effective achieving a bactericidal effect against *P. multocida* and *H. parasuis*, respectively.

DISCUSSION

In this study, we observed a marked potentiation effect of porcine serum on danofloxacin activities for *H. parasuis* but not for *P. multocida*. This finding was in agreement with the previous studies showing that incorporation of the increasing amounts of serum to broth progressively reduced macrolides MICs for seven bacterial species harvested from pigs (28, 30). On the contrary, reduced antimicrobial activity in serum was observed for moxifloxacin against *Staphylococcus aureus* (31). It is therefore likely that serum potentiation effect is strain- and drug-codependent (32). The complement and specific antibody are considered as the key factors responsible for increased serum activity (33). In general, a prolong PAE has a high likelihood of predicting favorable outcomes (34). The findings of our

TABLE 2 | PK/PD targets of danofloxacin in serum (AUC_{24h}/MIC) necessary to achieve the bacteriostasis, bactericidal, and eradication effects for the study organisms in piglets.

Organisms	MIC in serum (mg/L)	E_0	E_{max}	EC_{50}	N	R^2	Target values of AUC_{24h}/MIC ratio (h) in serum [a]		
							Bacteriostasis	Bactericidal	Eradication
P. multocida									
NM5-7	0.125	2.95	−4.29	38.9	3.13	0.93	33.1	58.7	86.8
CVCC434	0.031	1.29	−4.69	36.7	8.20	0.96	30.9	40.9	47.1
Mean	**NA**	**2.12**	**−4.49**	**37.8**	**5.67**	**NA**	**32.0**	**49.8**	**66.9**
SD	**NA**	**0.83**	**0.20**	**1.10**	**2.54**	**NA**	**1.10**	**8.91**	**19.8**
H. parasuis									
4–10	0.063	2.05	−5.24	31.8	1.64	0.97	16.8	50.5	79.3
5–2	0.063	2.88	−4.59	12.8	1.80	0.98	10.2	24.6	50.5
LM15	0.031	1.91	−4.83	25.4	2.31	0.97	16.8	38.4	58.9
Mean	**NA**	**2.28**	**−4.89**	**23.3**	**1.92**	**NA**	**14.6**	**37.8**	**62.9**
SD	**NA**	**0.43**	**0.27**	**7.89**	**0.29**	**NA**	**3.11**	**10.5**	**12.1**

[a] *The bacteriostasis, bactericidal and eradication effects were defined as the net static, 3-log_{10}, and 4-log_{10} kill endpoints over 24 h; $P < 0.05$ for bacteriostasis AUC/MIC target between P. multocida and H. parasuis (unpaired Student's t-test); Bold values indicate the means and standard deviations (SD); NA, not applicable.*

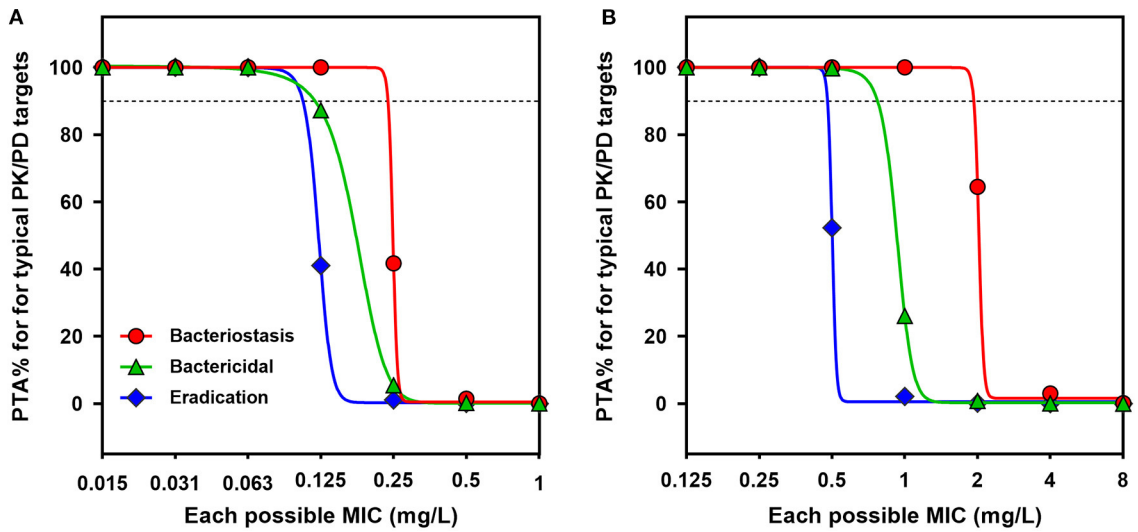

FIGURE 5 | PK/PD cutoff values for danofloxacin against *P. multocida* and *H. parasuis*. **(A,B)** Probability of target attainment (PTA) for typical AUC_{24h}/MIC targets (bacteriostatic, bactericidal and eradication effects) at each possible MIC when treated with danofloxacin at dose of 2.5 mg/kg against *P. multocida* **(A)** and *H. parasuis* **(B)** infections. Dotted lines denote the PTA of 90%.

study exhibiting the notable PAEs from 0.96 to 6.92 h in a concentration-dependent manner for both *P. multocida* and *H. parasuis*, point to clinical treatment with fluoroquinolones that cloud be administered at a longer dosing interval without loss of efficacy. It is well known that fluoroquinolones disrupt DNA synthesis by binding bacterial gyrase and topoisomerase, the PAEs induced by danofloxacin may represent the lag time for drug to dissociate from binding sites and to diffuse out of bacteria (35).

Similar to other fluoroquinolones, danofloxacin have good penetration into pulmonary epithelial lining fluid (ELF). A previous bronchopulmonary PK study with danofloxacin demonstrated higher peak concentration in ELF compared to plasma in pigs, with a mean ELF/plasma AUC ratio of 5.4 (9). Of note, the pharmacokinetic profiles of danofloxacin were linear and proportional in piglets as described by the result of linear regression analysis ($R^2 = 0.951$ for AUC_{24h}) (36). The similarity and proportionality of PK profiles potentially reflects passive diffusion of danofloxacin from plasma to ELF. In this case, serum could be used as a predictive surrogate for PD target assessment, although the value of PD target could be relatively high (37). The PD targets associated with bactericidal action in previous fluoroquinolone studies has been a total AUC_{24h}/MIC of 88 for *H. parasuis*, and values 1.5- to 5-fold higher (121–451) for gram-negative pathogens such as *Escherichia coli* and *Salmonella typhimurium* (38–40). In our study with danofloxacin, the

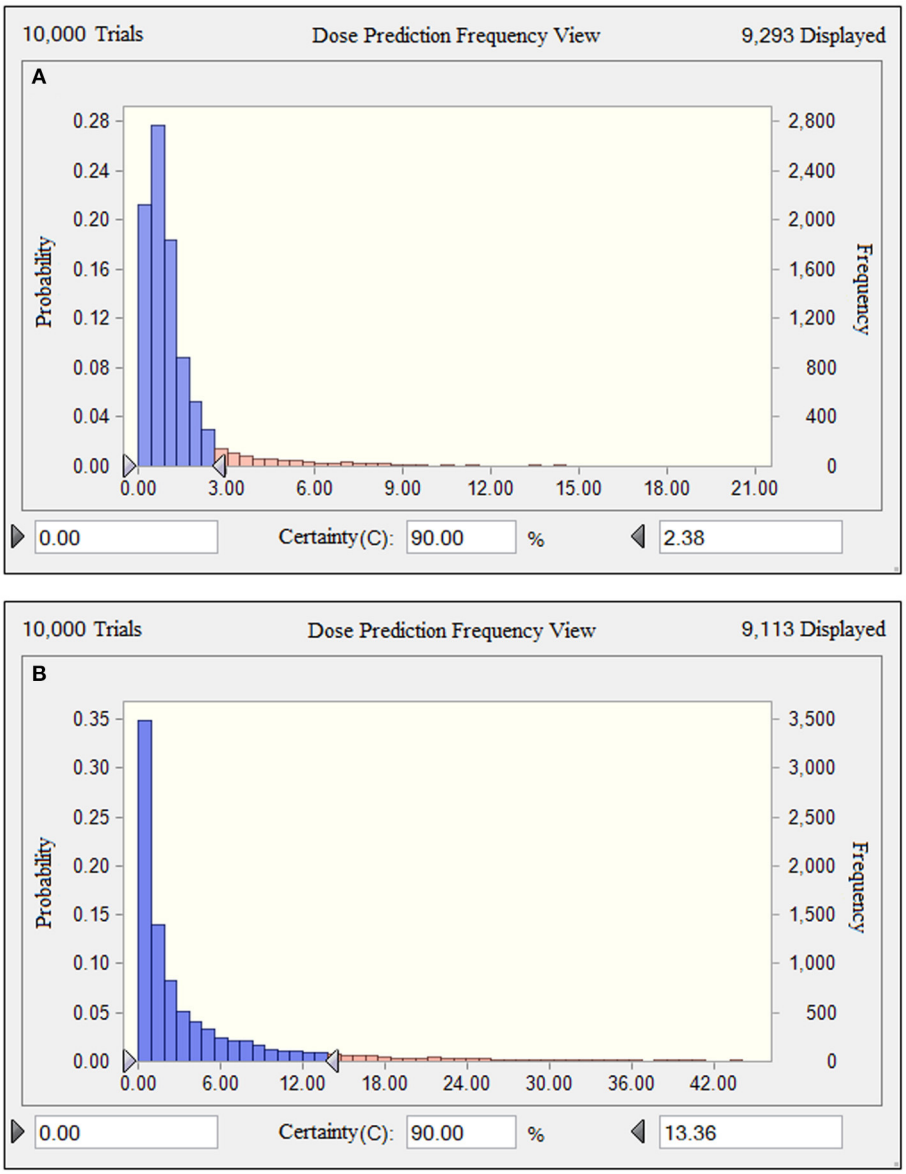

FIGURE 6 | Comparison of population distributions of danofloxacin doses for *P. multocida* **(A)** and *H. parasuis* **(B)**. The right triangle depicted the calculated target doses for a PTA of > 90% to cover the overall MIC population distributions in this study.

PD targets were lower for each of the bacteria tested. This difference was most profound for *H. parasuis* with a mean bactericidal AUC_{24h}/MIC of 37.8. Similarly, the AUC_{24h}/MIC target identified for *P. multocida* (49.8) was modestly lower than marbofloxacin with a bactericidal AUC_{24h}/MIC of 64.9 (41). These endpoints were roughly 2- to 9-fold lower than comparative PD studies for veterinary fluoroquinolones. The notable PK/PD efficacies for both *H. parasuis* and *P. multocida* provide a fairly robust option for treating SRD, especially in situations of bacterial coinfections due to mixed species.

At the current clinical dose of 2.5 mg/kg, the ECOFF and CO_{PD} values of danofloxacin against *P. multocida* were determined to be 0.03 and 0.125 mg/L, respectively. This is similar with the EUCAST MIC breakpoint (0.06 mg/L) used for levofloxacin and ciprofloxacin against *P. multocida* (42). The clinical trial has been previously conducted to investigate danofloxacin efficacy in Danish swine herds with a naturally occurring outbreak of acute *Pasteurella* pneumonia. A satisfactory response to treatment with 1.25 mg/kg danofloxacin was observed in 87% of the diseased pigs (43). For *H. parasuis*, danofloxacin CO_{PD} value (0.5 mg/L) was 8-fold greater than ciprofloxacin MIC breakpoint against *Haemophilus influenzae* (0.06 mg/L), but was equivalent to PK/PD breakpoints (0.25–0.5 mg/L) of other fluoroquinolones such as ofloxacin moxifloxacin (42). Of note, danofloxacin ECOFF for *H. parasuis* (4 mg/L) was higher compared to the corresponding CO_{PD} (0.5 mg/L) in this

study. The over-estimated ECOFF value could be due to other unknown resistance mechanisms and the limited number of strains collected. A similar cutoff value (≥2 mg/L) was observed for enrofloxacin against *Haemophilus somnus* (13). This result suggested that danofloxacin at 2.5 mg/kg may be insufficient to combat swine respiratory infections due to *H. parasuis* with high-level MICs of > 4 mg/L. Indeed, a higher danofloxacin dosage of 13.36 mg/kg is required to achieve a PTA of > 90% for bactericidal effect against the overall *H. parasuis* isolates collected in this study.

CONCLUSION

In summary, we have demonstrated a large potentiation effect of serum on the potency of danofloxacin for *H. parasuis*. Compared with *P. multocida*, the PAEs and PA-SMEs of danofloxacin were substantially longer for *H. parasuis*. The PK/PD targets and cutoff values identified in this study will be useful in guiding the optimum dosing regimen design for danofloxacin in the context of specific PK exposure and MIC distribution, and in the development of clinical breakpoints for the treatment of SRD involving these pathogens.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the South China Agricultural University (SCAU) Institutional Ethics Committee (Approval No. 2018014). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

Y-FZ and X-PL designed this study. Y-FZ and ZS wrote the manuscript. ZS, R-LW, J-GL, C-YN, X-AL, and Y-YF carried out the experiments. JS and Y-HL analyzed the data. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.811967/full#supplementary-material

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Impact of a Regulation Restricting Critical Antimicrobial Usage on Prevalence of Antimicrobial Resistance in *Escherichia coli* Isolates From Fecal and Manure Pit Samples on Dairy Farms in Québec, Canada

Maud de Lagarde[1,2], *John M. Fairbrother*[3,4,5], *Marie Archambault*[2,4,5], *Simon Dufour*[2,5], *David Francoz*[1,2], *Jonathan Massé*[2,5], *Hélène Lardé*[1,2,6], *Cécile Aenishaenslin*[5], *Marie-Ève Paradis*[7] *and Jean-Philippe Roy*[1,2]*

[1] *Department of Clinical Sciences, Faculty of Veterinary Medicine, Université de Montréal, Saint-Hyacinthe, QC, Canada,* [2] *Regroupement FRQNT Op+lait, Saint-Hyacinthe, QC, Canada,* [3] *OIE Reference Laboratory for Escherichia coli, Faculty of Veterinary Medicine, Université de Montréal, Saint-Hyacinthe, QC, Canada,* [4] *Swine and Poultry Infectious Research Center (CRIPA-FQRNT), Faculty of Veterinary Medicine, Université de Montréal, Saint-Hyacinthe, QC, Canada,* [5] *Department of Pathology and Microbiology, Faculty of Veterinary Medicine, Université de Montréal, Saint-Hyacinthe, QC, Canada,* [6] *Department of Clinical Sciences, Ross University School of Veterinary Medicine, Basseterre, Federation of Saint Christopher and Nevis,* [7] *Association des Médecins Vétérinaires Praticiens du Québec, Saint-Hyacinthe, QC, Canada*

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Edited by:
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Getahun E. Agga,
United States Department of Agriculture, United States

***Correspondence:**
Jean-Philippe Roy
jean-philippe.roy@umontreal.ca

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To tackle antimicrobial resistance (AMR), one of the major health threats of this century, the World Health Organization (WHO) endorsed a global action plan in 2015. This plan calls countries to develop national actions to address AMR. The province of Québec, Canada, adopted a new regulation on the 25th of February 2019, to limit the use in food animals of antimicrobials of very high importance in human medicine. We aimed to establish the impact of this regulation by comparing the AMR situation in dairy cattle in Québec ∼2 years before and 2 years after its introduction. We sampled calves, cows, and the manure pit in 87 farms. Generic and putative ESBL/AmpC *E. coli* were tested for susceptibility to 20 antimicrobials. Logistic regression was used to investigate whether the probability of antimicrobial resistance differed between isolates obtained from the pre and post regulation periods by sample type (calves, cows, manure pit) and in general. To identify AMR genes dissemination mechanisms, we sequenced the whole genome of 15 generic isolates. In the generic collection, at the herd level, the proportion of multidrug resistant (MDR) isolates, decreased significantly from 83 to 71% ($p = 0.05$). Folate inhibitor and aminoglycoside resistances demonstrated a significant decrease. However, when analyzed by sample type (calves, cows, manure pit), we did not observe a significant AMR decrease in any of these categories. In the ESBL/AmpC collection, we did not detect any significant difference between the two periods. Also, the general resistance

gene profile was similar pre and post regulation. We identified both clonal and plasmidic dissemination of resistance genes. In conclusion, as early as 2 years post regulation implementation, we observed a significant decrease in MDR in the dairy industry in Quebec in the generic *E. coli* collection with folate inhibitor and aminoglycoside resistances showing the most significant decrease. No other significant decreases were yet observed.

Keywords: ESBL/AmpC, cattle, calf/calves, bacterial clone, *Escherichia coli*, antimicrobial resistance

INTRODUCTION

Building sustainable food systems relies on effective antimicrobials being available to treat infections and ensure animal welfare. However, it is now well-recognized that antimicrobial resistance (AMR) threatens environmental, animal and public health and there is no more time to waste (1). Indeed, in 2015, recognizing the urgent need to tackle AMR, the membership of Food and Agriculture Organization (FAO), World Organization for Animal Health (OIE) and World Health Organization (WHO) endorsed a global action plan on AMR (GAP) (2). In 2016, the United Nations (UN) General Assembly reaffirmed the GAP as the guideline for fighting AMR and committed themselves to supporting and implementing it at the global, national, and regional levels (3). The GAP recognize in its fourth objective that one of the main actions for the different health actors, to contain AMR, is the judicious use of antimicrobials to reduce selective pressure on microorganisms. Indeed, all over the world, there is substantial misuse and/or overuse of antimicrobials in humans and food animals (4). WHO's GAP acknowledged laws and regulation as essential tools for ensuring the application of national standards to optimize the use of antimicrobials in human and animal health. This includes a call for all countries to develop and implement collaborative, multisectoral national action plans to address AMR in each country.

In this context, the province of Québec (Canada) adopted a new regulation on the 25th of February 2019, to restrict usage of category 1 antimicrobials of the Health Canada classification (5) in production animals (6). Briefly, Health Canada classified antimicrobials as function of their importance for human health. Category 1 antimicrobials are those of very high importance for humans based on two selective criteria: they are identified as the preferred option of treatment of serious human infections and there is no other (or limited) available alternative. The new regulation prohibits the use of these antimicrobials for preventive purposes in food-producing animals and restricts their usage for curative purposes unless it has been justified (e.g., with an antimicrobial susceptibility test) that there are no other effective alternative drugs available of lower importance (7). This regulation intends to limit the use of category 1 antimicrobials to rare curative cases. The goal is to limit selective pressure by antimicrobials, which should lead to an eventual decrease in AMR. However, implementation of rules without monitoring their effect in the field could result in applying restrictive and ineffective pressure on the food industry, which is always submitted to fierce competition.

In 2017, prior to the regulation implementation, the portrait of both antimicrobial use (AMU) and AMR in 101 dairy farms in Québec has been documented. It was demonstrated that the category 1 antimicrobials used on dairy farms were mainly third generation cephalosporins, fluoroquinolones and polymyxins. The intramammary route was the most frequently observed. The median herd was using 88 defined course doses (DCDbovCA) /100 cows-years of these latter antimicrobials (8). A change of AMU is expected after the regulation implementation and might influence AMR. Extended spectrum β-lactamase/AmpC (ESBL/AmpC) producing *E. coli* were found in either fecal or manure pit samples of 85% of these farms (9). As these results were published by our research team, we had an excellent comparison point to establish the impact of the regulation on the AMR in the dairy industry in Québec. Moreover, our team recently demonstrated an average category 1 AMU herd-level reduction of 80% of prescription following the implementation of the new regulation (10).

The objectives of the current paper were therefore: (i) to report the AMR situation ∼2 years after the regulation implementation; and (ii) to compare these results with the assessment that was performed 2 years prior to the regulation (9). The overall outcome was to determine if this regulation was beneficial for the dairy industry and to provide scientific evidence for others wishing to use a similar approach.

MATERIALS AND METHODS

Selection of Herds and Sample Collection

We used an observational descriptive cohort study on commercial dairy farms. Prior to initiating the research, the research protocol was approved by the Animal Use Ethics and the Research Ethics Committees of the Université de Montréal (20-Rech-2085). Written informed consent was obtained from the owners for the participation of their animals in this study. To enable a proper comparison between the period pre and post regulation implementation, we sampled the same herds as those sampled in 2017 to establish AMR prevalence in dairy farms in Québec (8, 9). The previous 101 farms, located in the three main dairy areas of Québec, Canada (Montérégie, Center-du Québec and Estrie), were contacted by a member of the previous research team (HL) in July 2020 and asked to participate to a second set of sampling. Following recruitment, two sampling

FIGURE 1 | Timeline of the sampling compared to the regulation implementation. Spring pre regulation and fall pre regulation were part of a previous study [Massé et al. (9)] that aimed at establishing the portrait of AMR in dairy farms in Québec, Canada. Spring post regulation and fall post regulation are the period of sampling ∼2 years after the implementation of the regulation in 87 of the same herds and used for comparison.

visits were made, firstly between August and September 2020 and secondly between February and March 2021. **Figure 1** clarifies the timeline of the four periods of sampling and the time of the regulation implementation.

The sampling protocol was followed as previously described (9). Briefly, on each visit, fecal samples were collected from five pre-weaned calves and mixed to obtain a composite sample. Then fecal samples of five lactating cows were also collected and mixed to obtain another composite sample. On each farm, a convenience sample was assembled based on accessibility of the calves and cows. Fecal samples were obtained directly from the rectum for calves and freshly voided cow feces were obtained from the floor. A composite manure sample was also collected from two convenient locations in the manure pit. For each of these six composite samples, approximately 25 g of feces or manure were placed in a 50 mL sterile tube and stored immediately on ice at the farm. Samples were processed in the laboratory within <24 h. A preservative medium (peptone water with 30% glycerol) was added to the sample at a 1:1 volume-to-weight ratio; samples were then homogenized and frozen at −80°C.

Bacterial Isolation and *Escherichia coli* Identification

Generic Collection

To accurately compare the period pre and post regulation implementation, we used the same protocol for bacterial isolation as the one we used in the initial AMR prevalence study (9). Briefly, 1 g of each composite sample was mixed in phosphate buffer saline and then streaked on MacConkey plates and incubated overnight at 37°C. One lactose positive colony was chosen for each composite sample was subcultured on Columbia agar with 5% sheep blood (Oxoid, Canada), and incubated overnight at 37°C. The identification of isolates as *E. coli* was confirmed by MALDI-TOF MS using a Microflex LT instrument (Bruker Daltonics, Germany).

ESBL/AmpC Collection

To allow an accurate comparison between the period pre and post regulation implementation, we also used the same protocol for bacterial isolation from our initial AMR prevalence study (9). Briefly, composite fecal samples were processed according to the laboratory protocol of the European Union Reference Laboratory on Antimicrobial Resistance which allowed the recovery of ESBL-, AmpC- and carbapenemase-producing *E. coli* from composite fecal samples. The protocol is available online at https://www.eurl-ar.eu/protocols.aspx. Briefly, 1 g of each composite fecal or manure sample was added to 9 mL of Buffered Peptone Water, then incubated at 37°C for 20 h. One loop (10 μl) was streaked onto a MacConkey agar plate containing 1 mg mL^{-1} of cefotaxime, then incubated at 44°C for 20 h. Lactose positive colonies were subcultured on Columbia agar with 5% sheep blood, and then incubated overnight at 37°C. Identification of *E. coli* was confirmed by MALDI-TOF MS. Composite samples with at least one *E. coli* colony isolated with this technique were labeled as presumptive ESBL/AmpC *E. coli*.

All *E. coli* selected in both collections were incubated for 24 h at 37°C in Luria-Bertani (LB) broth then mixed 50:50 with 30% glycerol and stored at −80°C.

Antimicrobial Susceptibility Testing

The minimum inhibitory concentrations (MIC) against 20 antimicrobials representing 12 classes of antimicrobials were determined on all isolates belonging to both collections. The broth microdilution method was used with the commercially available panels (Sensititre CMV4AGNF and BOPO7F) (Thermo Fisher scientific, Canada) following manufacturer recommendations in accordance with the Clinical Laboratory Standards Institute standards (11). For each antimicrobial, dilution range, class, breakpoint, MIC and category classification are available in **Table 1**. Isolates were defined as susceptible, intermediate, or resistant with the same criteria as previously described (9). Briefly, we used the CLSI M100 (12) (*Enterobacteriaceae*: amoxicillin/clavulanate, azithromycin, ampicillin, cefoxitin, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, meropenem, nalidixic acid, sulfisoxazole, tetracycline and trimethoprim/sulfamethoxazole), CLSI VET08 (13) (ceftiofur, danofloxacin, enrofloxacin, and spectinomycin), or CIPARS (14) (streptomycin) clinical breakpoints. A MIC breakpoint was not available for neomycin, thus the epidemiological cut-off value from European Committee on Antimicrobial Susceptibility Testing (EUCAST) was used (MIC ≥16 μg mL^{-1} was defined as resistant). There were no valid florfenicol clinical breakpoints

TABLE 1 | Minimum inhibitory concentration for medically important antimicrobials, according to the WHO, of 509 *Escherichia coli* isolated in the generic collection from calf or cow feces or manure pit of 87 dairy farms in Québec, Canada.

Importance for human medicine†	Antimicrobial Class	Antimicrobial agent	MIC (μg/ml) *																SUSC	INTER	RESIST
			0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	%	%	%
Critical - Highest priority	Cephalosporin 3rd generation	Ceftiofur					25.6	67.8	3.3	0.4	0.4	2.5							97.1	0.4	2.5
		Ceftriaxone					96.3	0.8	0.2			1.1	0.4	0.4	0.8				97.3	0.0	2.7
	Quinolone	Ciprofloxacin	95.5	2.1	0.8	0.6	0.4	0.2	0.2		0.2								99.8	0.0	0.2
		Danofloxacin				97.8	1.0	0.2	1.0										98.8	0.2	1.0
		Enrofloxacin				97.8	1.0	0.4	0.2	0.6									98.8	0.6	0.6
		Nalidixic acid							12.4	65.8	19.6	0.6	0.6	1.0					99.0	0.0	1.0
	Macrolide	Azithromycin						0.4	2.1	15.7	67.2	12.6	0.8	1.2					98.8	0.0	1.2
Critical	Aminoglycoside	Gentamicin						0.2	97.2	0.8	0.2		1.6						98.4	0.0	1.6
		Neomycin									93.5	0.4	0.2	5.9					93.9	0.0	6.1
		Streptomycin								1.6	50.5	27.9	3.0	1.7	15.3				84.7	0.0	15.3
	Carbapenem	Meropenem			99.6	0.4													100.0	0.0	0.0
	Penicillin + β-lactamase inhibitors	Amoxicillin/clavulanic acid							3.1	13.1	62.9	18.1	0.2	2.6					97.2	0.2	2.6
	Aminopenicillin	Ampicillin						0.4	3.9	31.8	47.2	4.5	11.8	0.4					87.8	11.8	0.4
High	Cephamycin	Cefoxitin							1.0	16.9	68.2	10.8	0.4	2.7					96.9	0.4	2.7
	Folate pathway antagonist	Sulfisoxazole											37.7	31.2	10.4	1.8	18.9		100.0	0.0	0.0
		Trimethoprime/ sulfamethoxazole				0.4				99.0	0.6								99.4	0.0	0.6
	Phenicol	Chloramphenicol								1.4	20.8	63.1	3.1	11.6					85.3	3.1	11.6
		Florfenicol					0.2		0.2	5.5	55.8	38.3							NA**	NA**	NA**
	Tetracycline	Tetracycline									78.4	0.4	0.8	20.4					78.4	0.4	21.2
Important	Aminocyclitol	Spectinomycin										4.7	75.3	10.8	9.2				90.8	9.2	0.0

**Numbers indicate percentages of isolates. White areas are concentrations of antimicrobials tested by the broth microdilution method. Dashed and plain lines represent threshold used to define intermediate and resistant clinical breakpoints, respectively. † Importance of antimicrobials according to World Health Organization. **Florfenicol has no valid clinical breakpoints for Enterobacteriaceae and the concentration of 0.25 to 4μg mL−1 did not include the European epidemiological breakpoint of 16 μg mL−1, thus no interpretation could be given.*

for *Enterobacteriaceae* and the tested concentrations (0.25–4 μg mL^{-1}) did not include the European epidemiological cut-off of 16 μg mL^{-1}, therefore no interpretation was attempted. For subsequent analyses, intermediate and resistant isolates were grouped together and labeled as resistant. *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853 were used as reference strains for batch controls. *Escherichia coli* ATCC 25922 was used as a daily control.

For statistical analyses, intermediate and resistant isolates were combined and designated as resistant. Multidrug resistance (MDR) was defined as acquired resistance to at least one agent in three or more antimicrobial classes and extensively drug resistance was defined as resistant to at least 1 agent in all but 2 or fewer antimicrobial classes (15) as previously defined (9).

In the ESBL/AmpC collection we determined the ESBL/AmpC phenotype based on the results of the MIC. An isolate was called "ESBL" if it was resistant to ceftriaxone or ceftiofur, susceptible to meropenem, susceptible to cefoxitin and susceptible to amoxicillin/clavulanic acid. An isolate was called "AmpC" if it was resistant to ceftriaxone or ceftiofur, susceptible to meropenem, resistant to cefoxitin and resistant to amoxicillin/clavulanic acid. An isolate was called "ESBL/AmpC" if it was resistant to ceftriaxone or ceftiofur, susceptible to meropenem, resistant to cefoxitin and susceptible to amoxicillin/clavulanic acid. An isolate was called "other phenotype" if it was resistant to ceftriaxone or ceftiofur, susceptible to meropenem, susceptible to cefoxitin and resistant to amoxicillin/clavulanic acid or if it was susceptible to ceftriaxone and ceftiofur, susceptible to meropenem, resistant or susceptible to cefoxitin and resistant to amoxicillin/clavulanic acid.

Antimicrobial Genotyping

Whole genome sequencing (WGS) was used on a subset of isolates of the generic collection to determine the genetic basis of the observed AMR. Due to financial and logistic restrictions, we sequenced 15 isolates in total. The selection was based on relevant phenotypes with the following criteria: isolates resistant to 8 or more antimicrobials classes (aminoglycosides and aminocyclitols were considered two different classes for this selection) ($n = 4$), isolates identified as harboring an ESBL ($n = 4$) or an AmpC ($n = 6$) phenotype, and an isolate resistant to danofloxacin and enrofloxacin. Briefly, genomic DNA was extracted using QIAamp DNA Mini Kit for DNA following manufacturer's guidelines (Qiagen, Hilden, Germany). We performed WGS on the Illumina (San Diego, CA) iSeq100 platform with 2 × 150 paired end runs after library preparation with the Illumina DNA prep kit (former Nextera Flex kit), according to the manufacturer's instructions. Illumina platform was used to assemble genomes using SPADES 3.9.0. An assembly was rejected if the number of contigs (>500 pb) was >400 or if the N50 was <50,000. Details of data assembly quality are available in **Supplementary Table S1**. To search AMR genes and point mutations, Res Finder 4.0 (16) and Point Finder (17)

bioinformatics tools from the Center of Genomic Epidemiology (CGE) platform (http://www.genomicepidemiology.org/) were used. To complete the analysis, we also used the CARDS database (18). Multi locus sequence typing (MLST) (19), O and H serotype (20) and core genome MLST (cgMLST) (21) were determined by the analysis of generated FASTA files using the Center of Genomic Epidemiology (CGE) platform (http://www.genomicepidemiology.org/ accessed on 11/15/2021). Phylogroups were determined with in-silico PCR using the Clermont Typing platform (http://clermontyping.iame-research.center/ accessed on 11/15/2021) (22).

Statistical Analysis

For all statistical analyses, the unit of analysis was the composite sample obtained from different origins (calves, cows, or manure pit), time periods (pre and post regulation), seasons (fall 1 and 2 or spring 1 and 2 visits), and herds. Each sample was represented by one *E. coli* isolate. We also conducted herd-period level analyses. For these latter analyses, if one of the 6 isolates obtained in each herd (pre or post regulation) was found positive for an outcome, the herd was considered positive for this outcome during that period.

Effect of Regulation Implementation on Antimicrobial Resistance

In the generic collection, we investigated whether the probability of resistance to a given antimicrobial or the probability for an isolate of being MDR differed between isolates obtained from the periods pre and post regulation. Because a season effect was detected in the previous study (9), we also compared the pre and post regulation periods by season (spring pre *vs.* spring post regulation and fall pre *vs.* fall post regulation). In the ESBL/AmpC collection, we investigated whether the probability of a sample to harbor a putative ESBL/AmpC *E. coli* differed between isolates obtained from the same periods. As calves demonstrated the greatest risk to present AMR in the previous study (9), we calculated the probabilities for each outcome by sample type (calves, cows, or the manure pit) and at the herd level. For all these analyses, we used a logistic regression model with susceptibility *vs.* resistance to a given antimicrobial or the MDR status or the growth of an *E. coli* on the cefotaxime plate as outcome variable. Sample type and periods (pre *vs.* post regulation) were used as fixed predictors and the model was considered to be a generalized mixed model in which a herd random effect was included to account for clustering of samples or isolates within herds (SAS, PROC GLIMMIX. Cary, NC, US). Tukey-Kramer adjustment was used to adjust for multiple comparisons. An alpha of 0.05 was chosen to define statistically significant results.

Effect of Regulation Implementation on Numbers of Antimicrobials to Which an Isolate Was Resistant to and on the ESBL Profile of Isolates From the ESBL/AmpC Collection

A generalized linear mixed model (SAS, PROC GLIMMIX, Cary, NC, US) was used to investigate whether the regulation implementation could influence the number of antimicrobials to which an isolate was resistant to and the ESBL profile of isolates from the ESBL/AmpC collection. In this model, a negative binomial distribution with a log link was used. The outcome was the number of antimicrobial classes to which an isolate was defined as resistant (0 to 10) or the ESBL profile (1–4). The predictor was either the origin of the samples (calves, cows, or manure pits) or the period (pre vs. post regulation implementation) and a random herd intercept was included to account for clustering of isolates by herd. A Tukey-Kramer test was applied to adjust for multiple comparisons and an alpha of 0.05 was used.

RESULTS

Selection of Herds and Sample Collection

Eighty-seven of the 101 farms accepted to participate in the post regulation set of sampling. Descriptive data for all samplings (pre and post regulation) on the 87 farms are available in **Table 2**.

For the sampling post regulation, manure pits were emptied approximately 4 months before the first visit. During the second visit, all manure pits were frozen, therefore we sampled the end of the drainpipe or the gutter. In this sampling, among the 516 fecal composite samples obtained, we recovered 509 *E. coli* isolates, in the generic collection. Indeed, 7 samples from manure pits, sampled in fall 2020, did not yield any lactose positive colonies. We also recovered 162 putative ESBL/AmpC *E. coli* in this putative ESBL/AmpC collection.

AMR Situation Approximately 2 Years After the Regulation Implementation

Generic Collection

Most isolates (69%) were pan susceptible as presented in **Table 1** and **Figure 2**. No resistance to meropenem (carbapenem class) was detected, although resistance to each of the other antimicrobials were observed at least once. Two isolates were considered extensively resistant (**Figure 2**). They were both identified in calves, however, not during the same period and not in the same farm.

As shown in **Figure 3D**, the highest levels of resistance, in herds, were toward tetracycline (76%), sulfisoxazole (70%), and streptomycin (63%). The most common AMR patterns were tetracycline (3.7%) and chloramphenicol (2.9%). The most prevalent MDR pattern was tetracycline-sulfisoxazole-streptomycin (1.6%). The most frequently observed resistance genes in tested isolates, identified with the WGS ($n = 15$), were responsible for tetracycline [*tet(A), tet(B)]*, sulfisozaxole (*sul1, sul2)*, and streptomycin *aph(6)-Id, aph(3')-Ia, aph(3")-Ib, aadA1, aadA2*. The AmpC phenotype was associated mainly with $bla_{CMY-2}(n = 6/8)$. A mutation in the promoter of the AmpC gene was responsible for the AmpC phenotype in the remaining isolates ($n = 2/8$). The ESBL phenotype was associated with $bla_{CTX-M-55}$ *(n = 3/4)* and $bla_{CTX-M-124}$ ($n = 1/4$). The bla_{EC} family was detected with CARDS in 7/15 isolates [bla_{EC-13} ($n = 2$), $_{14}$ ($n = 1$), $_{15}$ ($n = 2$), $_{18}$ ($n = 1$) and $_{19}$ ($n = 1$)]. All genes identified in the sequenced isolates are reported in **Figure 4**. There was a 100% correlation between phenotype resistance and associated resistance genes for all antimicrobials, except for

TABLE 2 | Descriptive statistics of cattle sampled in 87 farms dairy farms in Québec, Canada, per period of sampling (pre and post regulation).

	Calf					Cow				
	Number	**Mean age**	**Median age**	**Youngest (in days)**	**Oldest (in days)**	**Number**	**Mean lactation**	**Median lactation**	**Min lactation**	**Max lactation**
Fall pre regulation	339	27	21	1	150	435	2.6	2	1	10
Spring pre regulation	274	29	25	1	100	434	2.4	2	1	9
Fall post regulation	350	31	28	1	170	435	2.6	2	1	8
Spring post regulation	337	28	24	1	120	435	2.7	2	1	9

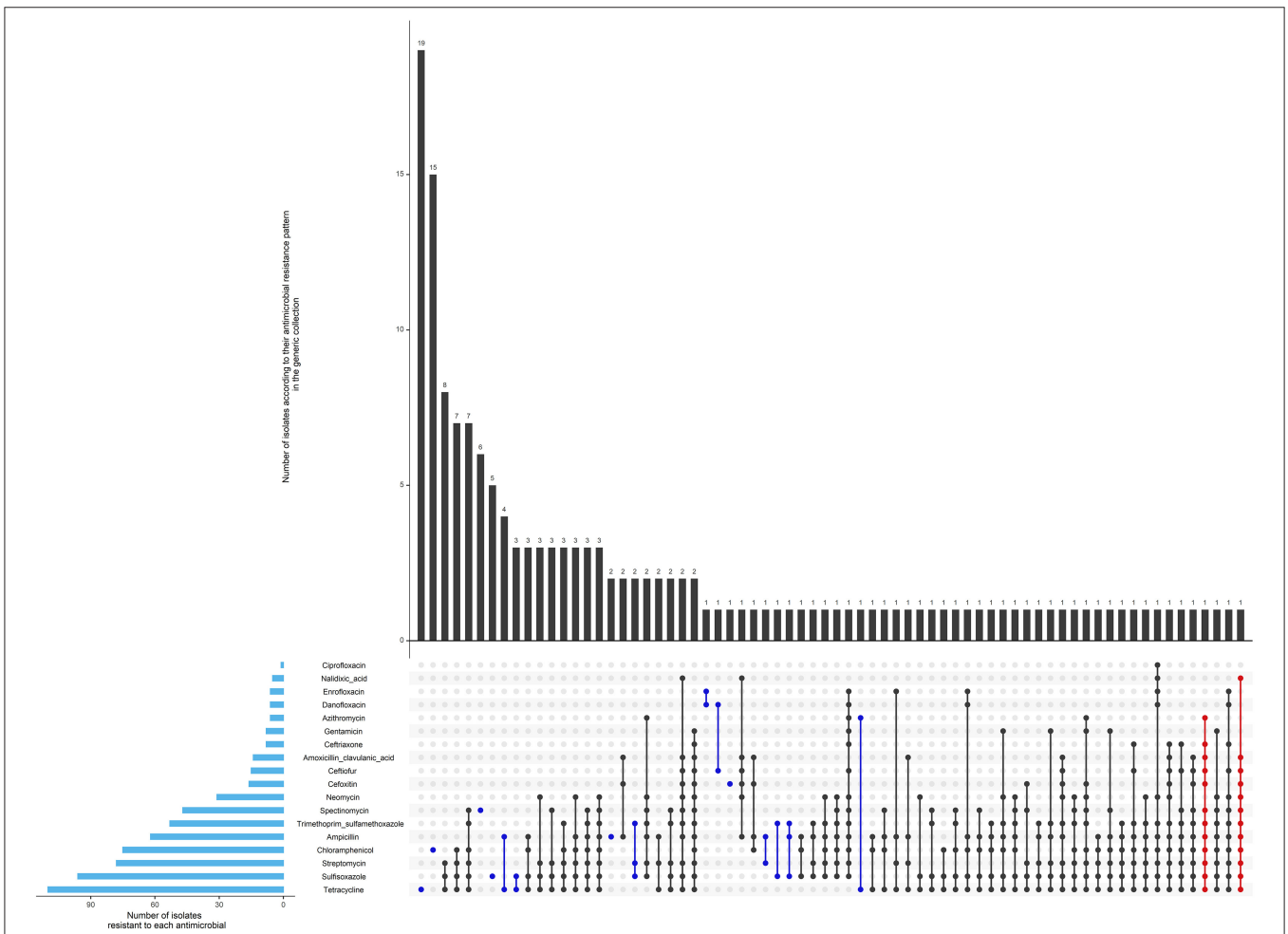

FIGURE 2 | Antimicrobial resistance pattern of 509 isolates of the generic collection from calf, cow feces or manure pit of 87 dairy farms in Québec, Canada in 2020–2021. The horizontal blue bars represent the frequency of isolates resistant to each antimicrobial. An antimicrobial pattern is represented by the linked dots. Black dots represent the MDR patterns, and red dots represent the XDR patterns. The vertical bars represent the frequency of isolates for each antimicrobial pattern. 352 (69%) isolates were susceptible to all antimicrobials (not represented in the figure).

azithromycin and danofloxacin with correlation of 80 and 93%, respectively. We also identified genes responsible for resistance to disinfectant (*qacE* or *sitABCD* or both) in 10/15 isolates that were sequenced.

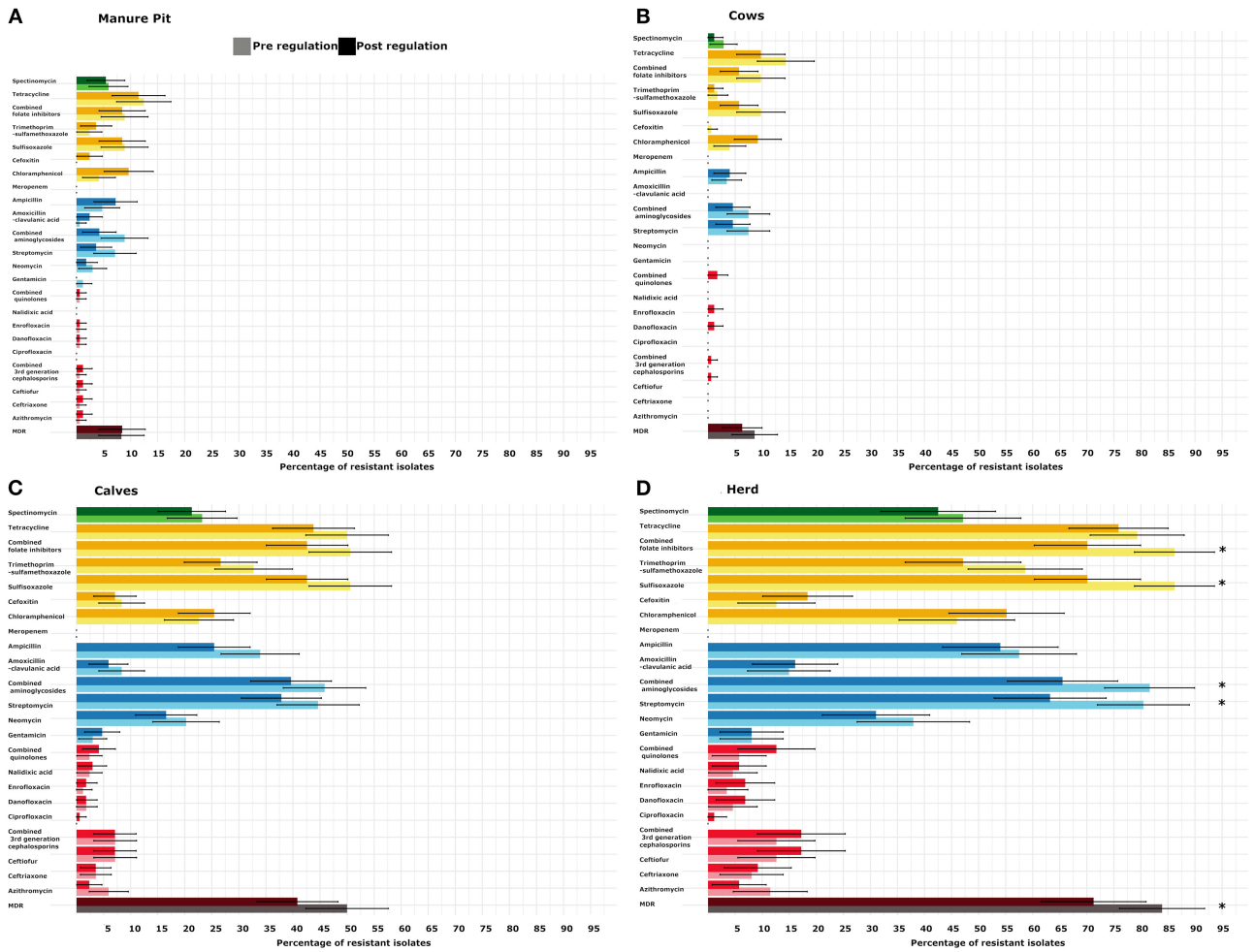

FIGURE 3 | Comparison of the proportion of isolates with at least one resistant (intermediate and resistant combined) *Escherichia coli* per period [pre- (light) and post-regulation (dark)] and sample type [manure pit **(A)**, cows **(B)**, calves **(C)**] or for the whole herd **(D)** from 87 dairy farms from Québec, Canada. On each farm, between 4 and 6 *E. coli* were tested for each antimicrobial. In dark and light burgundy, MDR: multidrug resistant (resistant to 3 classes of antimicrobial or more). In dark and light red, critical high priority antimicrobials for human medicine. In dark and light blue, critical priority antimicrobials for human medicine. In dark and light yellow, high priority antimicrobials for human medicine. In dark and light green: antimicrobials important for human medicine, SPE: spectinomycin. The importance of antimicrobial for human medicine was defined according to World Health Organization.

As illustrated in **Figure 4**, sequenced isolates showed a diversity of phylogroups, MLST, serogroup and cgMLST. However, three pairs of isolates had the same phylogenetic characteristics (107412A and 106712A, 108511A and 102911A, 108112A and 106012A). Based on this analysis they could be considered as clones. They belong to different farms, and periods, although they were all recovered in calf samples. This suggests a possible clonal dissemination of the most resistant isolates in the calf's population. Their replicon profile, illustrated in **Supplementary Figure S1**, was nevertheless not identical, thus explaining their differences in resistance profile. On the other hand, the replicon that we identified among other isolates, were often similar, with an omnipresence of the replicon IncFIB. IncFIA and IncFII were present in, respectively, 7/15 and 13/15 isolates. Although, we could not circularize the plasmid sequenced and, therefore, we could not assign one gene to one plasmid, these data suggest that, in this *E. coli* population, AMR genes were also spread through horizontal transfer.

ESBL/AmpC Collection

As shown in **Figure 5**, 82% (71/87) of herds were positive for ESBL/AmpC-producing *E. coli* in at least one sample during the post regulation period. According to our definitions 39% (63/161) of isolates had an ESBL profile, 48% (77/162) had an AmpC profile, 2% had an ESBL/AmpC profile and 11% (17/161) isolates had a profile designated as "other." Only 6 isolates were not MDR, and 6 isolates were extensively resistant (**Figure 6**).

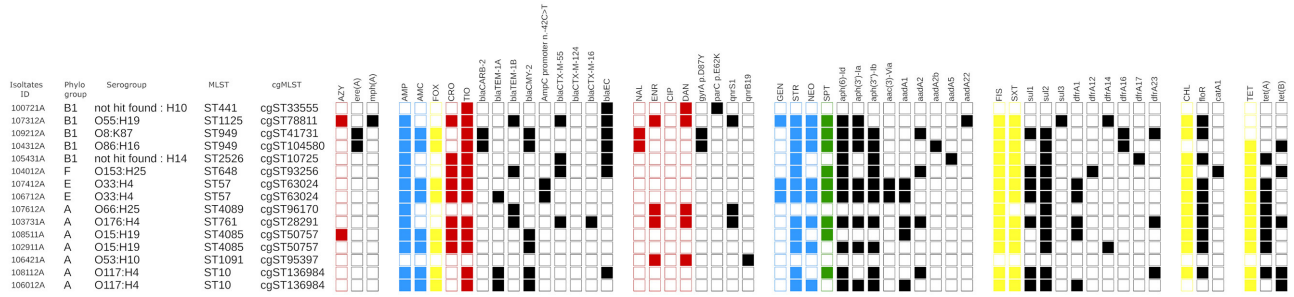

FIGURE 4 | Phylogenic, phenotypic, and genotypic characteristic of 15 isolates from the generic collection from 87 dairy farms from Québec, Canada in 2020–2021 determined by whole genome sequencing. In red, critical high priority antimicrobials for human medicine, AZM, azithromycin; CRO, ceftriaxone; TIO, ceftiofur; CIP, ciprofloxacin; DAN, danofloxacin; ENR, enrofloxacin; NAL, nalidixic acid; in blue, critical priority antimicrobials for human medicine; GEN, gentamicin; NEO, neomycin; STR, streptomycin; AMC, amoxicillin-clavulanic acid; AMP, ampicillin; MEM, meropenem. In yellow, high priority antimicrobials for human medicine, CHL, chloramphenicol; FOX, cefoxitin; FIS, sulfisoxazole; SXT, trimethoprim-sulfamethoxazole, FOL, combined folate inhibitors; TET, tetracycline; in green, antimicrobials important for human medicine, SPT, spectinomycin. The importance of antimicrobial for human medicine was defined according to World Health Organization. The presence of a characteristic is noted by a full square. In the bla_{EC} family we recovered bla_{EC-13} ($n = 2$), $_{14}$ ($n = 1$), $_{15}$ ($n = 2$), $_{18}$ ($n = 1$) and $_{19}$ ($n = 1$).

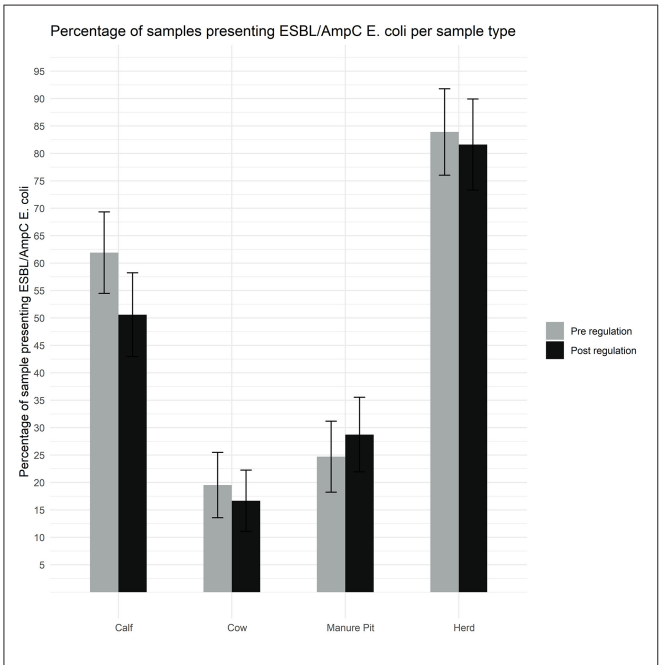

FIGURE 5 | Comparison of the proportion of isolates with a putative ESBL/AmpC *Escherichia coli* per sample type (manure pit, cows, calves, herd) from 87 dairy farms from Québec, Canada, pre (gray) and post (black) regulation implementation.

Impact of Regulation on the AMR Situation

For statistical comparison we excluded farms that were sampled only pre regulation, and therefore used 87 farms. In the generic collection, 511 and 509 *E. coli* isolates were available for the pre and post regulation period, respectively. In the ESBL/AmpC collection 181 and 162 *E. coli* isolates were available for the pre and post regulation period, respectively.

Generic Collection

As shown in **Figures 3A–C**, there were no statistical difference in the proportion of resistant isolates for antimicrobials tested between the pre and post regulation periods for the samples originating from manure pits, cows, or calves. However, at the herd level, the recovery percentage of MDR *E. coli* was statistically lower post regulation implementation (2.2 times lower odds; 95% CI (1.4–3.3); $p = 0.05$; **Table 3**). The recovery percentages of isolates positive for resistance to streptomycin and sulfisoxasole were also lower at the herd-level (odds ratio and *p*-value are available in **Table 3**).

Comparisons between pre *vs.* post regulation spring samples and pre *vs.* post regulation fall samples were also performed (**Supplementary Figures S2, S3**). No significant difference was observed between spring samples. However, the recovery percentages of herds positive for the presence of an MDR isolate and the resistance to streptomycin and sulfisoxasole and trimethoprim-sulfisoxasole were lower in post compared to pre regulation fall samples (odds ratio and *p*-value are presented in **Table 3**). Moreover, the recovery percentage of MDR isolates in calf samples and the resistance to sulfisoxasole were lower in the post *vs.* pre regulation fall samples (odds ratio and *p*-value are presented in **Table 3**).

The repartition of isolates resistant to a given number of classes of antimicrobials is presented in **Figure 7**. An isolate originating from a calf sample was, on average, resistant to 2.5 (CI95% 1.9–3.4) classes of antimicrobials before and 2.2 (CI95% 1.6–3.0) classes of antimicrobials after the regulation. There was no statistical difference between sample origin or between pre and post regulation periods in terms of number of resistances per isolate. Moreover, the genetic resistance profiles did not seem to have changed between the pre and post regulation periods.

ESBL/AmpC Collection

As illustrated in **Figure 5**, there was no statistical difference between the pre and post regulation period for the samples

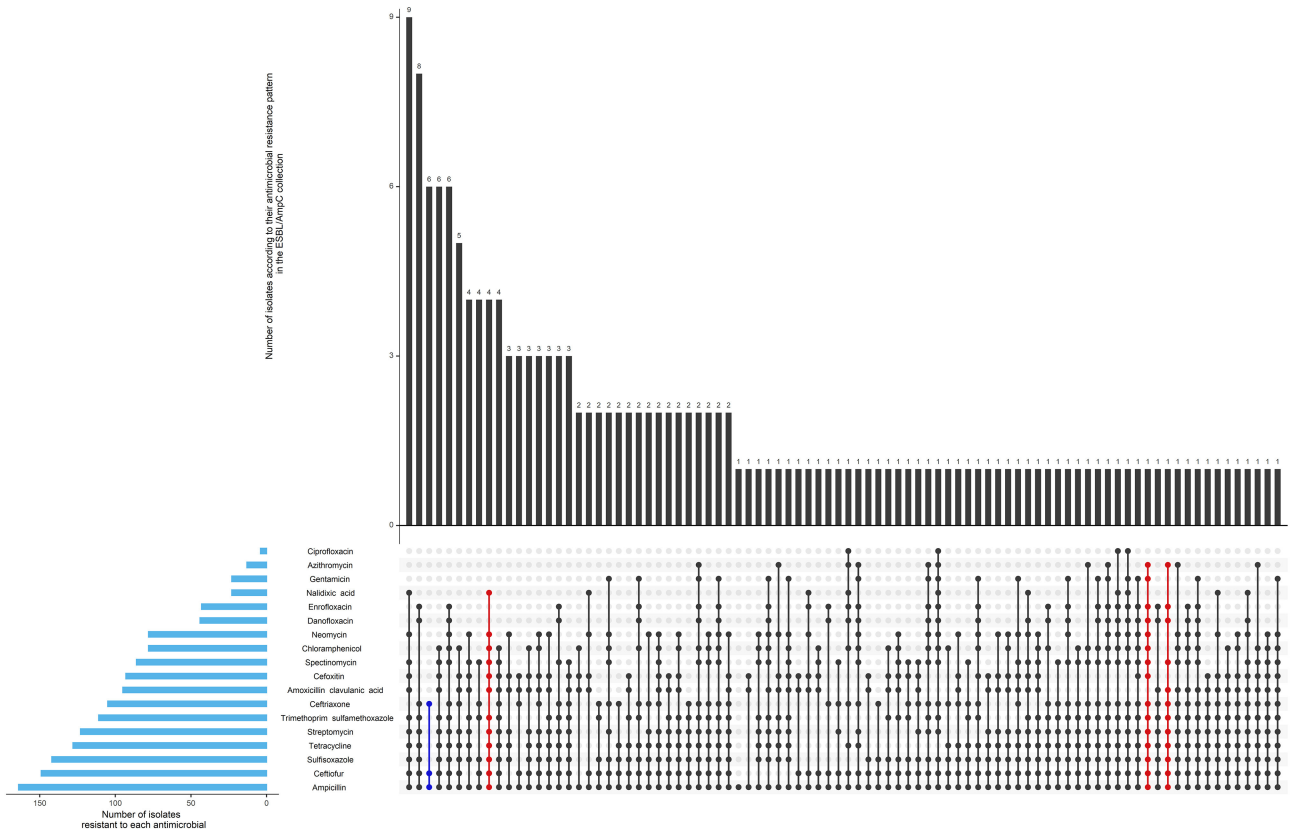

FIGURE 6 | Antimicrobial resistance pattern of 162 isolates of the ESBL/AmpC collection from calf, cow feces or manure pit of 87 dairy farms in Québec, Canada in 2020–2021. The horizontal blue bars represent the frequency of isolates resistant to each antimicrobial. An antimicrobial pattern is represented by the linked dots. Black dots represent the MDR patterns, and red dots represent the XDR patterns. The vertical bars represent the frequency of isolates for each antimicrobial pattern.

TABLE 3 | Parameter estimates and odds ratio from logistic regression models, for various outcomes, and using either the sample (feces from calves, feces from cows, manure pit sample) or herd as unit of analysis, based on the results of a cross-sectional study performed on 87 farms sampled in Québec, Canada between 2017 and 2021.

Outcome	Period compared	Sample type	Odd ratio	95% CI	P-value
MDR	Pre vs. post regulation	Herd	2.2	1.5–3.3	0.05
Streptomycin	Pre vs. post regulation	Herd	2.4	1.7–3.4	0.01
Sulfizoxasole	Pre vs. post regulation	Herd	2.9	1.9–4.6	0.01
MDR	Fall pre vs. fall post regulation	Calves	3.0	2.1–4.3	0.03
Sulfizoxasole	Fall pre vs. fall post regulation	Calves	2.8	2.0–3.9	0.04
MDR	Fall pre vs. fall post regulation	Herd	2.4	1.6–3.4	0.02
Streptomycin	Fall pre vs. fall post regulation	Herd	2.5	1.7–3.5	0.01
Sulfizoxasole	Fall pre vs. fall post regulation	Herd	2.1	1.6–3.0	0.02
Trimethoprim-sulfamethoxazole	Fall pre vs. fall post regulation	Herd	2.1	1.4–3.0	0.05

originating from manure pit, cows, calves, or for the herd in general, or per season, for the presence of a putative ESBL/AmpC *E. coli* (**Supplementary Figure S4**).

There was also no statistically significant difference in ESBL profile between pre and post regulation period, neither by sample type (**Figure 8**) nor by season (data not shown).

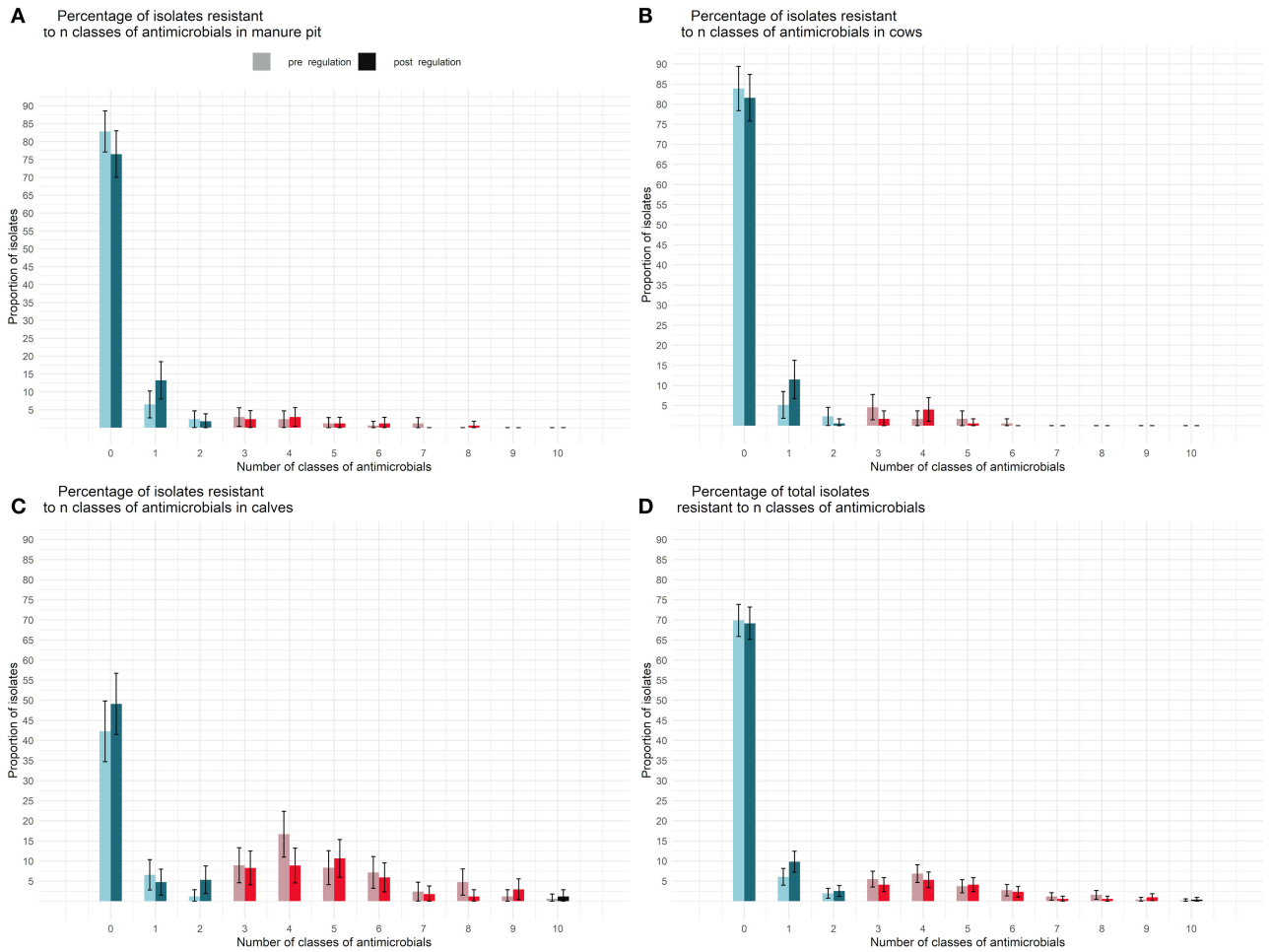

FIGURE 7 | Comparison of the percentage of *E. coli* isolates resistant to n classes of antimicrobial per sample type [manure pit **(A)**, cows **(B)**, calves **(C)**, herd **(D)**] from 87 dairy farms from Québec, Canada, pre (light) and post (dark) regulation implementation. On each farm, between 4 and 6 *E. coli* were tested for each antimicrobial. In dark and light blue, isolates are non MDR. In dark and light red isolates are MDR. In gray and black isolates are extensively resistant.

DISCUSSION

The main objective of this study was to establish the AMR situation in dairy cattle in Québec approximately 2 years after the implementation of a regulation limiting the use of category 1 antimicrobials according to Health Canada classification, and to compare this AMR situation to that of the period preceding the regulation implementation (9).

To the author's knowledge, Québec is a pioneer in Canada, regarding the implementation of a regulation restricting category 1 antimicrobial usage in production animals in February 2019. This study exploring the impact of such regulation on AMR in a Canadian context is also unique. Indeed, our research team was well-positioned to compare the AMR situation in dairy cattle in Québec post regulation implementation, as we collected data on AMR for a previous study in 2017, prior to the implementation of the regulation (9). The results of the present study demonstrate that the most significant decrease in the generic collection was for resistance to folate inhibitors and to aminoglycosides which led, consequently, to a decrease in MDR. On the other hand, we did not observe a significant decrease in resistance to any of the category 1 antimicrobials in the generic collection, nor in the ESBL/AmpC collection. These results are difficult to compare with any other previously conducted studies. Indeed, in 2016, several European countries, such as France and Belgium, had already banned the prophylactic use in animals of antimicrobials critical for public health, and the use of critical antimicrobial in production animal with some exceptions (such as emergency or if the veterinarian has proven with an antimicrobial susceptibility test that there is no other alternatives) (23, 24). In the Resapath annual report 2019 (25), which describes annually antimicrobial susceptibilities in animal pathogens from samples send to diagnostic laboratories in France, it seems that, in cattle, AMR toward cephalosporin and quinolones are decreasing. However, this tendency started in 2015 (prior to the ban), and statistical analyses were not performed on these data. Thus, it is difficult to attribute this impact to the regulation implementation vs. to the whole Ecoantibio plan (26). Indeed, this plan also involves the

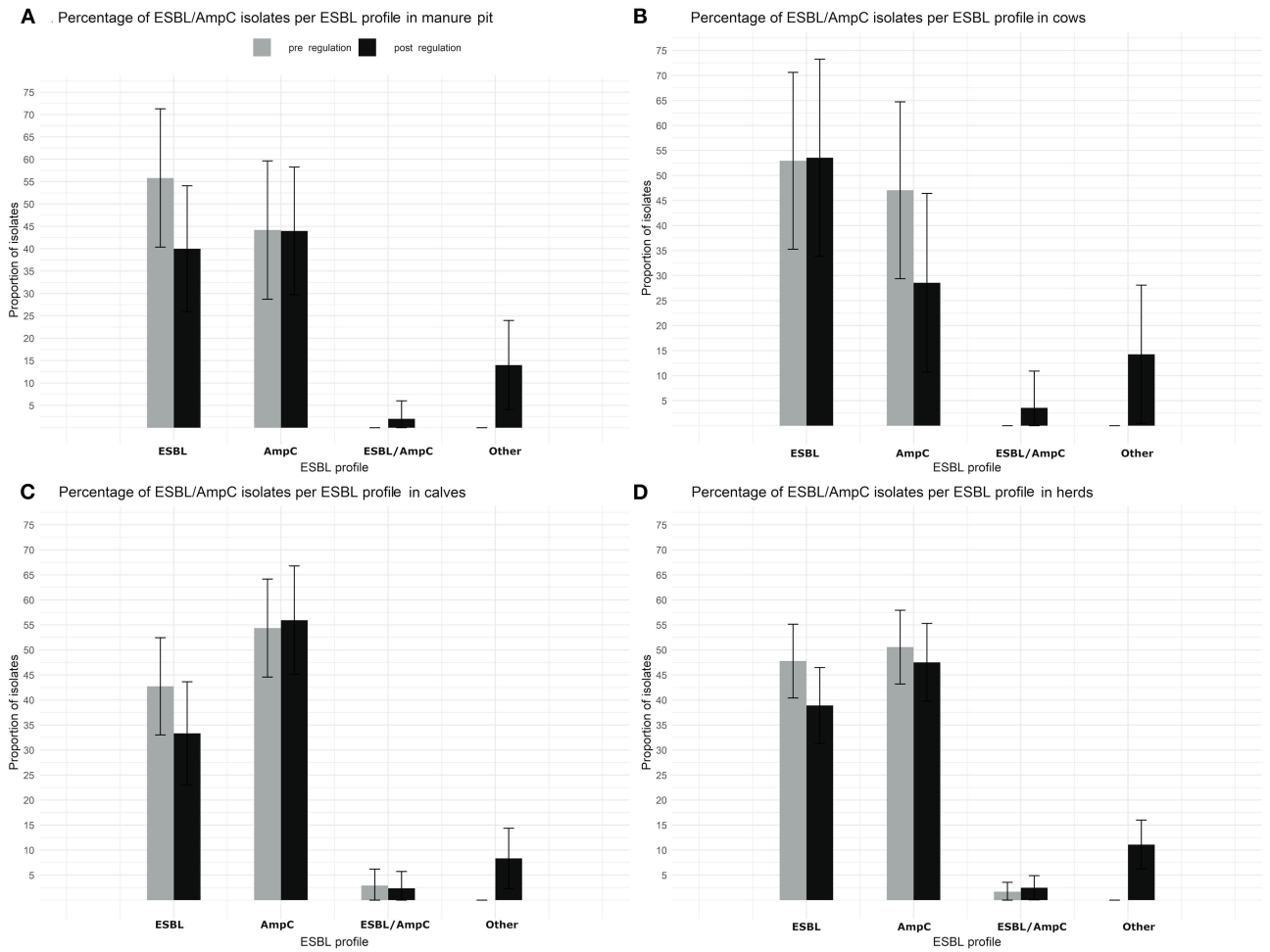

FIGURE 8 | Comparison of the proportion of isolates with a putative ESBL/AmpC *Escherichia coli* per ESBL profile and per sample type (manure pit, cows, calves, herd) from 87 dairy farms from Québec, Canada, pre (gray) and post (black) regulation implementation. **(A)** Percentage of ESBL/AmpC isolates per ESBL profil in manure Pit. **(B)** Percentage of ESBL/AmpC isolates per ESBL profil in cows. **(C)** Percentage of ESBL/AmpC isolates per ESBL profil in calves. **(D)** Percentage of ESBL/AmpC isolates per ESBL profil in herds.

prevention of infectious diseases, the communication on AMR fight and the provision of tools to follow up on AMU. We found no studies assessing AMR post implementation in Belgium.

The lack of significant decrease in resistance to category 1 antimicrobials was to be expected for several reasons. First, the resistance to these antimicrobials was not very high in the first place. Second, category 1 antimicrobials are used mainly intramammary in bovine. Therefore, the impact of the regulation on the fecal microbiota might be low. Third, we recruited 87 farms to participate in the second study, therefore we might have been lacking power to detect a decrease in AMR. Fourth, although it is of great importance to have a thorough follow up of the situation, the time between the regulation implementation and the second period of sampling (∼2 years) could be considered very short to capture a change in the AMR situation. There are very few data concerning the carriage duration for antimicrobial genes in cattle, as it is a complex question depending on the characteristics observed (genotype vs. phenotype), the variety of AMR, the mechanism of spread, the presence of co-selection, the selective pressure in the environment and even the microbiota of the individual animal (27). However, in humans the mean time to lose carriership of ESBL *E. coli* was determined to be 1.1 years (28). On the other hand, CTX-M ESBL-producing *E. coli* have been demonstrated to persist in fecal samples of calves for 69 weeks, specifically through the persistence of certain clonal lineages (29). A long-term AMR follow up is already planned, as a sentinel group of 30 dairy farms from Quebec (most of them were part of our study) was recently added to the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) program of the Public Health Agency of Canada (PHAC). Another explanation for the lack of significant decrease in resistance to category 1 antimicrobials may have been the context. Indeed, the COVID-19 pandemic started in February 2020 in Québec and has resulted in many supply difficulties, particularly for veterinary drugs. Consequently, several products were not available during this

period, especially some products containing category 2 or 3 antimicrobials. Therefore, during certain periods, dairy farmers and veterinarians had no other alternative than to use a category 1 antimicrobial. On the other hand, a decrease of 80% of category 1 antimicrobial usage in the dairy industry in Québec was reported (10) during the same period. The impact of the regulation on category 1 usage could have been higher if not hampered by these logistic considerations.

The seasonal effect is interesting to note. Indeed, we found no significant decrease of AMR between the spring 2017 and the spring 2021. In general, the levels of AMR observed during the spring seasons were low, thus limiting the statistical power of the study. On the other hand, there was a significant decrease in resistance to folate inhibitors and aminoglycosides both at the herd level and for the calf samples between the fall of 2017 and the one of 2020. It is well-described that horizontal gene transfer, and therefore genome mobility, increases with the temperature (30, 31). It is possible that during the fall season (samples gathered during the early fall reflect what happened during the summer), resistance genes are more susceptible to antimicrobial pressure (even if there is no reported difference in the AMU between season). Indeed, as genome plasticity increases, the variation (gain or loss of genes) of AMR genes prevalence might as well-increases.

The decrease of AMR was greater in calf samples. Even if the mechanisms are not completely elucidated, it is well-recognized that calves are more at risk to shed AMR bacteria than adults (32). Therefore, statistically, the decrease (if any) was more likely to be significant in the calf group. Given that for cows and manure pits, distribution of resistances was low, the study power was limited for these specific samples. The sampling post regulation in spring was performed early in the year. Therefore, we were not able to sample the manure pits as they were still frozen, and we sampled the end of the drainpipe. It has been reported that fresh manure samples have a higher alpha and beta diversity than manure pits (33). However, the same study demonstrated that there were no significant differences in AMR genes abundance or diversity between fresh manure and the manure pit samples. These results are supported by the fact that we did not find any significant differences between manure pit samples at any time in our studies and confirms that our comparisons between periods and sample types are reliable.

The decrease in folate inhibitor and aminoglycoside resistance is somewhat surprising because the regulation did not concern these categories of antimicrobials. The folate inhibitor and aminoglycoside resistance genes are often found on plasmids (34, 35). Therefore, due to co-resistances (other resistance genes present on the same plasmids), modification of AMU can have indirect effect on AMR. Moreover, some genes can be responsible for resistance to several antimicrobial classes. Therefore, the restriction of a specific class of antimicrobial can have repercussions on resistance to other antimicrobial classes. Furthermore, the regulation in Québec (as in other European countries) was accompanied by several other measures that probably contributed to the decrease of AMR. In particular, the veterinarians had access to complete training on the judicious usage of antimicrobials by several members of our research team (JPR, SD, DF, MA) in 2018–2019. Field veterinarians that followed this training then supervised dairy producer training. Consequently, the entire dairy industry had access to complete information on better usage of antimicrobials. It is very likely that this training had a role in the decrease we observed between the two periods. The discontinuation of the sale of a very popular intramammary formulation containing dihydrostreptomycin at the end of 2020 might also explain the decrease in resistance to streptomycin. Indeed, it was the only available product containing streptomycin and labeled for use in the bovine in Québec. However, even if its use was very prevalent (8), it is unlikely to be the only reason for this decrease as it was applied via the intramammary route, thus targeting a relatively narrow compartment with a relatively light density of microorganisms.

The frequency of *E. coli* producing ESBL/AmpC was much higher in the ESBL/AmpC than in the indicator collection. This was to be expected as healthy animals shed ESBL/AmpC isolates in small quantities (36). This data demonstrates the importance of improving detection sensitivity using enrichment with cefotaxime to allow more accurate estimation of the proportion of positive farms. Based on our results, $bla_{CTX-M-55}$ and bla_{CMY-2} seem to predominate and be linked, respectively to ESBL and AmpC phenotypes. Analysis of the fecal metagenome, to be able to quantify the genes present in the sample would be a good way to detect any decrease in resistance gene burden. The bla_{EC}gene family was not detected in the previous study because they were not included in the Resfinder database. This family of genes are class C beta-lactamases and are found in *E. coli.* They are not well-documented and not often reported. However, they have been observed in various environments such as in samples collected from human and cattle in Alberta and associated with β-lactam resistance (37); and in Gambia in non-human primates (38) where the phenotype was not documented. In one of our isolates, the presence of bla_{EC-14} seems the best explanation for the resistance to ceftiofur. It is also noticeable that the *qnrS* family genes, responsible for plasmidic resistance to fluoroquinolones (39), were not detected in the generic collection in the previous study (9), but were detected in this study. It might be a random finding, but the *qnrS* family genes should be monitored further in the next years. Indeed, even though they are usually known to be associated with a low degree of resistance (39), in our study 3 isolates presented clinically significant resistance to enrofloxacin and ciprofloxacin with no known mutation of the *parC* or *gyrA* genes. The presence of the *qnrS* genes was the only fluoroquinolone resistance determinant we detected.

The investigation on AMR gene dissemination mechanisms is essential because it helps in the fight to tackle AMR. It is often hypothesized that the relief of antimicrobial pressure will result in the loss of AMR genes, as some experiments in the 1970's showed (40). However, the reality is far more complex. Indeed, some genes carried by plasmids may impose little pressure on host strains and might be preserved even if the antimicrobial pressure is relieved (41). Other plasmids might carry resistance to other antimicrobials or even to disinfectant or heavy metals which would allow the plasmid to stay in the bacteria even if the antimicrobial pressure is removed. Therefore, in case of

plasmid carriage, other methods to diminish plasmid stability are needed to tackle persistence of AMR genes. According to our results, the dissemination mechanisms of resistance genes are a combination of clonal spread and horizontal gene transfer. First, three pairs of isolates (chosen among the most resistant in the generic collection) had the same phylogenetic characteristics (107412A and 106712A, 108511A and 102911A, 108112A and 106012A). The definition of a clone remains a challenge and depends on the method used to characterize the isolates (42). Based on cgMLST, which is a very discriminant method (21), they could be considered as clones. As they belong to different farms and periods, these data strongly suggest a clonal dissemination of the most resistant *E. coli* isolates in the dairy population in Québec. All these clones were identified in calf samples suggesting that calves are more susceptible to harbor MDR clones, as other studies have already proposed (29). The putative vectors for clonal dissemination are likely physical, such as transporters, material lending between farms, veterinarians, etc. The mechanisms by which calves harbor MDR clones are not clear but could be associated with greater levels of AMR such as microbiota immaturity and increased contact between individuals (32). It is very interesting to note that their replicon profiles and their resistance pattern (**Figure 4** and **Supplementary Figure S1**) are not identical, indicating that, in addition to this clonal dissemination, plasmids are also spreading resistance genes. Many of the replicons identified in this study belong to plasmid families known to carry AMR genes, and to be epidemic plasmids (43). However, our method of sequencing did not allow us to study the plasmids in greater depth.

In conclusion, ∼2 years after the regulation to limit the use of category 1 antimicrobials was implemented in the province of Québec, Canada in production animals, the proportion of MDR *E. coli* isolates decreased significantly in the dairy industry specifically due to a decrease in resistance to folate inhibitors and aminoglycosides. It is likely that the regulation and all other measures implemented to improve judicious use of antimicrobials played a role in this decrease. This study highlights the importance of monitoring the impact of such regulation to adjust restrictions and maximize their effectiveness. Also, the elucidation of AMR gene dissemination mechanisms is essential strengthen the fight to tackle AMR.

DATA AVAILABILITY STATEMENT

The whole genome sequences presented in this study can be found in online repositories. The names of the repository and accession number can be found at: https://www.ncbi.nlm.nih.gov/bioproject/ PRJNA783194 /.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Use Ethics and the Research Ethics Committees of the Université de Montréal (20-Rech-2085). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

MA, JF, DF, CA, SD, M-ÈP, JM, and J-PR conceived research project and designed experiments. ML, HL, and J-PR coordinated sample collection. ML conducted experiments. ML, SD, JF, and J-PR contributed to data analysis and interpretation. ML wrote the draft manuscript. All authors critically revised it and therefore contributed to the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.838498/full#supplementary-material

Supplementary Figure S1 | Replicon profile identified by whole genome sequencing of isolates of the generic collection ($n = 15$) from calf or cow feces or manure pit of 87 dairy farms in Québec, Canada in 2020–2021.

Supplementary Figure S2 | Comparison of the proportion of isolates with a least one resistant (intermediate and resistant combined) *Escherichia coli* per sample type [manure pit **(A)**, cows **(B)**, calves **(C)**, herd **(D)**] from 87 dairy farms from Québec, Canada, spring pre (light) and spring post (dark) regulation implementation. On each farm, between 4 and 6 *E. coli* were tested for each antimicrobial. In dark and light burgundy, MDR: multidrug resistant (resistant to 3 classes of antimicrobial or more). In dark and light red, critical high priority antimicrobials for human medicine. In dark and light blue, critical priority antimicrobials for human medicine. In dark and light yellow, high priority antimicrobials for human medicine. In dark and light green: antimicrobials important for human medicine, SPE: spectinomycin. The importance of antimicrobial for human medicine was defined according to World Health Organization.

Supplementary Figure S3 | Comparison of the proportion of isolates with a least one resistant (intermediate and resistant combined) *Escherichia coli* per sample type [manure pit **(A)**, cows **(B)**, calves **(C)**, herd **(D)**] from 87 dairy farms from Québec, Canada, fall pre (light) and fall post (dark) regulation implementation. On each farm, between 4 and 6 *E. coli* were tested for each antimicrobial. In dark and light burgundy, MDR: multidrug resistant (resistant to 3 classes of antimicrobial or more). In dark and light red, critical high priority antimicrobials for human medicine. In dark and light blue, critical priority antimicrobials for human medicine. In dark and light yellow, high priority antimicrobials for human medicine. In dark and light

green: antimicrobials important for human medicine, SPE: spectinomycin. The importance of antimicrobial for human medicine was mentioned according to World Health Organization.

Supplementary Figure S4 | Comparison of the proportion of isolates with a putative ESBL/AmpC *Escherichia coli* per sample type (manure pit, cows, calves, herd) from 87 dairy farms from Québec, Canada, pre (gray) and post (black) regulation implementation. **(A)** Comparison between spring 1 (pre) and spring 2 (post) regulation. **(B)** Comparison between fall 1 (pre) and fall 2 (post) regulation. No significant differences were identified between groups.

Supplementary Table S1 | Quality of assembly data of whole genome sequencing of isolates from the generic collection ($n = 15$) from calf or cow feces or manure pit of 87 dairy farms in Québec, Canada in 2020–2021.

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Reversal of Azithromycin Resistance in *Staphylococcus saprophyticus* by Baicalin

Jinli Wang, Jinwu Meng, Jinyue Zhu, Tianxin Qiu, Wenjia Wang, Jinxue Ding, Zhenguang Liu, Kun Li, Deyun Wang, Jiaguo Liu and Yi Wu**

MOE Joint International Research Laboratory of Animal Health and Food Safety and Traditional Chinese Veterinary Medicine Research Center, College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, China

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Xin Zhao,
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Francesca Paola Nocera,
Università degli Studi di Napoli Federico II, Italy
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Jingui Li,
Yangzhou University, China

***Correspondence:**
Jiaguo Liu
liujiaguo@njau.edu.cn
Yi Wu
wuyi2001cn@163.com

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In recent years, the efficacy of antibiotics has been threatened by the evolution of bacterial resistance. We previously demonstrated that baicalin (Bac) showed synergies with azithromycin (Azm) against Azm-resistant *Staphylococcus saprophyticus* (ARSS). The aim of this study was to explore the roles of Bac in reversing the resistance of ARSS to Azm. The ARSS was sequentially passaged for 20 days with the sub-MIC (minimum inhibitory concentration) of Bac. The strain that recovered sensitivity to Azm was named Azm-sensitive *S. saprophyticus* (ASSS). The sub-MIC of Bac reversed the resistance of ARSS to Azm. The MIC of Azm against the ASSS strain was 0.488 mg/l, and it was stable within 20 passages. The highest rate of resistance reversal reached 3.09% after ARSS was exposed to 31.25 mg/l Bac for 20 days. Furthermore, semiquantitative biofilm and RT-PCR assays reflected that the ability of biofilm formation and the transcript levels of *msrA*, *mphC*, and virulence-associated genes in the ASSS strain were significantly lower than those of the ARSS strain ($p < 0.05$). Simultaneously, Azm delayed the start time of death, alleviated the injury of the kidney, and decreased the bacterial burden in organs and cytokine levels in mice infected with ASSS. In contrast, Azm did not have a good therapeutic effect on mice infected with ARSS. Therefore, Bac has the potential to be an agent that reversed the resistance of ARSS to Azm.

Keywords: baicalin, *S. saprophyticus*, azithromycin, resistance, reversal

INTRODUCTION

Staphylococcus saprophyticus (*S. saprophyticus*), coagulase-negative coccus, causes urinary tract infections (1), francolin ophthalmia (2), and bovine mastitis (3). In poultry, macrolide antibiotics have often been used to treat infections induced with staphylococcus and streptococcus (4). It is reported that 60% of coagulase-negative staphylococci (CNS) isolated from various samples including central venous catheter tips, urine, and blood were highly resistant to penicillin (90%), ceftriaxone (40%), co-trimoxazole (60%), and azithromycin (Azm) (60%) (5).

Many resistance genes of macrolides are parts of either transposon, plasmids, phages, or genomic islands and, as such, can easily transfer across species, strain, and genus boundaries (6). Researchers are increasingly identifying multidrug-resistant *S. saprophyticus* from ready-to-eat food of animal origin (7). The presence of resistance in food could be a severe threat to public health due to the possible spread of antibiotic resistance (7). However, resistance rates continue to rise, and the rate of antibiotic discovery has decreased substantially. In recent years, the combination of antibiotic drugs and non-antibiotics for bacterial infection treatment appears promising (8, 9). However, drug-resistant bacteria remain a severe threat to the efficacy of antibiotics. One strategy to deal with this problem was to recover the sensitivity of old antibiotics to bacteria. The methods of preventing the spread of antibiotic resistance need to be prioritized (10, 11).

Regulation of virulence factor production is essential for bacterial colonization and pathogenesis (12). Virulence inhibitors are important therapeutic means to treat bacterial infections (13). Virulence inhibitors only inhibit the colonization and virulence of bacteria without exerting selective pressure to hinder the emergence of antibiotic resistance (14). In *S. saprophyticus*, urease (ureC), Uro-adherence factor (UafA), autolysis Aas, surface-associated protein of *S. saprophyticus* (ssp), and D-serine deaminase (dsdA) are associated with colonization and pathogenicity (1, 15, 16). The biofilm of *S. saprophyticus* could also cause persistent infection. Therefore, the development of effective virulence inhibitors is a potential method to deal with bacterial resistance.

Baicalin (Bac) is a potential candidate for reversing Azm resistance in Azm-resistant *S. saprophyticus* (ARSS), a flavonoid compound, extracted from *Scutellaria baicalensis* Georgi (17). It has been reported to restore the effectiveness of β-lactam antibiotics against β-lactam-resistant staphylococcus (17). We previously demonstrated that Bac combined with Azm exhibited synergistic activity against Azm-resistant *S. saprophyticus* (ARSS) (2). Although the synergy of Bac with Azm against ARSS has already been reported, its ability to reverse Azm resistance of ARSS and inhibit virulence has not previously been scrutinized.

Based on this information, we hypothesized that Bac had the potential to reverse the resistance of ARSS to Azm. Thus, the serial passage of ARSS exposure to the sub-MIC (minimum inhibitory concentration) of Bac was conducted. Moreover, the treatment efficacy of Azm to mice infected with the strain that recovered sensitivity to Azm by the serial passage was detected.

MATERIALS AND METHODS

Strains and Culture Conditions

Azm-resistant *S. saprophyticus* (Azm MIC of 1,000 mg/l determined by the broth micro-dilution method) was isolated from francolins suffering from ophthalmia in a francolin farm located in Jiangsu province, China (2). Furthermore, strains were cultured in Mueller–Hinton broth (MHB) or nutrient broth (NB, Hopebio, Qingdao, China) at 37°C with shaking at 180 rpm.

The Rate of Resistance Reversal

For the resistance reversal assay, 10^6 colony-forming unit (CFU) exponential-phase ARSS was inoculated into 1 ml of MHB containing Bac at sub-MICs: 250, 125, 62.5, 31.25 mg/l. After 24 h, the cultures allowed growth were further diluted at 1:100 with fresh MHB containing Bac. The passaging process was repeated for 20 days consecutively. Every five passages, approximately 1×10^3 CFU ARSS strain exposed to sub-MIC levels of Bac were cultured on mannitol salt agar (MSA) plates at 37°C. After 18 h of incubation, the colonies from the MSA plate were transferred onto a drug-free MSA plate and an MSA plate containing 250 mg/l Azm. Then the plates were cultured for 24 h at 37°C. The numbers of colonies surviving in the plate containing Azm and drug-free plate were respectively recorded as C_1 and C_2. Finally, the rate of resistance reversal was calculated based on the formula: resistance reversal rate (%) $= (1 - C_1/C_2) \times 100\%$. The strains that were recovered sensitivity to Azm were named Azm-sensitive *S. saprophyticus* (ASSS).

MIC Determination

The ASSS and ARSS strains in the logarithmic growth phase were diluted to a final concentration of 1×10^6 CFU/ml in MHB. Azm and Bac were diluted using a two-fold serial dilution to obtain the target concentration with MHB in a 96-well plate. A total of 50 μl of bacteria suspension was mixed with 50 μl of the compound and incubated aerobically for 18–24 h at 37°C. The lowest concentration which inhibited the visible growth of bacteria was defined as the MIC value. All of the assays were performed in triplicate independently with two samples.

The Stability of Resistance Reversal

The ASSS and ARSS strains were passaged in MHB without Bac. Briefly, exponential-phase bacteria (10^6 CFU/ml) were grown in MHB and cultured for 24 h. Then, the cultures were diluted at 1:100 into fresh MHB. The serial passages were conducted daily until the 20th passage. At least three independent biological replicates of each experiment were carried out. The MIC was determined as described as above every five passages.

Determination of Growth Curve

For the growth kinetic assay of ARSS and ASSS, overnight cultures were prepared and cultured in NB. The cell densities at 600 nm were measured at 0, 2, 4, 6, 8, 10, 12, and 24 h. For the growth curve under Bac, overnight ARSS cultures were prepared and cultured in MHB containing sub-MIC of Bac. The cell densities at 600 nm were measured at 0, 2, 4, 6, 8, 10, 12, 14, and 24 h.

Gene Transcription Levels Measured by RT-PCR

To measure the mRNA transcript levels of *msrA*, *mphC*, *dsdA*, *ureC*, *Aas*, *UafA*, and *ssp* genes, RT-PCR was conducted as previously described (18). The Bacteria RNA Extraction Kit (Angle Gene, Nanjing, China) was used to extract the total

Abbreviations: ARSS, Azm-resistant *S. saprophyticus*; ASSS, Azm-sensitive *S. saprophyticus*; Azm, azithromycin; Bac, baicalin.

RNA from bacterial cells in the mid-log phase. The values of A_{260}/A_{280} were 1.8–2.1. The RNA was used to reverse into cDNA using a HiScript II 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). Thereafter, the reverse transcription was performed at 50°C for 15 min and 85°C for 5 s. RT-PCR reactions were carried out in a StepOne PCR instrument (Applied Biosystems, Foster City, CA, USA) using ChamQ™ SYBR® qPCR Master Mix (Vazyme, Nanjing, China) as recommended by the manufacturer's instructions. The protocol of the RT-PCR reaction was as follows: first holding stage at 95°C for 3 min, followed by a cycling stage at 95°C for 10 s and 60°C for 60 s (40 cycles total), and a final melting curve stage at 95°C for 15 s, then 60°C for 60 s, and 95°C for 15 s. The housekeeping gene *16S rRNA* was chosen as the internal control gene. The method of $2^{-\Delta\Delta CT}$ was used to analyze the data which were presented relative to the ARSS group. Primer sequences used are shown in **Table 1**.

Biofilm Assay

Biofilm formation assays were performed following a previously published protocol with slight modifications (19). Briefly, a 96-well plate was inoculated with 1×10^5 CFU/ml ARSS or ASSS suspension (200 μl/well) and incubated at 37°C without shaking. After 24 h, the supernatants were removed, and wells were washed with PBS twice. Afterward, the attached bacteria were fixed by 2.5% glutaraldehyde for 1.5 h and then air-dried at room temperature. The wells were stained by adding 200 μl 1% (wt/vol) crystal violet for 20 min and then rinsed thoroughly with PBS until the negative control wells (without bacteria) became colorless. Finally, 200 μl of 33% glacial acetic acid was added to the wells and the absorbance values were detected at 570 nm using a Thermo™ Multiskan™ FC enzyme-labeled instrument. The data were calculated by subtracting the values in the negative control from all the experimental groups.

TABLE 1 | Oligonucleotide primers used in this study.

Target gene	Primer	Sequence (5′-3′)	Source
16S rRNA	*16S rRNA*-F	AGTTGTTCTCAGTTCGGATT	This study
	16S rRNA-R	ATACGGCTACCTTGTTACG	
msrA	*msrA*-F	GCTCTACTGAATGATTCTGATG	This study
	msrA-R	TGGCATACTATCGTCAACTT	
mphC	*mphC*-F	GAGACTACCAAGAAGACCTGACG	This study
	mphC-R	CATACGCCGATTCTCCTGAT	
dsdA	*dsdA*-F	GTGGAAGTCATAGAACATCAG	This study
	dsdA-R	GCGTCATCATACCTAATAGC	
ureC	*ureC*-F	ACACATATCGGTGGCGGTACAG	This study
	ureC-R	GGTTTACAGCTTGCCCTTTACCAG	
Aas	*Aas*-F	GCCGACTACGCAGCAACTAAC	This study
	Aas-R	CCATGAGGGTCAGAGTGGTCAG	
uafA	*uafA*-F	TTCGGTGGTTATGTATGGTT	This study
	uafA-R	CAGTGTTGTTCGCTTGTG	
ssp	*ssp*-F	ACTTCGGTCTATCTCAATGG	This study
	ssp-R	ACATCTGTTGCTTCGGTATA	

Mouse Model of ASSS or ARSS Infection

For the experiment, 100 female ICR mice (18–20 g) were randomized into five groups: ASSS group, ASSS-Azm group, ARSS group, ARSS-Azm group, and blank control (BC) group. For mice in the ASSS and ASSS-Azm groups, they were intraperitoneally injected with 3.6×10^9 CFU of the ASSS strain in sterile normal saline. The mice in the ARSS and ARSS-Azm group were challenged with 2×10^9 CFU of the ARSS strain in sterile normal saline. In the BC group, mice were incubated with equal-volume normal saline. Two hours later, mice in the ASSS-Azm and ARSS-Azm groups were orally administrated with Azm at a dosage of 75 mg/kg, once a day for 3 days. To ensure consistency across tests, mice in the other groups were treated with equal-volume normal saline. At 10 h postinfection, five mice were anesthetized and blood was collected *via* retro-orbital bleeding. Then, clotted blood was centrifuged at 3,000 rpm for 15 min and serum was collected. The levels of IL-6, IL-8, and TNF-α in serum were measured with ELISA kits (Angle Gene, Nanjing, China). The liver, spleen, kidney, and bladder were removed after thoracotomy, weighed, and homogenized in sterile saline solution. The serially diluted samples were plated onto MSA plates for CFU enumeration. For hematoxylin and eosin (H&E) staining and examination, the kidney was collected and fixed in 4% paraformaldehyde. The mice were monitored for a survival rate up to 58 h.

Statistical Analysis

Mean ± standard deviation (SD) was used to express the data. Duncan's multiple-range tests and independent-sample tests were performed to analyze the data among groups using SPSS software (IBM SPSS Statistics 20.0). The statistical significance level was P-value ≤ 0.05.

RESULTS

The Growth Curve of ARSS Under sub-MIC of Bac

Previously, we indicated that the MIC of Bac against ARSS was 500 mg/l (2). In this experiment, the growth curves of ARSS under sub-MIC of Bac were measured. As shown in **Figure 1**, 250 mg/l Bac inhibited the growth of ARSS in MHB. The start time of the exponential phase of the 250-mg/l Bac group was 2 h later than the other groups. In the 250-mg/l Bac group, the OD_{600nm} values were three-fold lower than those of the control group at 12 h. 125 and 62.5 mg/l Bac moderately inhibited the growth of ARSS. However, we can detect a marked multiplication of strain in the 31.25-mg/l Bac and untreated groups.

The Effect of Bac Reversed the Resistance of ARSS to Azm

As shown in **Figure 2A**, the rates of resistance reversal gradually increased in the presence of sub-MIC of Bac over time. Importantly, in the 20th passage, the rate of resistance reversal was the highest, reaching 3.09% in the 31.25-mg/l Bac group. The

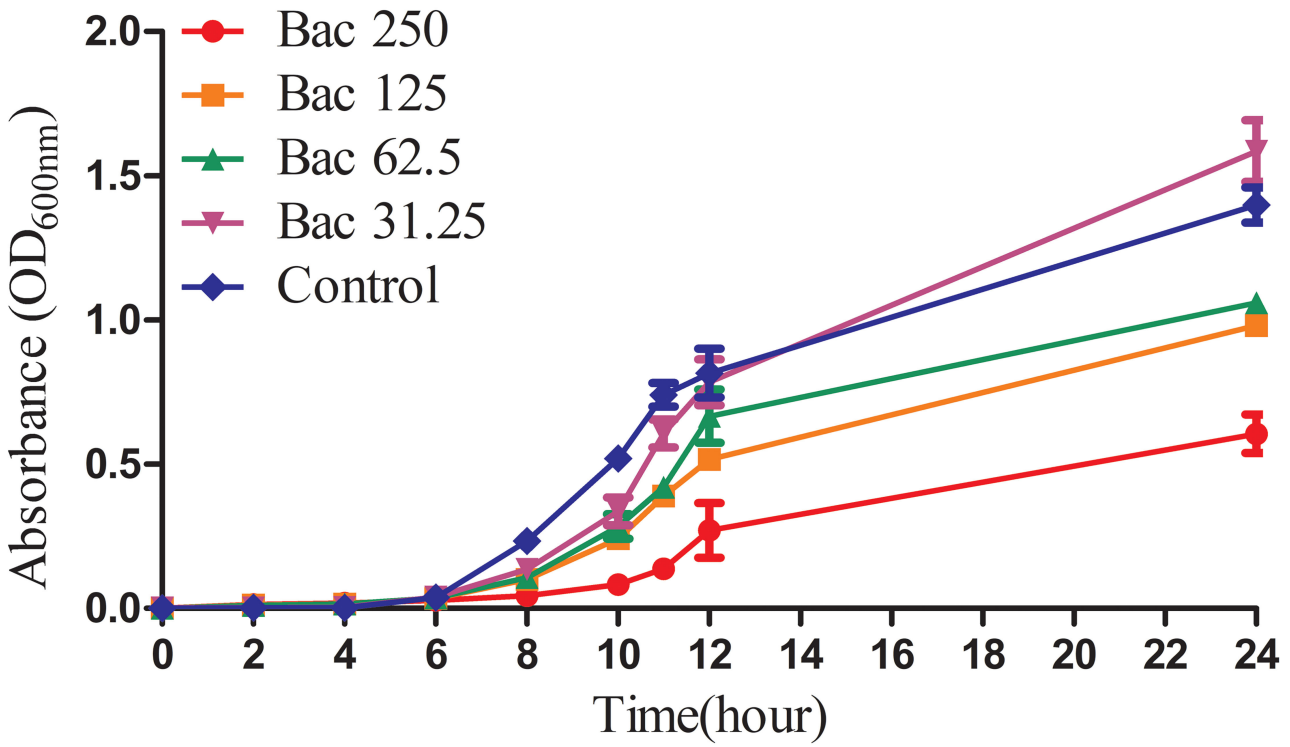

FIGURE 1 | The growth curves of ARSS in MHB under the sub-inhibitory concentration of Bac. OD_{600nm} values were the means ± SD from three independent experiments.

strain that recovered sensitivity to Azm was named ASSS. Then, the MIC and stability of resistance reversal were determined. The MIC of Azm against ASSS decreased 2,049-fold to 0.488 mg/l compared with the ARSS strain. The MICs of Bac against ARSS and ASSS were 500 mg/l (**Table 2**), and the MICs of Azm against ASSS and ARSS were stable within 20 passages in non-drug MHB (**Figure 2B**). Simultaneously, the growth curves of ARSS and ASSS were similar in NB (**Figure 2C**).

TABLE 2 | Minimal inhibitory concentrations (mg/L) of Azm and Bac.

Strain	MIC (mg/L)	
	Azm	Bac
ARSS	1,000	500
ASSS	0.488	500

Azm, azithromycin; Bac, baicalin.

The Changes of the Transcript Levels of Resistance-Associated Genes and Biofilm Formation Ability

To measure the mRNA transcript levels of resistance-associated genes in ARSS and ASSS, RT-PCR was performed. As shown in **Figure 3A**, the mRNA transcript levels of *msrA* and *mphC* genes in the ASSS group were prominently lower than those in the ARSS group ($P < 0.05$). Biofilm also confers resistance to antibiotics. Therefore, the ability of biofilm formation was measured in ARSS and ASSS. The A_{570} values of the ASSS group were remarkably reduced with a 2-fold decrease compared to the ARSS group (**Figure 3B**, $P < 0.05$).

The mRNA Transcript Levels of Virulence Genes

To investigate the changes of the mRNA transcript levels of virulence genes, RT-PCR was carried out. Compared to the ARSS strain, the transcript levels of *ureC*, *Aas*, *uafA*, and *ssp* genes of the ASSS strain significantly degraded ($P < 0.05$). However, the RT-PCR data of *dsdA* gene manifested that no pronounced differences were observed between the ARSS and ASSS groups (**Figure 4**, $P > 0.05$).

The Clinical Curative Effect of Azm to ASSS or ARSS-Associated Infection

The clinical curative effect of Azm is presented in **Figure 5**. In the ASSS group, infection with the ASSS strain resulted in rapid mouse death, with a 0% survival rate occurring within 25 h. However, in the ASSS-Azm group, the first death occurred at 14 h postinfection, and approximately 6.7% of mice remained alive at 58 h. However, the survival rate of the ARSS-Azm group (33.3%) was similar to that of the ARSS group (26.7%).

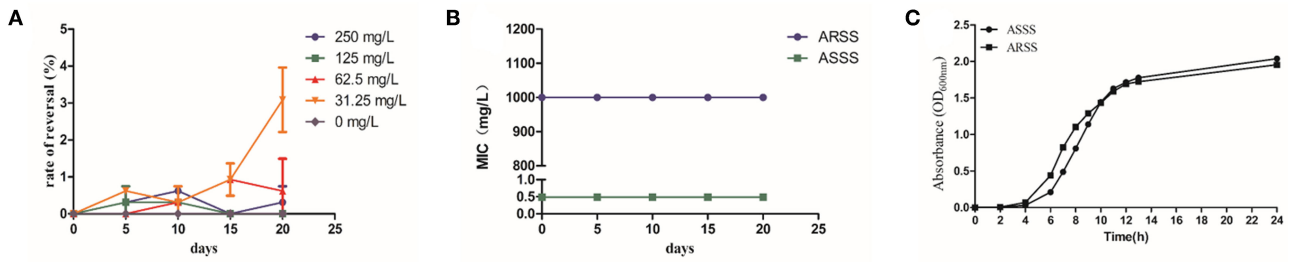

FIGURE 2 | Bac reversed the resistance of ARSS to Azm. **(A)** Rate of resistance reversal development of ARSS to Azm during serial passaging in the presence of sub-inhibitory concentrations of Bac. **(B)** The MICs of Azm against ARSS and ASSS during passaging under non-drug MHB. **(C)** The growth curve of ARSS and ASSS. OD_{600nm} values are the means ± SD. All tests were done with three biological replicates.

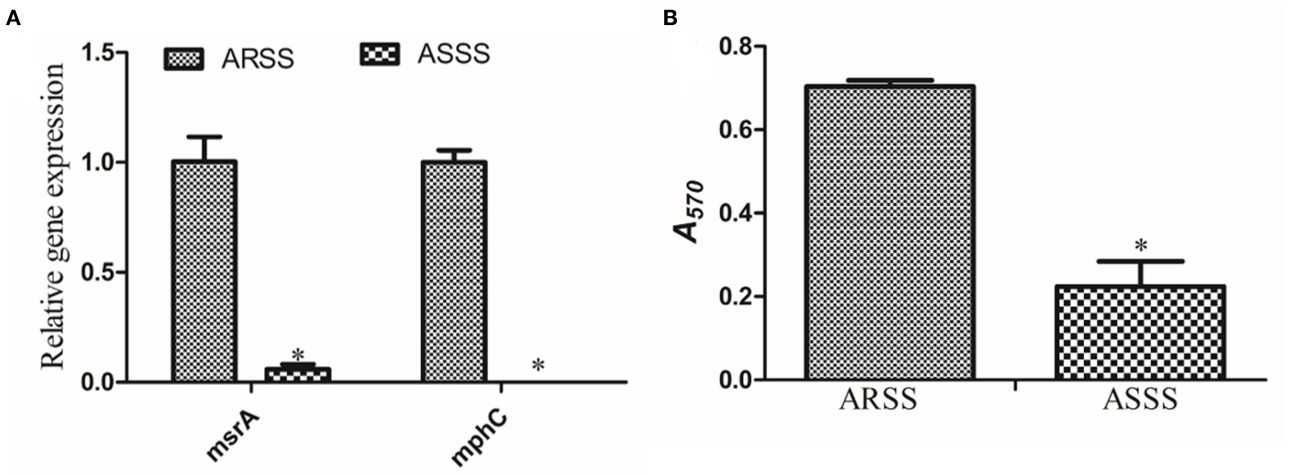

FIGURE 3 | The changes of the transcript levels of resistance-associated genes and biofilm formation ability. **(A)** The transcript levels of *msrA* and *mphC* genes were detected by the RT-PCR method. **(B)** The biomass of ARSS and ASSS was assessed by the crystal violet assay. *$p < 0.05$.

The Changes of Bacterial Burdens

In order to explore the influences of Azm during ASSS or ARSS infection, the bacterial burdens were calculated in the liver, spleen, kidney, and bladder. Post 8 h infection, compared to the ASSS group, the proliferation level of the ASSS strain in the ASSS-Azm group was significantly reduced (**Figures 6A–D**). Additionally, the bacterial distribution data showed that no statistically obvious difference was found between the ARSS and ARSS-Azm groups (**Figures 6E–H**). These results revealed that Azm efficiently inhibited the proliferation of the ASSS strain in the tissues rather than the ARSS strain.

Pathological Changes of the Kidney

The kidney histological changes of each group observed by HE staining are summarized in **Figure 7**. As shown in the figures, no lesion was observed in the kidney of the BC group. However, the histopathology of the mice in other groups showed that atrophic glomerulus (indicated by arrows) was observed at 8 h postinfection. Furthermore, kidney histological changes of the ASSS-Azm group were significantly alleviated with an obvious decrease in the atrophic glomerulus. In contrast, the histological changes of the ARSS-Azm group were similar to those of the ARSS group.

Serum Cytokine Levels

ELISA was carried out to detect the levels of IL-6, IL-8, and TNF-α in serum at 8 h postinfection. As illustrated in **Figure 8**, in the BC group, the levels of IL-6, IL-8, and TNF-α were remarkably lower than those in the other groups ($P < 0.05$). Compared with the ASSS group, Azm significantly induced a decrease of IL-6, IL-8, and TNF-α in the ASSS-Azm group ($P < 0.05$). In addition, the results of cytokine levels indicated that there were no pronounced differences between ARSS-Azm and ARSS groups (**Figure 8**).

DISCUSSION

Global healthcare was threatened by the rapid emergence and spread of multidrug-resistant bacteria. Coupled with the fact that the development of novel antibiotics is very slow, it is necessary to develop antibiotic adjuvants to reverse antibiotic resistance (9). Bacteria that are previously sensitive to commonly used antibiotics became resistant by stepwise

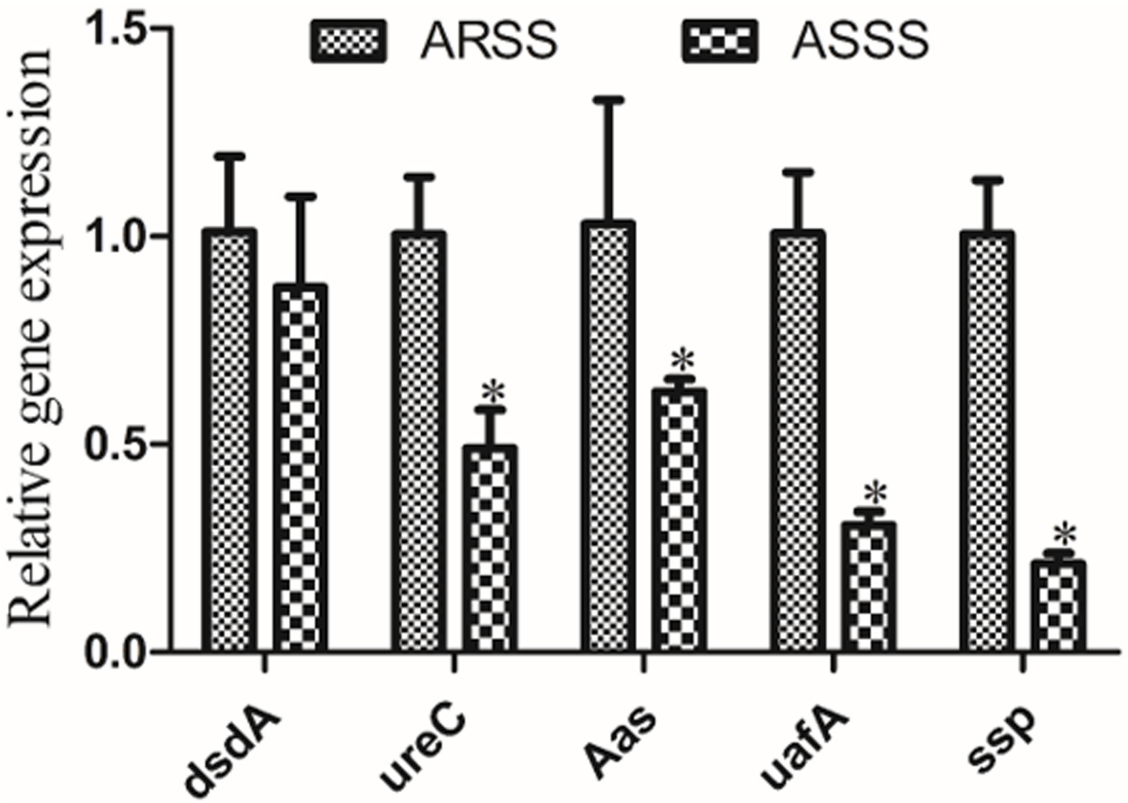

FIGURE 4 | RT-PCR measurement of the differentially virulence-associated genes in the ARSS and ASSS strains. Five genes, i.e., *dsdA*, *ureC*, *Aas*, *uafA*, and *ssp*, were selected and amplified using RT-PCR. The 16*S rRNA* gene was used as the internal control. Significant differences are marked by asterisks (*$p < 0.05$).

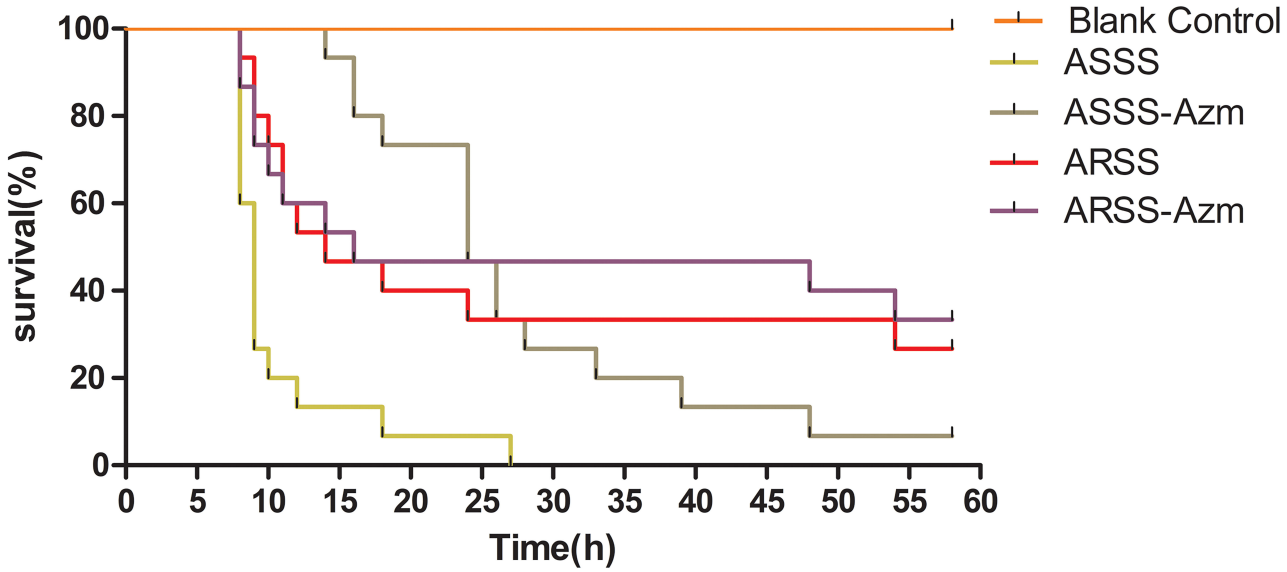

FIGURE 5 | The survival rate of mice infected by the ARSS or ASSS strains. Mice were i.p. injected with the indicated strains, and the mice were monitored for mortality up to 58 h.

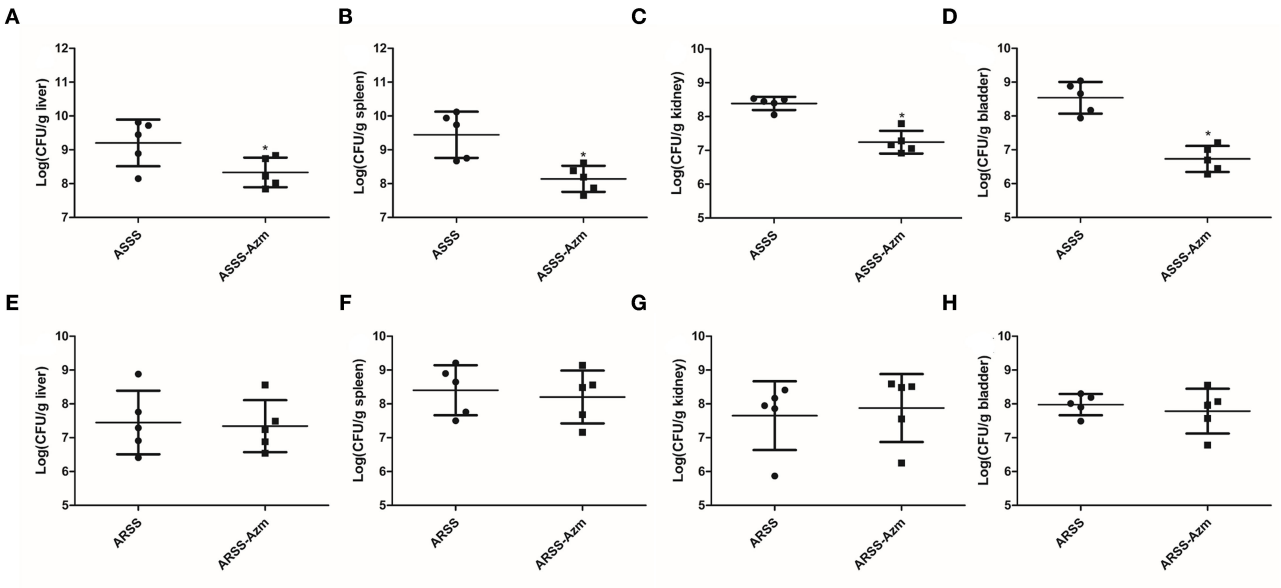

FIGURE 6 | *In vivo* bacterial distribution and burden in organs of mice infected with the ARSS or ASSS strain. At 8 h postinfection, liver **(A,E)**, spleen **(B,F)**, kidney **(C,G)**, and bladder **(D,H)** were collected for measuring the total number of CFU. The number of CFU from each organ was calculated as per gram of tissue. Bars represent the means for five infected mice. Error bars represent standard deviation (SD). Significant differences are marked by asterisks between groups (*$p < 0.05$).

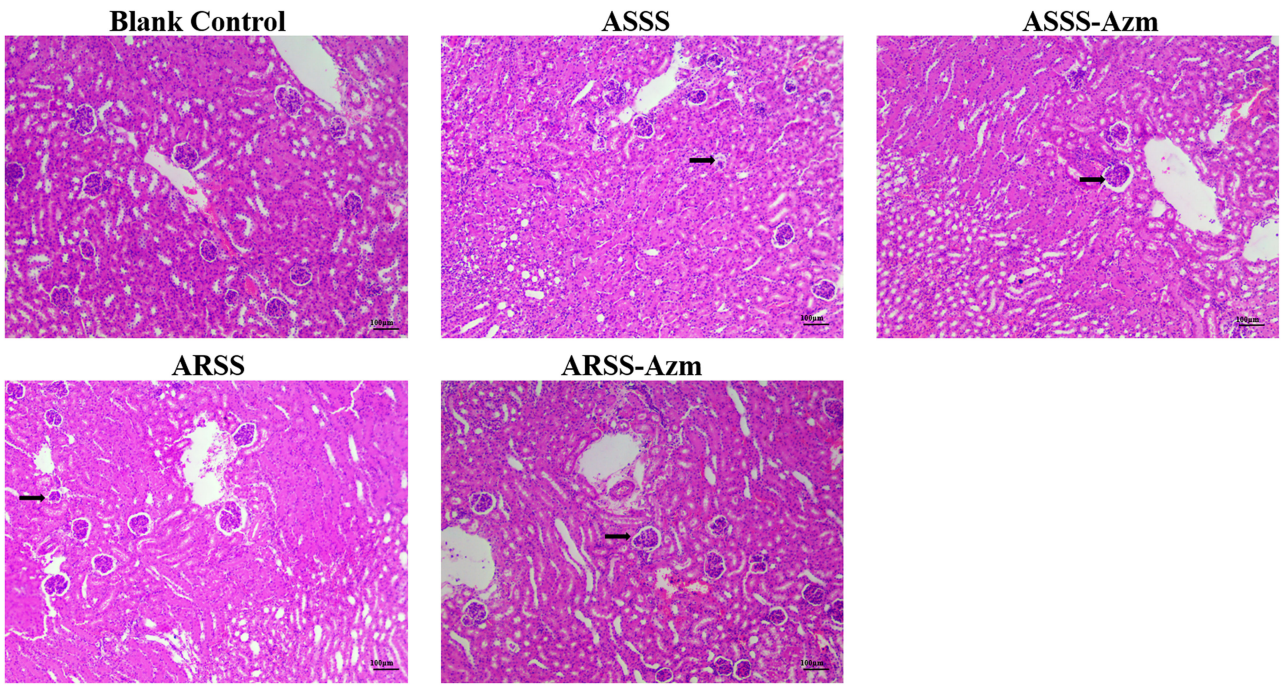

FIGURE 7 | Kidney histological changes of each group at 8 h postinfection (HE stain, ×200). Kidney tissues were randomly isolated and collected from five mice in each group at 8 h postinfection. A portion of each isolated kidney tissue was instantly fixed in 4% paraformaldehyde, followed by hematoxylin and eosin (H&E) stain. Arrows indicate atrophic glomerulus.

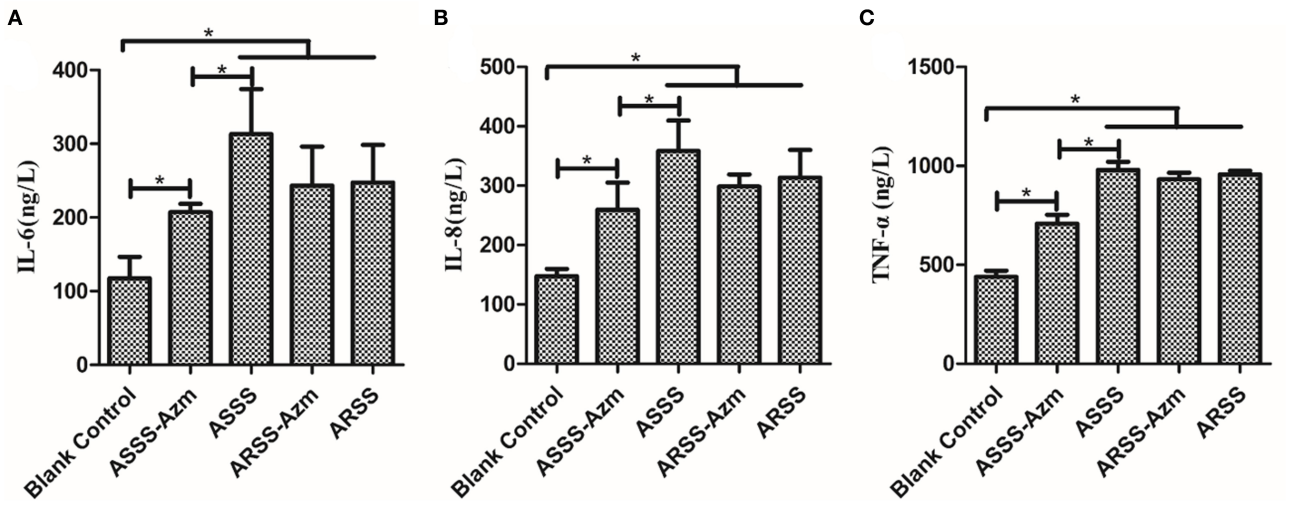

FIGURE 8 | Cytokine levels in the serum of mice i.p. injected with ASSS or ARSS. IL-6 **(A)**, IL-8 **(B)**, and TNF-α **(C)** levels in the serum were measured by ELISA at 8 h postinfection. Values were presented as the mean ± SD. Significant differences between groups are linked by brackets. *$p < 0.05$.

exposure to these compounds, and efflux pump inhibitors could reverse this resistance (20). Our previous investigation demonstrated that Bac could hinder the effect of MsrA efflux pump in ARSS by decreasing the ATP content and the mRNA transcript levels of the *msrA* gene (18). Simultaneously, ARSS recovered the sensitivity to Azm under the exposure of sub-MIC of Bac (**Figure 2**). The strain that recovered susceptibility was renamed ASSS. The mRNA transcript level of the *msrA* gene in the ASSS strain was lower than the ARSS strain (**Figure 3A**). What is important is that the rate of resistance reversal was highest in the 31.25-mg/l Bac group (**Figure 2A**). The reason for the phenomenon is that 62.5–250 mg/l Bac moderately inhibited the growth of ARSS (**Figure 1**). The MICs of Azm against ARSS and ASSS were identical within 20 passages (**Figure 2B**). These results reflected that Bac is a potential antibiotic adjuvant of reversing resistance.

The major mechanisms of macrolide resistance to staphylococci involve (i) modification by methylation of 23S ribosomal rRNA, (ii) Msr family efflux pump upregulation, and (iii) macrolide inactivation by phosphotransferases or esterases (21). Also, biofilm is also the main factor influencing the efficiency of antibiotics. In this investigation, the ability of biofilm formation and the transcript levels of *mphC* and *msrA* efflux genes simultaneously decreased in the strain that recovered susceptibility to Azm (ASSS) compared with the ARSS strain (**Figure 3**). The expression of *mphC* in *S. aureus* was shown to be highly dependent on the presence of a portion of the gene encoding the MsrA efflux pump (22). It has been proposed that the transcriptional level of biofilm matrix components and biofilm formation could be decreased by genetic inactivation and inhibitor of efflux pumps (23). We previously indicated that Bac inhibited biofilm formation by modulating the MsrA efflux pump of ARSS (18). Therefore, we surmised that along with the decrease of transcript levels of the *msrA* gene, the transcript level of the *mphC* gene and biofilm formation ability dwindled under the exposure of sub-MIC of Bac. The mechanisms needed to be further investigated.

At present, the therapeutics of anti-virulence have shown potential in preventing the bacteria from acquiring antibiotic resistance (24). It is reported that Bac suppressed the relative expression of virulence-related genes in *S. aureus* (25) and protected mice from lethal Shiga-like toxin 2 (Stx2) challenge by inducing Stx2 to form inactive oligomers (26). In this study, serial passage of ARSS under sub-MIC of Bac over 20 days failed to produce resistant mutants (**Table 2**). Simultaneously, compared with the ARSS strain, the mRNA transcript levels of *dsdA*, *ureC*, *Aas*, *uafA*, and *ssp* genes were decreased in the ASSS strain (**Figure 4**). The activities of ureC and dsdA are essential for successful colonization and pathogenicity in *S. saprophyticus* (15). UafA of *S. saprophyticus* was a cell wall-anchored protein with an LPXTG motif (15). Proteins associated with the surface of *S. saprophyticus* (ssp) (1) and a fibronectin-binding autolysis Aas (16) were non-covalently surface-associated proteins. Therefore, we inferred that the colonization ability and pathogenicity of ASSS were lower than those of ARSS. When mice were infected with ARSS and ASSS in $OD_{600\,nm}$ of 2.71, the mortalities were 83.3 and 54.0%, respectively (data not shown). Then we extended our study on the protective effects of Azm on the treatment of ASSS or ARSS infections in mice. In the verification experiment, Azm delayed the start time of death, alleviated the injury of the kidney, and decreased the bacterial burden in tissues and cytokine levels in mice infected with ASSS. In contrast, Azm could not treat the infection caused by the ARSS strain (**Figures 5–8**). These results suggested that Azm exhibited a better therapeutic efficacy on ASSS infection than ARSS infection.

CONCLUSION

In conclusion, the sub-MIC of Bac reversed the resistance of ARSS to Azm. The strain that recovered sensitivity to Azm after being exposed to sub-MIC of Bac was named ASSS. The ability of biofilm formation and the mRNA transcript levels of *msrA*, *mphC*, and virulence genes were decreased in the ASSS strain. Azm successfully treated the infection caused by ASSS rather than ARSS. These results indicated that Bac could be expected to be developed into a new adjuvant of resistance reversal drug.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) set by Nanjing Agricultural University (approval number: PTA030). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

JW, JM, YW, and JL designed the experiment. JW, JZ, TQ, JD, and WW conducted the research. JW, ZL, and KL analyzed the data and wrote the manuscript. YW and DW revised the manuscript. The final manuscript has been read and approved by all authors.

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Characterization of Bacterial Microbiota Composition in Healthy and Diarrheal Early-Weaned Tibetan Piglets

Qinghui Kong[1,2†], Wenqian Zhang[2†], Miao An[2†], Muhammad Fakhar-e-Alam Kulyar[2], Zhenda Shang[1,3], Zhankun Tan[1,3], Yefen Xu[1], Jiakui Li[1,2*] and Suozhu Liu[1,3*]*

[1] College of Animal Science, Tibet Agricultural and Animal Husbandry University, Linzhi, China, [2] College of Veterinary Medicine, Huazhong Agricultural University, Wuhan, China, [3] Tibetan Plateau Feed Processing Engineering Research Center, Linzhi, China

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***Correspondence:**
Qinghui Kong
770337011@qq.com
Suozhu Liu
346249610@qq.com
Jiakui Li
lijk210@sina.com

[†] These authors have contributed equally to this work

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The occurrence of diarrhea in Tibetan piglets is highly notable, but the microorganisms responsible are yet unclear. Its high incidence results in serious economic losses for the Tibetan pig industry. Moreover, the dynamic balance of intestinal microflora plays a crucial role in maintaining host health, as it is a prime cause of diarrhea. Therefore, the present study was performed to analyze the characteristics of bacterial microbiota structure in healthy, diarrheal and treated weaned piglets in Tibet autonomous region for providing a theoretical basis to prevent and control diarrhea. The study was based on the V3–V4 region of the 16S rRNA gene and gut microbiota functions following the metagenome analysis of fresh fecal samples ($n = 5$) from different groups. The Shannon and Simpson indices differed substantially between diarrheal and treated groups ($p < 0.05$). According to our findings, the beta diversities, especially between healthy and diarrheal groups, were found different. Firmicutes, Bacteroidetes and Proteobacteria were the dominant phyla in three groups. Furthermore, the abundance of Fusobacteria in the diarrheal group was higher than the other groups. The dominant genera in the diarrheal group were *Fusobacterium, Butyricimonas, Sutterella, Peptostreptococcus, and Pasteurella*. Moreover, *Lactobacillus, Megasphaera* and *Clavibacter* were distinctly less abundant in this group. It is noteworthy that the specific decrease in the abundance of pathogenic bacteria after antibiotic treatment in piglets was noticed, while the level of *Lactobacillus* was evidently increased. In conclusion, fecal microbial composition and structure variations were discovered across the three groups. Also, the ecological balance of the intestinal microflora was disrupted in diarrheal piglets. It might be caused by a reduction in the relative number of beneficial bacteria and an increase in the abundance of pathogenic bacteria. In the context of advocating for non-resistant feeding, we suspect that the addition of probiotics to feed may prevent early-weaning diarrhea in piglets. Moreover, our findings might help for preventing diarrhea in weaned Tibetan piglets with a better understanding of microbial population dynamics.

Keywords: microbial diversity, bacteria, 16S rRNA, Tibetan piglets, diarrhea

INTRODUCTION

The Tibetan pig is a valuable indigenous specie, as it is the only one that can survive in China's high altitude. Tibetan pigs are mainly found in semi-grassland and semi-farmland regions of Tibet (1). Under long-term harsh environmental conditions, Tibetan pigs have developed resistance against cold and diseases by developing different characteristics. These characteristics have made them indispensable for pig production in the plateau (2).

Early weaning is often used in intensive pig production, both at home and on farms (3). Meanwhile, weaning is an important turning point for piglet's growth to reduce the rate of vertical disease transmission and helps in the overall improvement of a pig farm. Conversely, earlier weaning caused psychological, environmental and nutritional stress in piglets, which induced diarrhea, dystrophia and slow growth (4) resulting in significant economic losses for the pig industry. Studies have argued that the diarrhea of weaned piglets is caused by infection with multiple pathogenic factors (bacteria, virus, etc.) and the intestinal dysfunction of piglets (5). Also, the imperfect immune system of the piglets, environmental changes, dietary changes, and improper feeding methods are conducive to the invasion of pathogenic strains, e.g., *Escherichia coli* (6).

The dynamic balance of intestinal microbiota plays an important role in the immune regulation of animals (7). Normal intestinal microbiota can stimulate the animal intestinal immune system by improving the intestinal self-recognition and immune ability of different bacteria. The intestine also serves as a barrier that can reduce the probability of host infection. Furthermore, weaning stress has disrupted the natural gut balance, reducing helpful microorganisms (8).

Due to the harsh cultural environment and intensive breeding strategies, Tibetan piglets frequently suffer from diarrhea after weaning. These factors are significantly decreasing the production performance and economics. The present study was performed to analyze the microbial diversity of different bacterial strains in healthy and diarrheal Tibetan piglets in Nyingchi, Tibet autonomous region, to investigate the etiology of diarrhea and develop a theoretical framework for it's prevention and treatment.

MATERIALS AND METHODS

Animal Feeding and Sample Collection

The experimental animals for this study were taken from five healthy sows (The sows were raised at Tibetan Pig Collaborative Research Center of Tibet Agriculture and Animal Husbandry University) maintained at similar conditions. The sows gave birth on the same day. Tibetan piglets and sows were bred together in a farrowing house (the temperature of the farrowing house was ∼21°C, and the farrowing bed was strictly cleaned and disinfected). The feed was given to the piglets when they were 3 weeks old. At the age of 6 weeks, healthy Tibetan piglets were weaned and transferred to the nursery house (the temperature of the nursery house was 16°C).

Hermann-Bank (9) test criteria were used to determine healthy and diarrheal Tibetan piglets. The feces of healthy Tibetan piglets (piglets without any clinical symptoms) were granular or stripe-shaped for more than 2 days. While the feces of diarrheal piglets were thin and unformed for more than 2 days. The fecal samples of healthy and diarrheal piglets (the diarrheal early-weaned Tibetan piglets not birthed by the same sow) were simultaneously collected. All samples were transferred from the ranch to the laboratory using a vehicle-mounted refrigerator (−15°C). Then the samples were stored at −20°C for further evaluation. The marked diarrheal Tibetan piglets were treated with 1 mL of Gentamycin (4%) sulfate through intra muscular route (HuaXu Company, China, Product number: 17925752842). Five fecal samples from healthy piglets (group A; marked as A1, A2, A3, A4, and A5), five fecal samples from diarrheal piglets (group B; marked as B1, B2, B3, B4, and B5), and five fecal samples from post-treatment piglets (group C; marked as C1, C2, C3, C4, and C5) were selected.

DNA Extraction

The microbial DNA was extracted from 15 fecal samples of piglets using QIAamp Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) as per the manufacturer's recommendations. The concentration and quality of DNA were detected with a nucleic acid detector (Nanodrop, Thermo Scientific NC2000, USA) and 1.2% agarose gel electrophoresis, respectively.

16S rRNA Amplification

The standard bacteria V3–V4 hypervariable region gene PCR primers (forward primer: ACT CCT ACG GGA GGC AGC A; reverse primer: GGA CTA CHV GGG TWT CTA AT) were used. AxyPrep DNA Gel Extraction Kit (Axygen, CA, USA) and the 2% agarose gel electrophoresis were used for target fragment recovery and evaluation of PCR amplification product. Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Waltham, Massachusetts, USA) was used to detect the recovered PCR products. Moreover, TruSeq Nano DNA Low Throughput Library Prep Kit (Illumina, CA, USA) was implied for sequence library construction. Amplified products' sequence ends were repaired by End Repair Mix2. PCR amplification was carried out to enrich the sequencing library template, and the library enrichment product was purified again via BECKMAN AMPure XP Beads. The library's final fragment-selection and purification were performed using 2% agarose gel electrophoresis.

The quality of libraries was examined on Agilent Bioanalyzer using Agilent High Sensitivity DNA Kit before sequencing procedure. The libraries with only one peak signal and no linker signal were considered for the process. Moreover, the libraries were quantified using Quant-iT PicoGreen dsDNA Assay Kit on Promega QuantiFluor fluorescence quantification system. The qualified library concentration was more than 2 nM. These qualified libraries were gradient diluted and mixed in proportion according to required sequencing. The MiSeq Reagent Kit V3 (600 cycles) was used to perform 2 × 300 bp paired-end sequencing on the MiSeq sequencing machine after the mixed libraries were denatured into single strands by sodium hydroxide.

Sequence Data Processing and Statistical Analysis

Sequences analysis was established as operational taxonomic units (OTUs) via Uclust with over 97% similarity (10). The

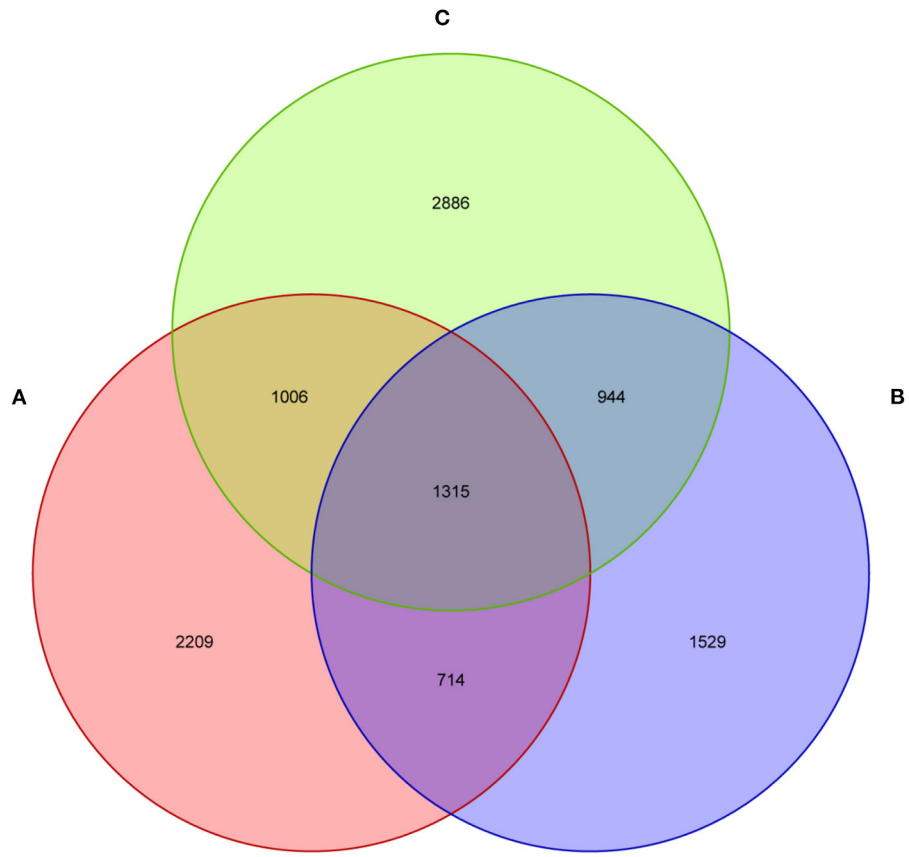

FIGURE 1 | The fecal microflora of weaned piglets, analysed by a Venn diagram. **(A)** Healthy piglets; **(B)** Diarrheal piglets; **(C)** Treated piglets.

highest abundant sequence in each OUT was selected as the representative sequence (11). Then, OTUs were taxonomically classified and grouped by comparing with those in the Unite database (12). Micro microflora's richness and evenness index was calculated using the measurement indexes (Chao1, ACE, Shannon, and Simpson). Beta diversity based on the weighted UniFrac distance matrices were calculated with QIIME (Version 1.7.0), while the Cluster analysis was preceded by principal coordinate analysis (PCA) (13). The metastatic statistical algorithm was used to analyze the discrepancy in microbial communities between groups at the phylum and genus levels (14). The heat map was created via R software (v3.0.3), and all the data were evaluated statistically by one-way analysis of variance through SPSS 20.0 software (SPSS Inc., Chicago, Illinois 60606-6307, USA).

RESULTS

Sequencing Results and OTU Cluster's Statistical Analysis

The current study subjected 15 fecal samples collected from Tibetan piglets to the high-throughput sequencing analysis. After optimizing the preliminary data, a total of 413,584, 427,913, and 408,109 high-quality valid sequences were obtained from the A, B and C groups, respectively. As shown in the dilution curve of species observation index, with the deepening test depth, its slope gradually decreased and reached the plateau stage. This finding indicated that the sequencing quantity of the samples was saturated, and the majority of bacteria were covered (**Supplementary Figure 1**). The sequences were established at the phylum, class, order, family, genus and species levels as OTUs via Uclust with over 97% similarity (**Supplementary Figure 1**). The three groups shared 1,315 bacteria species, as found by Venn map/diagram analysis (**Figure 1**). The diarrheal piglets showed 1,529 common bacteria species, which were not found in the healthy and antimicrobial-treated piglets. A total of 2,209 bacteria species were found to be common among the healthy piglets.

Effects of Microbial Community Diversity

The alpha diversity of fecal microbiota was evaluated by using Chao1, ACE, Shannon and Simpson. The Simpson and Shannon index demonstrated that there was no striking difference in the micro microflora abundance between group A (0.887, 5.962) and B (0.834, 4.97) ($p > 0.05$) (**Figure 2**), whereas C (0.945, 6.582) group was significantly higher than that of the B group ($p < 0.05$) (**Figure 2**). The ACE and Chao1 indices showed that A group and C group had higher richness than B group, whereas no

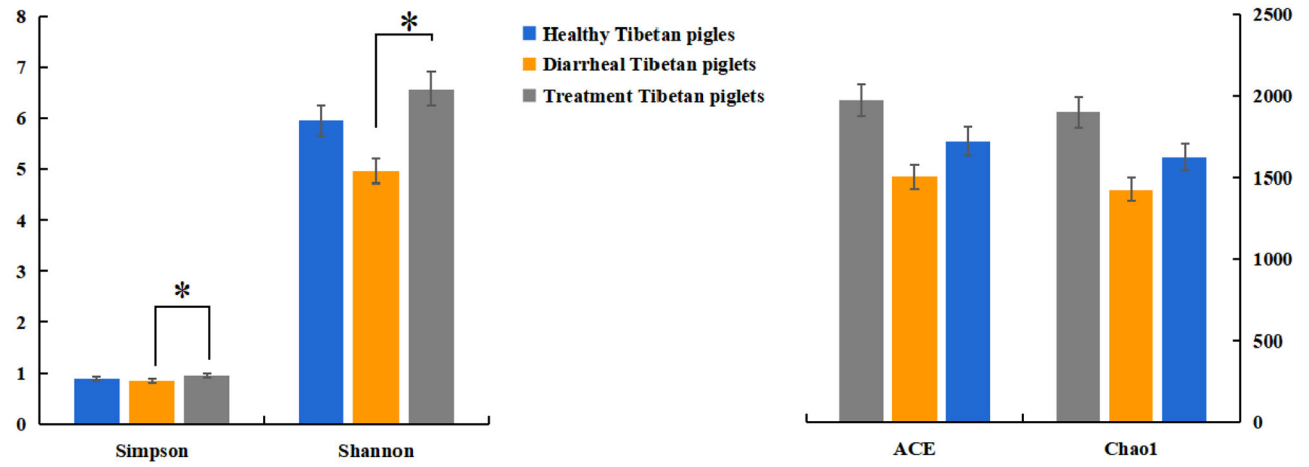

FIGURE 2 | Diversity indices of the fecal microbiota in different Tibetan piglets. Chao1, ACE, Shannon, and Simpson indices were used to evaluate the alpha diversity of the fecal microbiota. The results were evaluated through one-way ANOVA. All of the data represent means ± SD. *$p < 0.05$.

striking difference in the microflora richness was noticed among the three groups ($p > 0.05$) (**Figure 2**). Specifically, The Chao1 index amounted to 1,625.74, 1,427.63, and 1,899.73 in groups A, B, and C, while the ACE index reached 1,721.43, 1,504.51, and 1,972.87 in groups A, B, and C, respectively. However, significant differences were found in the microbial community structure by principal component analysis (PCA) in different groups, especially among healthy piglets, as compared with other two groups (**Figure 3**).

Composition Analysis of the Microbial Community Structure in Different Groups

The bacterial community in the three groups were assessed at different taxonomical levels. Firmicutes (75.28 ± 12.70% in group A, 62.78 ± 15.75% in group B, 72.16 ± 12.65% in group C) and Proteobacteria (10.36 ± 8.48% in group A, 13.76 ± 18.62% in group B, 13.66 ± 15.87% in group C) were dominant in all samples at the phylum level (**Figure 4A**). Other phyla, including Bacteroidetes and Actinobacteria, presented a lower abundance (<8% of all samples) (**Figure 4A**). Interestingly, Fusobacteria in group B (13.02 ± 8.82%) was higher as compared to group A (0.08 ± 0.13%) and group C (2.58 ± 4.16%). *Peptostreptococcaceae* (21.92 ± 22.13%), *Enterobacteriaceae* (11.32 ± 18.75%), *Streptococcaceae* (12.32 ± 19.45%), *Collinsella* (3.86 ± 7.75%), *Dorea* (2.26 ± 2.09%) were predominant in the B group, whereas *Psychrobacter* (4.30 ± 9.61%) and *Clostridium* (2.04 ± 2.34%) in the C group at the genus level (**Figures 4C,D**). In addtion, *Lactobacillus* (47.10 ± 15.31% in group A, 2.00 ± 0.78% in group B, 12.22 ± 2.18% in group C) and *Akkermansia* (4.38 ± 9.68% in A group) were predominant bacteria genera. The relative abundance of genera *Prevotella*, *Roseburia* and *Bacteroides* were <2% in all samples (**Figures 4C,D**).

The relative abundance of Fusobacteria in group B was significantly higher as compared to group A ($p < 0.01$) and group C ($p < 0.05$) at the phylum level (**Figure 5A**). The abundance of Elusimicrobia ($p < 0.05$) in the C group was significantly higher than group A and group B (**Figure 5A**). Furthermore, *Fusobacterium*, *Butyricimonas*, *Sutterella*, *Peptostreptococcus*, *Pasteurella* and *Veillonella* were the most abundant genus in group B, which were significantly higher than in other groups ($p < 0.05$) (**Figure 5B**). In contrast, *Megasphaera* and *Clavibacter* were less abundant in diarrheal piglets than healthy piglets ($p < 0.05$) (**Figure 5B**). *Lactobacillus* in the A group was significantly higher ($p < 0.01$) than in the B and C groups, whereas the abundance in group C was also significantly higher than that in group B ($p < 0.01$) (**Figure 5B**). Moreover, the relative abundance of *Klebsiella*, *Bilophila*, *Roseburia*, *1–68*, *Clostridium*, *Sutterella* and *Tissierella_Soehngenia* in group C ($p < 0.05$) were significantly higher than in group A at the genus level (**Figure 5B**).

We also performed Linear discriminant analysis effect size (LEfSe) tests to compare further intestinal microflora differences among the three groups (**Figure 6**). When comparing different Tibetan piglets, we found 4, 7 and 11 bacterial taxa that were abundant in healthy, diarrheal, and treated piglets. Furthermore, healthy piglets had the most enriched phylotypes from the phylum *Lactobacillus*, whereas diarrheal piglets had the most *Sutterella*, *Fusobacterium*, and *Pasteurella* phylotype.

DISCUSSION

Piglet diarrhea is a common issue throughout the pig breeding process. The reasons for piglet diarrhea are quite a lot, such as weaning, nutritional, environmental and physiological stress on piglets (15). In addition, pathogenic bacteria, stress, management factors and excessive feed intake are also associated with piglet diarrhea (16). Moreover, intestinal epithelial mucosal barrier is the first line of defense that animals use to resist in such adverse conditions as it plays an important role in animals' normal intestinal functioning. Therefore, the changes in intestinal microbiota diversity would affect the intestinal function and cause diseases. This study evaluated the fecal microflora structure

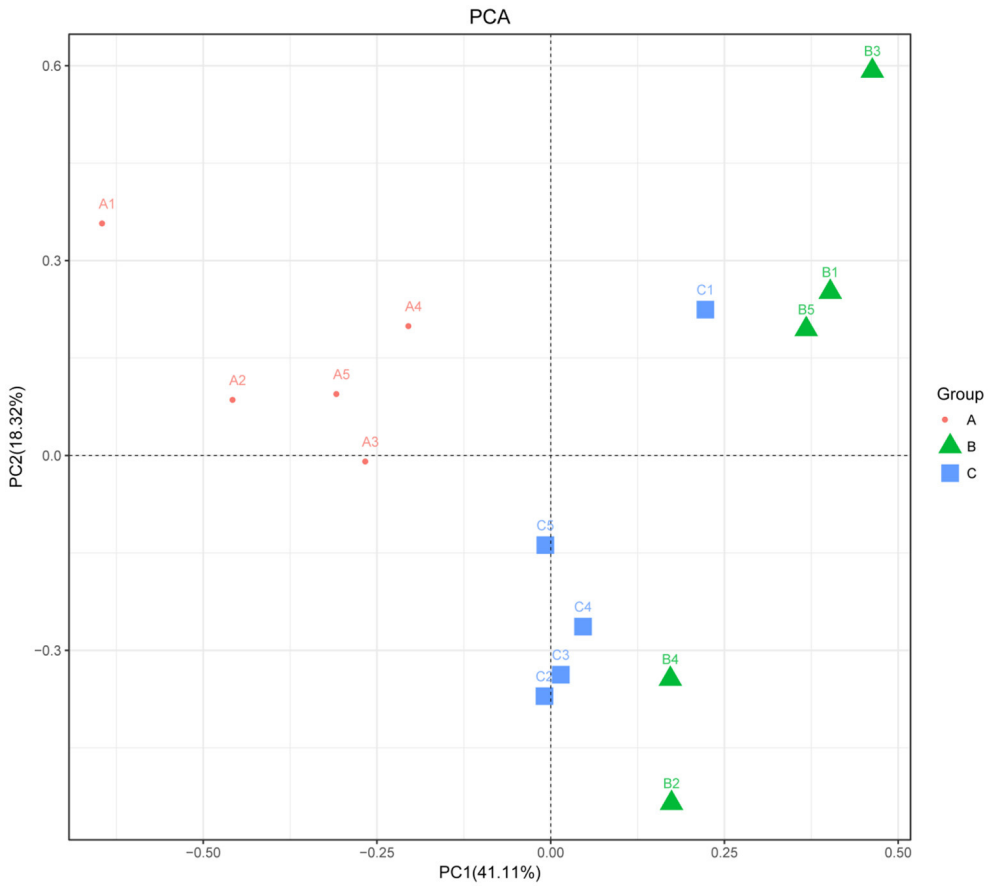

FIGURE 3 | Principal component analysis of the fecal microbiota. PCA map based on Euclidean distance. Each point indicates one sample. The distance of the two points indicates the difference in fecal microbiota. A: Healthy piglets; B: Diarrheal piglets; C: Treated piglets.

in healthy, diarrheal and treated piglets of Tibet autonomous region. The findings showed that diarrhea altered the bacterial microbiota structure of Tibetan pigs and impacted the variety of fecal microflora. There were a variety of bacteria in the feces of the Tibetan piglets. By Venn diagram analysis, 1,529 bacterial species were shared among the diarrheal piglets, which were not found in the healthy and treated piglets. Whereas, 2,209 bacterial species were found in the healthy group. PCA analysis showed a significant difference in bacterial community structure among the three groups, especially between healthy and diarrheal piglet groups based on Euclidean distance.

Generally, species are phylogenetically affiliated to phyla Firmicutes, Proteobacteria, and Bacteroidetes, which are abundant in Large White and Chinese Shanxi Black pigs (17). Our results indicated that Firmicutes, Bacteroidetes and Proteobacteria were the most dominant phyla in three groups of Tibetan piglets, which were consistent with previous observations in pig (18), bovine (19), sheep (20) and yak (21). Actinobacteria were mainly distributed in the stomach of herbivores, and they promote fiber decomposition and help in the digestive function of these animals (22). It is noteworthy that Actinobacteria was dominant phylum in Tibetan piglets, which was identified with predecessor's research in wild pigs (23). This phenomenon may be related to the herbivorous nature of Tibetan piglets. Some studies suggest that the abundance of Fusobacteria (24) and Fusobacteria phyla activate host inflammatory responses in order to protect against pathogens that promote tumor growth (25). Remarkably, the higher abundance of Fusobacteria in the fecal microbiota of diarrheal piglets may induce an immune response and increase the risk of pathogen infection of the host. Our results manifested that the Elusimicrobia level in the C group showed an upward trend as compared to the A and B groups, while it was known as an enigmatic bacterial phylum previously. The first representatives were termite gut-associated (26) isolated from the rumen (27) and the environment (28), that comprised of Planctomycetes, Verrucomicrobia, Chlamydia, Omnitrophica, Desantisbacteria (29), Kiritimatiellaeota (30) and Lentisphaerae (31). Cultivation and genome-based studies revealed that some species belonging to Elusimicrobia that are capable of glucose fermentation (32) with the ability to fix nitrogen (28).

Fusobacterium is being unveiled pathogen of gastrointestinal disorders. Previous research indicated that *Fusobacterium* plays

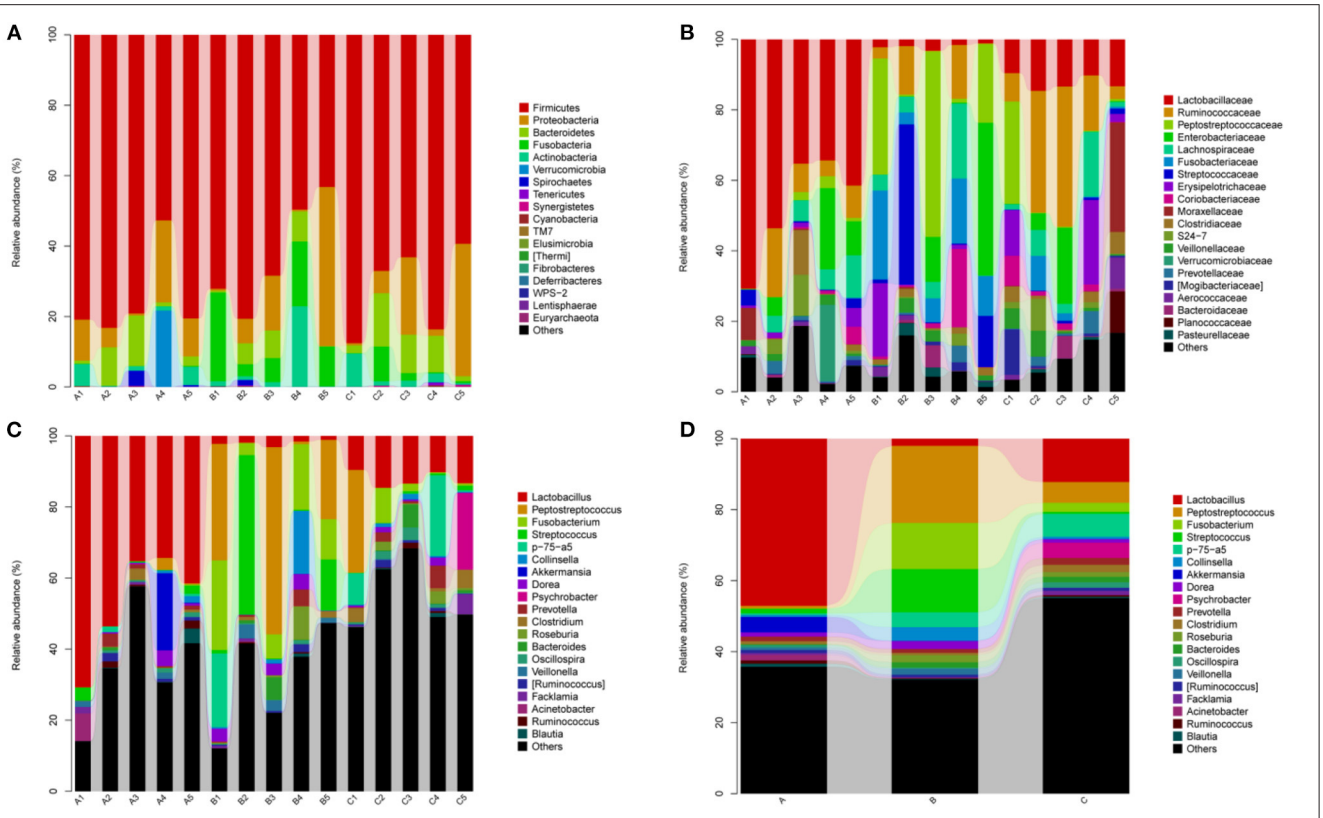

FIGURE 4 | Relative abundance of gut bacterial taxa among different groups. **(A)** Phylum level; **(B)** Family level; **(C)** Genus level; **(D)** Taxonomic assignment at genus level. A1–A5: Healthy piglets; B1–B5: Diarrheal piglets; C1–C5: Treatment piglets.

a role in the pathogenesis of ulcerative colitis (33) and exert potentially carcinogenic (colorectal cancer) effects on the host (34). *Butyricimonas* bacteraemia has been described in patients with colon cancer (35) and patients with posttraumatic chronic bone and joint infections (36). It was isolated from a stool sample of a morbidly obese French patient living in Marseille, using the culturomics approach, which is critical to deciphering the links among gut microbiota and obesity (37). Recent reports link *Sutterella* with gastrointestinal diseases to induce substantial inflammation; rather, it can degrade IgA (38). *Peptostreptococcus* promotes colorectal carcinogenesis and modulates tumor immunity (39).

On the contrary, indole acrylic acid produced by commensal *Peptostreptococcus* species suppresses inflammation (40). Nevertheless, we observed that *Peptostreptococcus* was significantly higher in the diarrheal Tibetan piglets than in the other two groups. Its exact mechanism in Tibetan pigs needs to be further studied. *Pasteurella* are one of the important pathogens that infect a wide range of animals, including swine atrophic rhinitis (41), porcine respiratory disease complex (42), bovine hemorrhagic septicemia (43, 44), avian cholera (45–47) and rabbit respiratory disease (48, 49). A specific decrease in the abundance of *Lactobacillus* in diarrheal Tibetan piglets increased after antibiotic treatment in Tibetan piglets. *Lactobacillus* has been widely recognized for its role in gut microbiota, metabolism, immunity, and health maintenance (50–52).

Additionally, *Lactobacillus* is widely used in animal production because of its antibacterial activity and various biological characteristics (53). *Megasphaera* is a lactate-utilizing bacterium whose ruminal abundance is significantly elevated during fat milk depression (54), producing several short-chain fatty acids (SCFAs). These SCFAs serve as an energy source for host animals and play an important role in gut health (55). The genus *Clavibacter* harbors economically important plant pathogens, infecting crops such as potato and tomato (56, 57). Thus, our results conveyed important information that the relative abundances of pathogenic bacteria (such as *Fusobacterium*, *Butyricimonas*, *Sutterella*, *Peptostreptococcus*, *Pasteurella*) increased in the diarrheal Tibetan piglets, which disrupted the normal dynamic balance of the intestinal microbiota and led to a competitive decrease in the abundance of beneficial bacteria (*Lactobacillus*, *Megasphaera*). This phenomenon may also be the main cause of diarrhea in weaned Tibetan piglets. In addition, the intestinal microbial structure was changed by antibiotic treatment in weaned Tibetan piglets. Moreover, abundance of *Lactobacillus* was also increased significantly after antibiotic treatment.

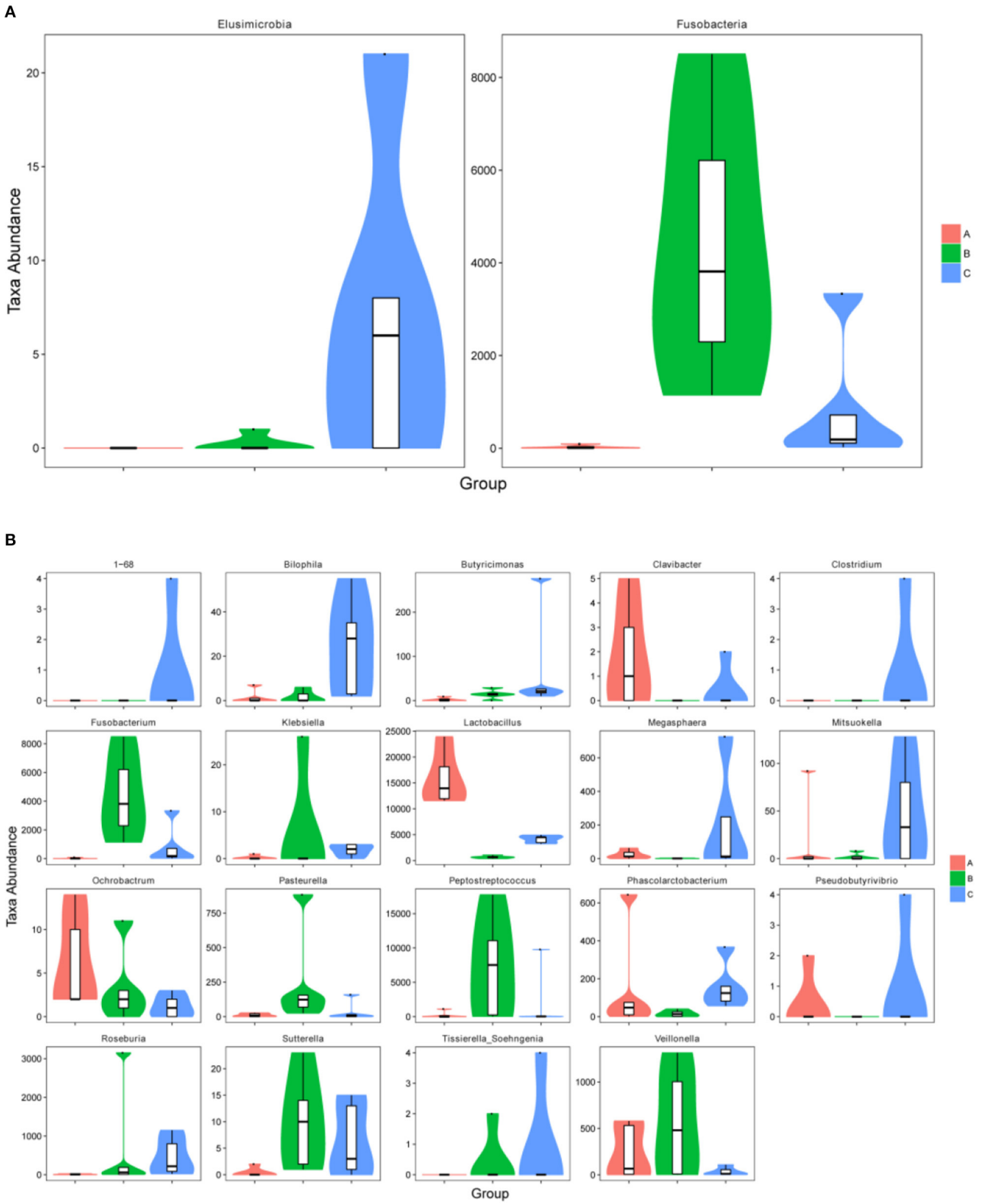

FIGURE 5 | The metastatic composition of microbial diversity. **(A)** Microbial diversity at phylum level, **(B)** microbial diversity at genus level. A: Healthy piglets; B: Diarrheal piglets; C: Treated piglets.

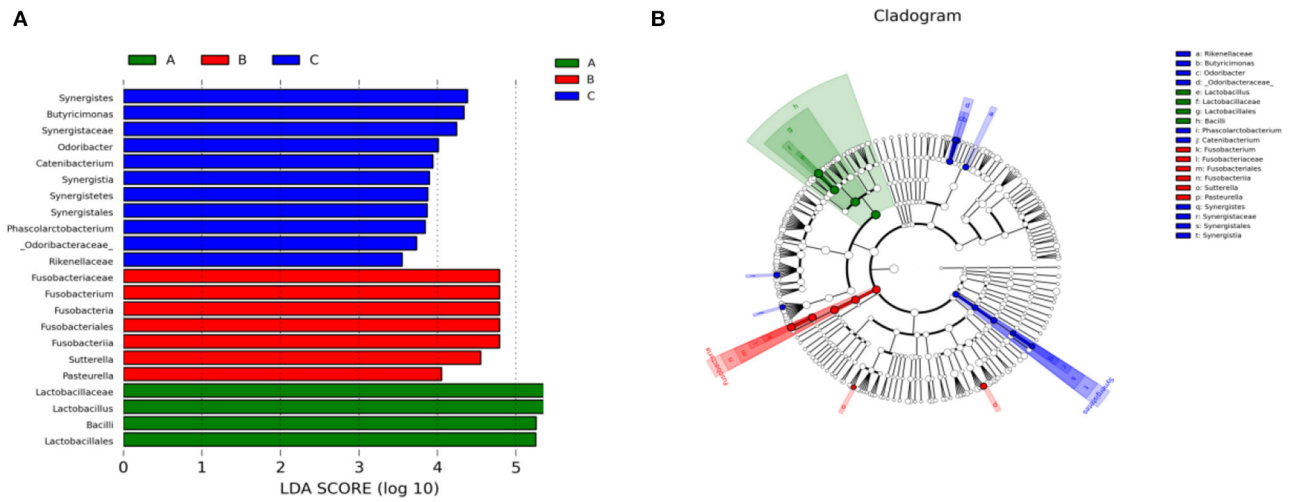

FIGURE 6 | Linear discriminant analysis effect size (LEfSe) analysis of fecal microbiota composition in different groups of Tibetan piglets. **(A)** Histogram of the Linear Discriminant Analysis (LDA) scores computed for bacterial taxa differentially abundant among different groups. **(B)** A cladogram showing statistically and physiologically consistent distinctions among different groups. A: Healthy piglets; B: Diarrheal piglets; C: Treated piglets.

Overall, there were significant difference in gut microbial composition and structure among the groups. Hence, the current study suggested that the decreased relative abundance of beneficial bacteria and increased relative abundance of pathogenic bacteria might cause diarrhea in Tibetan piglets. Therefore, this study provides a better insight into microbial population structure in order to prevent diarrhea in weaned Tibetan piglets.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA739650.

ETHICS STATEMENT

Ethical review and approval were not required for the animal study because the present used only fecal samples of Tibetan piglets. Fresh feces were collected by the Animal Care Staff (keepers) during their routine cleaning of the enclosure or directly from the soil without influencing the animals.

AUTHOR CONTRIBUTIONS

QK: conceptualization and writing original draft. WZ and MA: methodology. ZS, ZT, and YX: formal analysis and investigation. MK: review and editing. JL and SL: supervision, technical assistance, and funding. All authors participated in the writing of the manuscript, read, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.799862/full#supplementary-material

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Multidrug-Resistant *Klebsiella pneumoniae* Complex From Clinical Dogs and Cats in China: Molecular Characteristics, Phylogroups, and Hypervirulence-Associated Determinants

Zhenbiao Zhang[1,2], *Liu Zhang*[1], *Hegen Dai*[2], *Haixia Zhang*[2], *Yu Song*[2], *Qi An*[2], *Jianzhong Wang*[1]* and *Zhaofei Xia*[2]*

[1] College of Veterinary Medicine, Shanxi Agricultural University, Jinzhong, China, [2] College of Veterinary Medicine, China Agricultural University, Beijing, China

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Edited by:
Patrick Rik Butaye,
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Ulises Garza-Ramos,
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Omotayo Opemipo Oyedara,
Osun State University, Nigeria

***Correspondence:**
Jianzhong Wang
wjz@sxau.edu.cn;
jianzhongwang@cau.edu.cn
Zhaofei Xia
zhaofeixiacau@126.com

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Klebsiella pneumoniae complex is an increasingly important bacterial pathogen that is capable of causing severe organs and life-threatening disease. This study aimed to investigate the multidrug resistance, phylogroups, molecular characterization, and hypervirulence-associated determinants of the complex, which were isolated from clinical diseased dogs and cats. A total of 35 *K. pneumoniae* complex (2.3%; 95% confidence interval, 1.6–3.2) isolates were identified from 1,500 samples, all of which were collected randomly from veterinary hospitals in the 12 regions across China. Antimicrobial susceptibility testing showed that isolates were extremely resistant to amoxicillin–clavulanate (82.9%) and trimethoprim–sulfamethoxazole (77.1%). The rate of multidrug-resistant reached an astonishing 82.9% and found a carbapenemase-producing strain carrying IncX3-bla_{NDM-5} derived a cat from Zhejiang. The prevalence rates of extended-spectrum β-lactamase gene bla_{CTX-M} and plasmid-mediated quinolone resistance gene *aac(6')Ib-cr* were 51.4% and 45.7%, respectively. The resistance gene *aph(3')-Ia* of isolates from cats was more significantly ($p < 0.05$) prevalent than that from dogs. Likewise, *K. pneumoniae* complex harbored hypervirulence-associated genes *ybt* (11.4%), *iuc* (5.7%), and *iroB* (2.9%). Three (8.6%) of the 35 isolates were determined as hypermucoviscous by the string test. Lipopolysaccharide serotype O1v2 had the highest percentage of 25.7%, but capsular serotypes presented diversity distribution among the isolates. The core–genome phylogenetic tree demonstrated most of the isolates belonged to the *KpI* phylogroup (91.4%). Multilocus sequence typing analysis identified 25 different STs; ST15 and ST37 were the most abundant accounting for isolates, followed by ST307, ST656, ST1408, and ST4566. In addition, the prevalence of IncFIB-type plasmid for cat isolates was significantly higher ($p < 0.05$) than that for dogs. Sequences of IncX3 in bla_{NDM-5}-positive strain contained regions showing >99% nucleotide sequence identity to the reference plasmid pNDM-MGR194 from the human.

Keywords: ***Klebsiella pneumoniae*** **complex, dog, cat, whole-genome sequence, multidrug resistance**

INTRODUCTION

Klebsiella species, gram-negative opportunistic pathogens, commonly caused acquired antimicrobial-resistant infections in hospitals or communities. They are belonging to the Enterobacteriaceae, which includes *Escherichia*, *Salmonella*, and *Shigella*. In companion animals, *K. pneumoniae* has been reported to colonize hosts and causes extraintestinal infections, such as urinary tract infections, pyometra, upper respiratory tract infections, and bloodstream infection (septicemia) (1, 2). The treatment of these infections was often difficult because of the emergence of antibiotic resistance, which may be associated with high morbidity and mortality rate (3). In recent years, multidrug-resistant (MDR), carbapenem-resistant *K. pneumonia* (CRKP), and hypervirulent strains (hvKP) spread widely as a critical public health threat in China and even the world (4).

The high incidence of *K. pneumoniae* antimicrobial resistance rate (AMR) received increasing attention, mainly due to the continuous increase in deaths associated with AMR clone produced by CRKP and hvKP. Most of the new AMR genes discovered in the past two decades were first detected and then spread widely among gram-negative bacterial pathogens, including the extended-spectrum β-lactamase (ESBL) forms of $bla_{\mathrm{CTX-M}}$ and bla_{SHV}, the carbapenemases bla_{KPC} and bla_{NDM}, and most recently *mcr-1*, the first plasmid-borne gene associated with colistin resistance (5). The emergence of these AMR genes from *K. pneumoniae* not only increases the risk of failure for human antibacterial treatment but also affects that for companion animals. Unfortunately, if the bacteria were transmitted from the pets to their owners, the antimicrobial bacteria from companion animals may have an important impact on human public health (6).

The new technology of molecular strain typing based on DNA sequencing provides various opportunities for elucidating the structure of the *K. pneumoniae* population (7). Multilocus sequence typing (MLST) provided a standardized and replicable system for *K. pneumoniae* identification and naming, which is based on chromosomally encoded seven housekeeping genes (*rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB*, and *tonB*) (8). Whole-genome sequencing (WGS) could identify closely related species in clinical and research laboratories that have an average nucleotide homology of 95–96% with *K. pneumoniae* through biochemical or proteomic analysis (9, 10). Sequencing of *wzi* alleles was a marker of capsule serotype (KL), which is highly predictive of capsule (K) serotype and had a strong correlation with KL/K type (11, 12). While O antigen of lipopolysaccharide (LPS) has been defined by sequence identity in the conserved *wzm* and *wzt* genes (13).

The members of the *K. pneumoniae* complex were first distinguished based on the *gyrA* and were designated as the phylogenetic group of *K. pneumoniae*. WGS confirmed that the average nucleotide consistency of the whole genome is ≥3%, which is sufficient to specify new species and help identify other member species: *Klebsiella pneumoniae* (Kp1/*KpI*), *Klebsiella quasipneumoniae* subspecies *quasipneumoniae* (Kp2/*KpIIa*), *K. quasipneumoniae* subspecies *similipneumoniae* (Kp4/*KpIIb*), *Klebsiella variicola* subspecies *variicola* (Kp3/*KpIII*), *K. variicola* subspecies *tropica* (Kp5), *Klebsiella quasivariicola* (Kp6), and *Klebsiella africana* (Kp7) (14, 15). *K. variicola*, an emerging pathogen in humans and had been reported numerous infections worldwide but with a lower frequency in wild and companion animals, can also display the hypermucoviscous (hmKv) and/or hypervirulent (hvKv) phenotypes (16, 17). *K. quasipneumoniae* is a new species discovered in recent years. It has extensive kinship with *K. pneumoniae* and *K. variicola*. *K. quasipneumoniae* is also viscous and easy to acquire resistance and has certain high virulence characteristics (18). In this study, we represented a molecular characterization of *K. pneumoniae* complex isolates collected from diseased companion animals as part of a national surveillance program from different regions in China and examined their epidemiological relatedness.

MATERIALS AND METHODS

Sample Collection

Between November 2017 and October 2019, a total of 1,500 clinical specimens with suspicious bacterial infections were collected from dogs ($n = 835$) and cats ($n = 665$) in veterinary hospitals. These hospitals were distributed in the following 26 regions in China: Guangdong, Shandong, Shanghai, Tianjin, Liaoning, Jiangsu, Hubei, Henan, Hebei, Sichuan, Hunan, Zhejiang, Fujian, Heilongjiang, Inner Mongolia, Shanxi, Shaanxi, Chongqing, Ningxia, Anhui, Jilin, Guangxi, Jiangxi, Hainan, Gansu, and Xinjiang. The sampling sites were not disinfected before collection, and the hosts of all samples came to the veterinary hospital for the first time, or they had been more than 2 months since the last antibiotic administration. In addition, the sampling of companion animals was conducted following the principles of the China Agricultural University Animal Ethics Committee document (no. AW01017102-2). All samples consisted of urine (37.1%, 557/1,500), abscess (14.3%, 215/1,500), skin (8.8%, 132/1,500), ear swabs (5.9%, 88/1,500), nasal swabs (5.4%, 81/1,500), coelomic fluid (5.2%, 78/1,500), throat swabs (4.7%, 71/1,500), surgical infection (4.7%, 70/1,500), tracheal lavage (2.7%, 41/1,500), pyometra (1.2%, 18/1,500), oral swabs (1.2%, 18/1,500), blood (1.1%, 16/1,500), vaginal swabs (0.9%, 14/1,500), eye secretion (0.9%, 13/1,500), and other samples (5.86%, 88/1,500) that contained anal swabs, prostatic fluid, synovial fluid, foreskin swabs, cerebrospinal fluid, and bile.

Bacterial Isolation and Identification

The collected samples were evenly inoculated on sterile MacConkey inositol adonitol agar (HopeBio, Qingdao, China) containing 100 mg/L carbenicillin and then incubated at 37°C overnight. Clones with the red center were inoculated into a 1-mL volume of brain–heart infusion broth medium (Land Bridge, Beijing, China) and cultured 12 h at 37°C with shaking (200 revolutions/min). DNA of the bacterial solution was extracted by TIANamp Bacteria DNA Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. Subsequently, the DNA was used as the template for polymerase chain reaction amplification of 16S rDNA gene as previously described (19), and amplicons were sequenced to confirm bacterial genus using the BLAST algorithm.

Antimicrobial Susceptibility Testing

All *K. pneumoniae* complex isolates were subjected to antimicrobial susceptibility testing using the agar/broth dilution method with 16 clinically relevant antibiotics of 10 categories (amoxicillin–clavulanate, piperacillin–tazobactam, ceftazidime–avibactam, cefotaxime, cefepime, meropenem, imipenem, aztreonam, ciprofloxacin, enrofloxacin, gentamicin, amikacin, doxycycline, colistin, florfenicol, and trimethoprim–sulfamethoxazole). *Escherichia coli* ATCC 25922 was used as a quality control organism. Results of minimum inhibitory concentrations (MICs) were explained according to the breakpoints of Clinical and Laboratory Standards Institute documents VET08-ED4:2018 /M100-ED30:2020 and European Committee on Antimicrobial Susceptibility Testing (EUCAST) documents (version 9.0, 2019).

String Test

String test was conducted to define hypermucoviscous phenotype as previously described (20). All tested strains were cultured on 5% sheep blood agar plates and incubated overnight at 37°C. A standard bacteriological inoculation loop was used to touch the colonies lift gently. A viscous string ≥5 mm in length by stretching bacterial colonies was defined as string test positive.

WGS and Molecular Analysis

Genomic DNA was extracted from all *K. pneumoniae* complex using TIANamp Bacteria DNA Kit according to the manufacturer's instruction. DNA library was established using KAPA HyperPrep kit (Roche, Basel, Switzerland), and sequencing was performed on the HiSeq X Ten platform (Illumina) with 150-bp paired-end reads by Annoroad Genomics Co., Ltd. The draft genomes were assembled using SPAdes (version 3.9.0) (21). All WGS data for this work were deposited in the GenBank and under BioProject accession no. PRJNA685900. Plasmid Inc types, antibiotic resistance genes, and virulence genes were identified using abricate (https://github.com/tseemann/abricate). Sequence types (STs) of *K. pneumoniae* were determined by the bioinformatics tool at https://cge.cbs.dtu.dk/services/MLST, and *K. variicola* was evaluated through the professional *K. variicola* MLST system (http://mlstkv.insp.mx) (22). Harvest package (version 1.1.2) (23) was used to demonstrate a phylogenetic tree of core–genome alignments for all assembled genomes and visualized using Interactive Tree of Life (http://itol.embl.de/) with the corresponding features of each isolate. Capsule serotype and O antigen were analyzed using Kleborate (https://github.com/katholt/Kleborate). A minimum spanning tree of all STs was generated by BioNumerics version 7.6 (Applied Maths, Belgium) using the BURST algorithm. BLAST Ring Image Generator (24) was used to compare the genetic background of different bla_{NDM}-carrying plasmids.

Statistical Analysis

Statistical significance was determined using χ^2 test and Fisher exact test in SPSS Statistics (version 22; IBM Corporation), and the level of significance was set at $p < 0.05$.

RESULTS

Prevalence and Distribution of *K. pneumoniae* Complex

Overall, 35 *K. pneumoniae* complex [35 of 1,500; 2.3%; 95% confidence interval (CI), 1.6–3.2] isolates were identified from 1,500 samples, which collected randomly from veterinary hospitals in the 12 regions across China (**Figure 1**), including 19 from dogs [19 of 835 (2.3%); 95% CI, 1.4–3.5] and 16 from cats [16 of 665 (2.4%); 95% CI, 1.4–3.9]; no significant difference among animals ($p = 0.868$) (**Table 1**). Two [2 of 35 (0.06%); 95% CI, 0.01–0.19] of *K. variicola* (Kp34 and Kp87) were, respectively, isolated in urine (cat from Shanghai) and skin swab (dog from Shandong). The *K. quasipneumoniae* (Kp36) was obtained from urine of dog from Guangdong (**Figure 1**). In female companion animals, the isolated rate of *K. pneumoniae* complex from cats was significantly higher than that from dogs ($p = 0.038$). Among samples from cats, most *K. pneumoniae* complex was isolated from urine [8 of 325 (2.5%); 95% CI, 1.1–4.8]. The most strains were isolated from throat swabs of dogs [6 of 56 (10.7%); 95% CI, 4.0–21.9]. Regarding the regions, majority of *K. pneumoniae* complex was isolated from Guangdong (**Figure 1**), where the largest number of samples was collected. The complex isolates were six of 306 (2.0%; 95% CI, 0.7–4.2) from dogs and 10 of 348 (2.9%; 95% CI, 1.4–5.2) from cats ($p = 0.451$), respectively. No significant difference was found in the prevalence of *K. pneumoniae* complex from different sources and regions between dogs and cats ($p > 0.05$) (**Table 1**).

Antimicrobial Susceptibility and Resistance

Of these *K. pneumoniae* complex from companion animals, resistance detection rate for dogs (1.9%; 95% CI, 1.1–3.1; $n = 16/835$) was not statistically different from the rate of cats (2.4%; 95% CI, 1.4–3.9; $n = 16/665$) ($p = 0.514$). Antimicrobial susceptibility testing of the 35 isolates showed that they were extremely resistant to amoxicillin–clavulanate (82.9%, $n = 29/35$) and trimethoprim–sulfamethoxazole (77.1%, $n = 27/35$) (**Table 2**). There were 29 isolates resistant to more than three categories of antibiotics; the rate of MDR reached an astonishing 82.9%, and the MDR rate was no different between dogs and cats ($p = 0.187$). MIC_{50} and MIC_{90} of strains from cats were generally greater than or equal to dogs. Among them, the MIC_{50} of amoxicillin and that of cefepime to cat strains were twice that of dog strains; cefotaxime and amikacin were four times, and aztreonam and enrofloxacin were 32 times. However, the florfenicol-MIC_{50} of dog isolates was 16 times higher than that of cats. Besides, the MIC_{90} values of ciprofloxacin and amoxicillin–clavulanate of strains from cats were two and four times higher than those of dog strains, respectively. *K. pneumoniae* complex from cats showed higher resistance frequencies against all antibiotics than that from dogs ($p > 0.05$) (**Table 2**). Unfortunately, there was one CRKP isolated from a cat in Zhejiang, which was concurrently resistant to all tested antibiotics except aztreonam and colistin. Also, two strains were resistant (MIC = 32 mg/L and MIC = 8 mg/L) to colistin according to EUCAST.

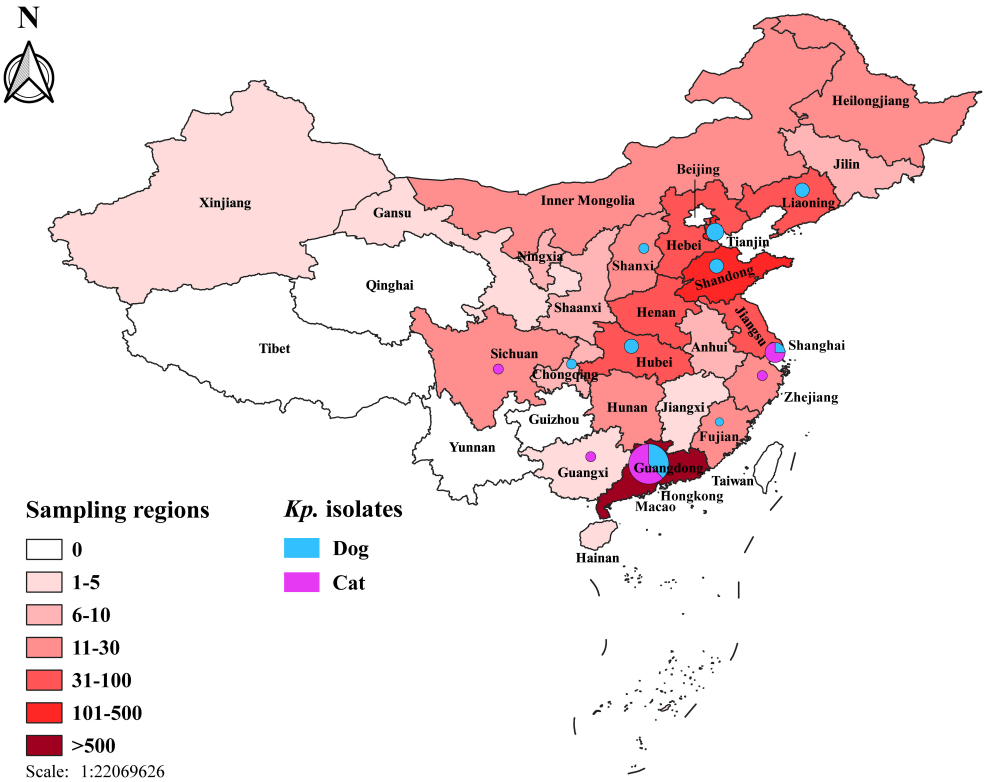

FIGURE 1 | Map of the sampling locations and the prevalence of *K. pneumoniae* complex isolates among dogs and cats in the 26 regions across China. The color gradation stands for the number of samples in different regions. Numbers and the percentages of *K. pneumoniae* complex were demonstrated using isometric pie charts.

Genotypes of Resistance and Virulence

Carbapenem resistance gene $bla_{\mathrm{NDM-5}}$ was harbored in one isolate of abscess from cat in Zhejiang, but it was not detected in dogs, and other bla_{NDM} variants were not found (**Figure 2**). The bla_{SHV} (91.4%, $n = 32/35$) were the most prevalent resistant genes in companion animals. ESBL gene $bla_{\mathrm{CTX-M}}$ was the second commonly present in dogs (22.9%, $n = 8/35$) and cats (28.6%, $n = 10/35$), respectively ($p = 0.315$). There were seven CTX-M genotypes (−3, −14, −15, −27, −55, −65, −122), dominated by $bla_{\mathrm{CTX-M-15}}$ (22.9%, $n = 8/35$) and $bla_{\mathrm{CTX-M-55}}$ (10.3%, $n = 4/35$). There was one strain with coexisting $bla_{\mathrm{CTX-M-55}}$ and $bla_{\mathrm{CTX-M-122}}$, which were isolated from tracheal lavage of a dog in Guangdong. The other ESBL and AmpC-containing isolates harbored bla_{OXA} (25.7%, $n = 9/35$) and bla_{DHA} (5.7%, $n = 2/35$). The bla_{LEN} ($n = 2$) and bla_{OKP} ($n = 1$) were only harbored by *K. variicola* (Kp34 and Kp87) and *K. quasipneumoniae* (Kp36). Aminoglycoside-non-susceptible isolates frequently harbored *aph(3″)-Ib* (45.7%, $n = 16/35$) and along with *aph(6)-Id*. The resistance gene *aph(3')-Ia* of isolates from cats was more significantly ($p = 0.042$) prevalent than that from dogs. Among the assessed plasmid-mediated quinolone resistance genes, *aac(6')Ib-cr*, *qnrB*, *qnrS*, and *oqxAB* were 16 (45.7%, $n = 16/35$), 11 (31.4%, $n = 11/35$), 11 (31.4%, $n = 11/35$), and 35 (100%, $n = 35/35$), respectively. The prevalence of other resistance genes that we obtained was also severely resistant gene; *fosA*, *dfrA*, and *sul1* were prevalent in isolates (**Figure 2**).

In addition, *K. pneumoniae* complex harbored 11.4% ($n = 4/35$) yersiniabactin (*ybtA/E/P/Q/S/T*), 5.7% ($n = 2/35$) aerobactin (*iucA/B*), and 2.9% ($n = 1/35$) salmochelin (*iroB/C/D/N*), but other key virulence genes such as colibactin (*clb*) and regulators of mucoid phenotype genes (*rmpA/A2*) were not detected. Gene *ybt* and *iuc* coexisted in strain Kp96 (ST24), which was from an ear swab of a dog in Shandong (**Figure 2**). Besides, virulence-associated genes such as enterotoxins (*astA*, *entA/B*), ferrienterochelin receptor (*fepC*), yersiniabactin receptor (*fyuA*), yersiniabactin biosynthesis (*irp1/2*), aerobactin receptor (*iutA*), outer membrane protein (*ompA*), and common pili (*ecpA/B/C/D/E/R*) were identified using abricate and demonstrated in **Figure 2**, and there was no significant difference in virulence-associated genes between dogs and cats ($p > 0.05$).

Hypermucoviscosity, Capsule Serotype, and O Antigen of *K. pneumonia* Complex

Three of the 35 isolates (8.6%) were determined to be hypermucoviscous *K. pneumonia* (hmKp) by the string test. One of the hmKp that indicated strong virulence had a KL2 capsular serotype and O1v1 LPS serotype, and the remaining two strains were serotype KL9/O2v2 and KL127/OL101 (**Figure 2**).

TABLE 1 | The prevalence and distribution of *K. pneumoniae* complex from dogs and cats.

Parameters	Category	Dogs		Cats		*p*-value‡
		No. of samples (%)	*Kp* isolates (%, 95% CI)	No. of samples (%)	*Kp* isolates (%, 95% CI)	
Origin	Animals	835 (55.7)	19 (2.3, 1.4–3.5)	665 (44.3)	16 (2.4, 1.4–3.9)	0.868
Gender	Male	475 (56.9)	17 (3.6, 2.1–5.7)	478 (71.9)	10 (2.1, 1.0–3.8)	0.167
	Female	360 (43.1)	2 (0.6, 0.1–2.0)	187 (28.1)	6 (3.2, 1.2–6.9)	0.038*
Source	Urine	232 (27.8)	5 (2.2, 0.7–5.0)	325 (48.9)	8 (2.5, 1.1–4.8)	0.813
	Throat swab	56 (6.7)	6 (10.7, 4.0–21.9)	15 (2.3)	1 (6.7, 0.2–31.9)	1.000
	Abscess	138 (16.5)	4 (2.9, 0.8–7.3)	77 (11.6)	1 (1.3, 0–7.0)	0.784
	Nasal cavity	39 (4.7)	0	42 (6.3)	1 (2.4, 0.1–12.6)	1.000
	Ear swab	49 (5.9)	1 (2.0, 0.1–10.9)	39 (5.9)	0	1.000
	Tracheal lavage	30 (3.6)	1 (3.3, 0.1–17.2)	11 (1.7)	0	1.000
	Coelomic fluid	34 (4.1)	0	44 (6.6)	1 (2.3, 0.1–12.0)	1.000
	Skin	97 (11.6)	2 (2.1, 0.3–7.3)	35 (5.3)	2 (5.7, 0.7–19.2)	0.613
	Surgical infection	50 (6.0)	0	20 (3.0)	1 (5.0, 0.1–24.9)	0.286
	Eye secretion	7 (0.8)	0	6 (0.9)	1 (16.7, 0.4–64.1)	0.462
	Other specimens	103 (12.3)	0	51 (7.7)	0	—
Regions	Guangdong	306 (36.6)	6 (2.0, 0.7–4.2)	348 (52.3)	10 (2.9, 1.4–5.2)	0.451
	Shanghai	52 (6.2)	1 (1.9, 0–10.3)	84 (12.6)	3 (3.6, 0.7–10.1)	0.975
	Tianjin	87 (10.4)	3 (3.4, 0.7–9.7)	40 (6.0)	0	0.551
	Hubei	23 (2.8)	2 (8.7, 1.1–28.0)	13 (2.0)	0	0.525
	Liaoning	43 (5.1)	2 (4.7, 0.6–15.8)	17 (2.6)	0	1.000
	Shandong	114 (13.7)	2 (1.8, 0.2–6.2)	54 (8.1)	0	1.000
	Fujian	7 (0.8)	1 (14.3, 0.4–57.9)	15 (2.3)	0	0.318
	Guangxi	2 (0.2)	0	3 (0.5)	1 (33.3, 0.8–90.6)	1.000
	Shanxi	14 (1.7)	1 (7.1, 0.2–33.9)	2 (0.3)	0	1.000
	Sichuan	15 (1.8)	0	13 (2.0)	1 (7.7, 0.2–36.0)	0.464
	Zhejiang	17 (2.0)	0	6 (0.9)	1 (16.7, 0.4–64.1)	0.261
	Chongqing	6 (0.7)	1 (16.7, 0.4–64.1)	2 (0.3)	0	1.000
	Other regions	149 (17.8)	0	68 (10.2)	0	—

Data is the prevalence and distribution of samples and K. pneumoniae complex from companion animals. The p-values are for comparisons of K. pneumoniae complex between dogs and cats.

‡*p-values were determined by Chi-square (χ^2) and Fisher's exact test in SPSS Statistics.*

*$p < 0.05$.

**$p < 0.01$.

—, *no data.*

Isolates covered 26 capsular serotypes and presented diversity distribution. KL19 was the most abundant (8.6%, $n = 3/35$), accounting for three isolates, followed by KL2, KL25, KL51, KL53, KL102, and KL107, and two *K. pneumonia* strains with KL14 but match confidence was none (**Figures 2**, **3A**). While O1v2 had the highest percentage of 25.7% ($n = 9/35$) and then O3b, O2v2, O1v1, and OL101 (**Figure 3B**). The O antigens of three-string test–positive isolate were O1v1, O2v2, and OL101, respectively. O antigen named OL101 onward was defined on the basis of gene content and was not yet associated with a specific serologically defined O type.

MLST and Phylogenetic Analysis

Core–genome phylogenetic tree demonstrated most of the isolates belonged to *KpI* phylogroup (91.4%, $n = 32/35$), with only one being *KpIIa* and two appertained to the *KpIII* phylogroup (**Figure 2**). MLST analysis identified 25 different STs among the 35 *K. pneumoniae* complex; the diversity was similar to the capsular serotypes, and five of them were novel STs (ST4566, ST4568, ST4569, ST4571, ST4581). ST15 and ST37 (11.4%, $n = 4/35$) were the most abundant accounting for isolates and followed by ST307, ST656, ST1408, and ST4566. The STs of *K. variicola* Kp34 and Kp87 were ST42 and ST54; they were from the urine of cat and a skin swab of dog, respectively (**Figure 2**). ST of bla_{NDM-5}-positive *K. pneumoniae* was ST11. Apart from the *KpIIa* phylogroup, each lineage comprised strains from dogs and cats. Minimum spanning tree analysis further supported the commonality of *K. pneumoniae* complex from different sources and regions with the same STs (**Figures 3C,D**).

Plasmid Profiles and Genetic Context of bla_{NDM}

The backbone sequences were assembled, and all contigs and gaps were identified by WGS analyses. Through comparison

TABLE 2 | Antimicrobial resistance of clinical *K. pneumoniae* complex isolates from dogs and cats.

Antimicrobial agents	All isolates ($n = 35$)	Dog ($n = 19$)			Cat ($n = 16$)			*p*-value‡
	Resistance, %	MIC_{50}	MIC_{90}	Resistance, %	MIC_{50}	MIC_{90}	Resistance, %	
Amoxicillin-clavulanate	29 (82.9)	32/16	64/32	14 (73.7)	64/32	>256/128	15 (93.8)	0.187
Piperacillin-tazobactam	14 (40.0)	4/4	>256/4	5 (26.3)	32/4	>256/4	9 (56.3)	0.094
Ceftazidime-avibactam	1 (2.9)	0.25/4	0.5/4	0	0.25/4	1/4	1 (6.3)	0.457
Cefotaxime	22 (62.9)	64	>256	10 (52.6)	256	>256	12 (75.0)	0.293
Cefepime	17 (48.6)	4	>256	9 (47.4)	8	256	8 (50.0)	1.000
Meropenem	1 (2.9)	0.06	0.125	0	0.06	0.125	1 (6.3)	0.457
Imipenem	1 (2.9)	0.5	1	0	0.5	1	1 (6.3)	0.457
Aztreonam	20 (57.1)	4	>256	9 (47.4)	128	>256	11 (68.8)	0.306
Ciprofloxacin	22 (62.9)	2	128	10 (52.6)	32	256	12 (75.0)	0.293
Enrofloxacin	21 (60.0)	1	64	9 (47.4)	32	64	12 (75.0)	0.166
Gentamicin	22 (62.9)	64	>256	11 (57.9)	64	>256	11 (68.8)	0.727
Amikacin	11 (31.4)	2	>256	4 (21.1)	8	>256	7 (43.8)	0.273
Doxycycline	23 (65.7)	32	64	11 (57.9)	32	64	12 (75.0)	0.476
Colistin	2 (5.7)	2	2	0	2	2	2 (12.5)	0.202
Florfenicol	23 (65.7)	256	>256	13 (68.4)	16	256	10 (62.5)	0.736
Trimethoprim-sulfamethoxazole	27 (77.1)	>32/608	>32/608	14 (73.7)	>32/608	>32/608	13 (81.3)	0.700

‡*p-values were determined by Fisher's exact test in SPSS Statistics.*
*$p < 0.05$.
**$p < 0.01$.

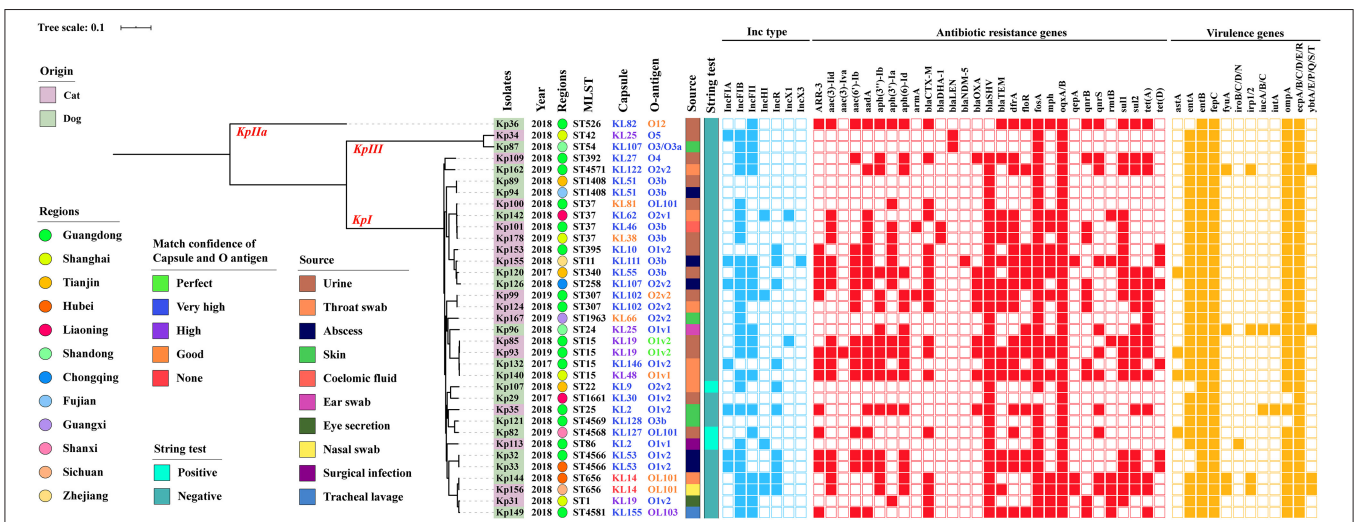

FIGURE 2 | Core genome-based phylogenetic tree and distribution of *K. pneumoniae* complex phylogroups, origins, isolated years, collected regions, MLST, capsular serotype, LPS O antigen, isolate sources, string test, Inc-type plasmids, antibiotic resistance genes, and virulence genes among isolates from companion animals. The origins, regions, KL/O match confidence, sources, and string test of *K. pneumoniae* complex are differentiated by color. Inc-type plasmids, antibiotic resistance genes, and virulence genes are denoted by filled squares for the presence and empty squares for absence.

and monitoring, we obtained seven Inc-type plasmids, which were dominant by IncFIB (77.1%, $n = 27/35$), IncFII (54.3%, $n = 19/35$), IncR (28.6%, $n = 10/35$), followed by IncFIA (20%, $n = 7/35$), IncHI (14.3%, $n = 5/35$), IncX1 (5.7%, $n = 2/35$), and IncX3 (2.9%, $n = 1/35$) (**Figure 2**). The prevalence of IncFIB plasmid for cat isolates was significantly higher than that for dogs ($p = 0.047$). In addition, BLASTn results demonstrated that bla_{NDM-5} was harbored on IncX3-type in plasmid bla_{NDM-5}-positive strains (Kp155), which also contains IncFIA, IncFIB, IncHI, and IncR (**Figure 2**). Sequences of IncX3 in Kp155 (ST11), which is a source of abscess derived from cat in Zhejiang, contained regions showing >99% nucleotide sequence identity to the reference plasmid pNDM-MGR194 (46253bp, GenBank accession no. KF220657). And bla_{NDM-5} was included in an insertion sequence (IS) cassette (ΔIS*Aba125*-IS*5*-bla_{NDM}-*ble-trpF-dsbC*-IS*26*) compared by ISfinder, which

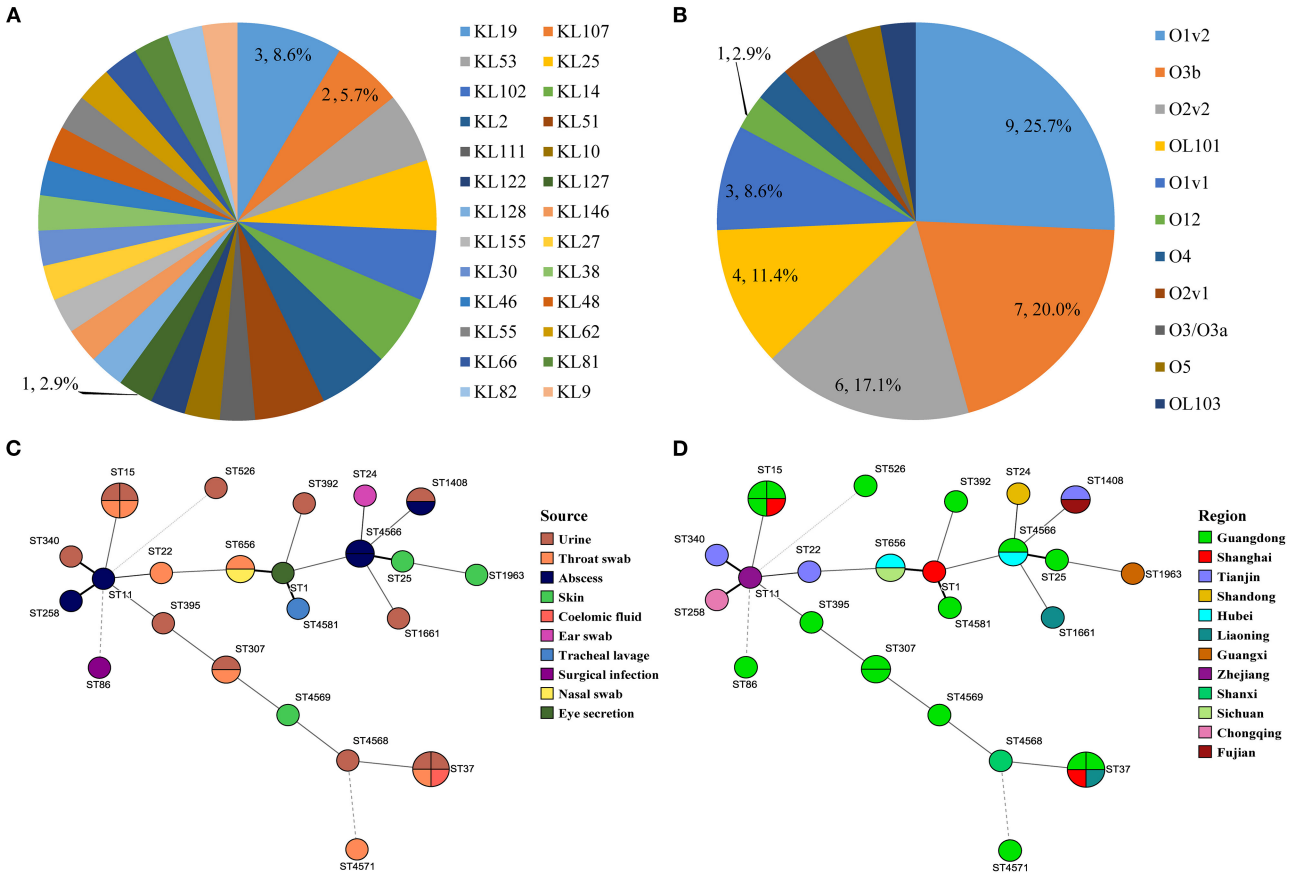

FIGURE 3 | Serotype distribution of capsular serotype and O antigen **(A,B)**. Minimum spanning trees of MLST data (except *K. variicola*) based on source and region **(C,D)**.

was consistent with bla_{NDM-5}-carrying plasmids originating from human (pQDE2-NDM, MH917280), dog (pP16NDM-502, MN701974), chicken (p1079-NDM, MG825384), and goose (pL65-9, CP034744) (**Figure 4**).

DISCUSSION

China Antimicrobial Surveillance Network (CHINET) has well-documented the antimicrobial resistance of humans in China (http://www.chinets.com/). However, investigations on companion animals are still lacking in this regard in China. In the current study, we collected 1,500 clinical samples of companion animals for the isolation of *K. pneumoniae* complex and investigated the prevalence of antibiotic resistance, virulence, and molecular typing through whole-genome analysis. Thirty-five *K. pneumoniae* (2.3%) complex was identified from 1,500 samples, which was a lower isolation rate compared with Italy (3.53%) (25). There was no significant difference among animals, 19 from dogs and 16 from cats; the isolation rate of *K. pneumoniae* complex from cats was significantly higher than that from dogs in female animals. But the rate of MDR, 82.9%, was higher than that reported in Singapore (50%) (26). MIC_{50} and MIC_{90} of antibiotics to *K. pneumoniae* complex from cats were generally greater than or equal to that from dogs, suggesting that the resistance of feline strains was more severe than that of dogs. The results were consistent with previous investigations in Iberian Peninsula (27) and China (28). *K. pneumoniae* complex from cats showed no significant difference ($p > 0.05$) against all antibiotics compared with that from dogs, which was consistent with the previous study in South Korea (29). In our study, the resistance of amoxicillin–clavulanate and trimethoprim–sulfamethoxazole were 82.9% and 77.1%, all generally higher than those reported in Portugal (30). One CRKP was resistant to meropenem and imipenem and harbored carbapenemase gene bla_{NDM-5}, which has been reported in humans (31) and other animals (32), but rarely detected in companion animals.

We found a higher prevalence of ESBLs in *K. pneumoniae* complex clinical isolates (57.1%, $n = 20/35$), compared with those from companion animals in Japan (34.8%) (1), Italy (21.4%) (25), Germany, and other European countries (7.6%) (33). In this study, ESBLs were CTX-M-genotypes (−3, −14, −15, −27, −55, −65, −122) and SHV-genotypes (−41, −42), which was not quite the same as that in previous reports (34). Among CTX-M genotypes, which were dominant by

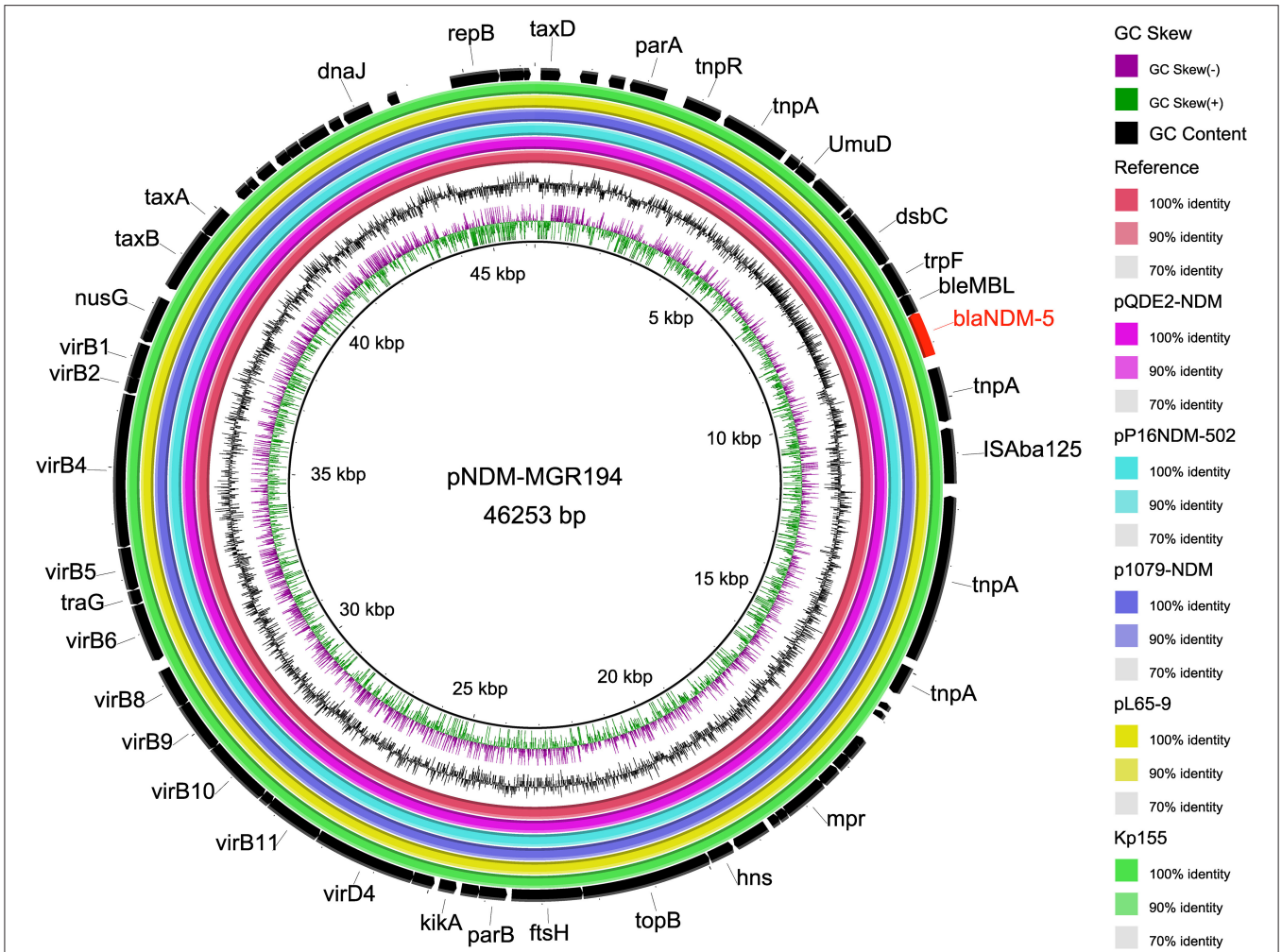

FIGURE 4 | Comparison of whole-genome sequences of plasmid IncX3 carrying bla_{NDM-5}. Each ring represents an isolated strain. The internal ring is the reference sequence of NDM-5 carrying plasmid pNDM-MGR194 (46253bp, GenBank accession no. KF220657), and the outside rings are the other five plasmids, including Kp155 from this study. From inside to outside, the rings were derived from human (pQDE2-NDM, MH917280), dog (pP16NDM-502, MN701974), chicken (p1079-NDM, MG825384), and goose (pL65-9, CP034744).

$bla_{CTX-M-15}$ (22.9%, n = 8/35) and $bla_{CTX-M-55}$ (10.3%, n = 4/35), there was one strain with coexisting $bla_{CTX-M-55}$ and $bla_{CTX-M-122}$. In particular, the CTX-M ESBL genes were widely present in the field of human medicine, and the CTX-M-15–producing *K. pneumoniae* complex was the most frequently detected genotype associated with extended-spectrum antibiotic resistance in humans and animals (35). Because of the close contact between companion animals and humans, the genotypes of *K. pneumoniae* complex from companion animals in this study were similar to those of humans in China. Otherwise, bla_{SHV} (91.4%) was the most prevalent resistant genes, which were constituted by SHV-187 (28.6%, n = 10/35), −106 (17.1%, n = 6/35), −110 (14.3%, n = 5/35), −182 (11.4%, n = 4/35), −145 (5.7%, n = 2/35), and SHV-11,−28,−41,−42, and−62 (each 2.9%, 1/35) in our study. The other ESBL and AmpC-containing isolates harbored bla_{OXA} (25.7%, n = 9/35) and bla_{DHA} (5.7%, n = 2/35). The bla_{LEN} (n = 2) and bla_{OKP} (n = 1) were only harbored by *K. variicola* (Kp34 and Kp87) and *K. quasipneumoniae* (Kp36). *K. variicola* has been widely recognized as an important opportunistic human pathogen commonly involved in hospital-acquired infections; multiple antibiotic resistance genes have been shown to exist, such as clinically relevant resistance determinants such as bla_{CTX-M}, bla_{DHA}, and bla_{LEN}. The bla_{LEN} gene corresponds to an intrinsic chromosomal β-lactamase in the *K. variicola* genome (36). Meanwhile, bla_{DHA} has been indicated to cause the resistance of *K. pneumoniae* complex in companion animals from a veterinary hospital in Switzerland (37). Population diversity studies have shown that *K. pneumoniae* is phylogenetically closely related to *K. variicola* and *K. quasipneumoniae* (38). The bla_{OKP} β-lactamases, closely related to bla_{SHV}, and the OKP type enzyme were also clearly found in the phylogenetic group *KpII* of *K. quasipneumoniae* (39). Some variant *K. variicola* has been identified from multiple sources, including

environments; humans; animals such as dogs, birds, monkeys, and cattle (mastitis); and plants such as coriander (food supply). As potential pathogens of zoonotic, *K. variicola* from companion animals could be transmitted to humans (16).

Hypervirulence and hypermucoviscosity are two different *K. pneumoniae* phenotypes; they could predict positive value by the string test and molecular markers such as the virulence genes *ybt*, *clb*, *iuc*, *iro*, *rmpA*, and *rmpA2* (40). Three of (8.6%, $n = 3/35$) the 35 isolates were determined to be hypermucoviscous by string test in our study. Kleborate is a tool to screen genome assemblies of *K. pneumoniae* complex and the complex for integrative conjugative element–associated virulence loci (*ybt*, *clb*) and virulence plasmid–associated loci (*iro*, *iuc*, *rmpA*, *rmpA2*) (41). In this study, one isolate of ST24 harbored *ybt* and *iuc*, which derived an ear swab from a dog in Shandong, and its virulence gene score was evaluated by Kleborate as four. Also, one other strain scored 3 because it harbored *iuc*, and three isolates appeared *ybt*, so it was scored 1. The capsular polysaccharide is located outside the outer membrane; the most common hvKp capsule types are K1, K2, K5, K20, K54, and K57, of which K1 and K2 account for ∼70% of hvKp isolates. Otherwise, hvKp strain also has the O antigen, which is part of LPS. K1 and K2 capsule types are usually related to the O1 O antigen type (42). In the current study, capsular presented diversity distribution and covered 26 serotypes, which were predominated by KL19. One of the hmKp that indicated strong virulence had a KL2 capsular serotype and O1v1 LPS serotype. While LPS serotype O1v2 had the highest percentage of 25.7%, the O antigens of three-string test–positive isolate were O1v1, O2v2, and OL101, respectively.

Global problem clones have been isolated from a series of animals, such as ST11 in poultry, ST15 in companion animals, ST23 in non-human primates and horses, and ST25 in pigs (15). Not surprisingly, ST15 and ST37 (11.4%, $n = 4/35$) were the most abundant accounting for isolates in this study. Previous researches on dairy farms in the United States (43), canals or farms in Thailand (44), and farms in the North of England (45) had revealed a large amount of diversity between clinical isolates, and non-human samples. MLST analysis identified 25 different STs among the 35 *K. pneumoniae* complexes in our research, which diversity was similar to the capsular serotypes, and five of them were novel STs (ST4566, ST4568, ST4569, ST4571, ST4581). The STs of *K. variicola* Kp34 and Kp87 from companion animals were ST42 and ST54, respectively. ST42 was mainly prevalent in humans of Mexico; ST54 was dominantly found in the United States and Vietnam according to previous research (22). In China, *K. variicola* from both humans and plants has been described, with ST65 and ST92 corresponding to human isolates described in different reports (22). However, the report of *K. variicola* from the source of companion animals is the first. CRKP or MDR ST11 *K. pneumoniae* harboring KL64 or KL47 and virulence plasmids carrying *iuc* (or *rmpA2*) is widely spread in China (46). Coincidentally, the ST of plasmid IncX3-bla_{NDM-5}-positive and MDR *K. pneumoniae* was ST11 in our results. Otherwise, some researchers believe that hvKP could accumulate plasmids carrying virulence and resistance genes, which can continuously enhance its resistance to major antibiotics (47). Inc-type plasmids were analyzed and suggested that there was some correlation between MDR and plasmid type. These findings indicated that the variants of *K. pneumoniae* complex resistance genes, virulence genes, and mobile plasmid elements are not limited to certain hosts, emphasizing the need for coordinated control in the concept of One Health.

CONCLUSION

We found a high prevalence of MDR *K. pneumoniae* complex isolates from sick dogs and cats in different regions of China. The abuse of combination medications was likely contributed to that, and especially widespread use of amoxicillin–clavulanate and trimethoprim–sulfamethoxazole in the veterinary hospital. These strains harbored bla_{SHV}, bla_{CTX-M}, and bla_{NDM}, and most promoted an MDR profile. Meanwhile, the emergence of hvKP and epidemic clones has increased the risks of veterinarians. Diversity analysis of the core–genomes and STs of this clinical *K. pneumoniae* complex from different sources and regions suggested that the dissemination of Inc-type plasmids has broad reservoirs in *K. pneumoniae* complex. Relevant measures must be formulated to suppress or block transmission of high-risk *K. pneumoniae* complex clonal lineages to ensure the safety of companion animal practitioners and public health.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA685900.

ETHICS STATEMENT

The animal study was reviewed and approved by China Agricultural University Animal Ethics Committee document (No. AW01017102-2). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

ZX and JW: conceived and designed study, collected, complied, and analyzed data. LZ, HD, and HZ: statistical analyses. YS and QA: collected the clinical samples. ZZ and JW: drafted and edited manuscript. All authors contributed to the article and approved the submitted version.

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Longitudinal Monitoring Reveals Persistence of Colistin-Resistant *Escherichia coli* on a Pig Farm Following Cessation of Colistin Use

Nwai Oo Khine[1,2], *Kittitat Lugsomya*[3], *Waree Niyomtham*[1], *Tawat Pongpan*[4], *David J. Hampson*[5] *and Nuvee Prapasarakul*[1,4]*

[1] *Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand,* [2] *The International Graduate Program of Veterinary Science and Technology (VST), Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand,* [3] *Department of Infectious Diseases and Public Health, Jockey Club College of Veterinary Medicine and Life Sciences, City University of Hong Kong, Kowloon, China,* [4] *Center of Excellence in Diagnosis and Monitoring of Animal Pathogens (DMAP), Bangkok, Thailand,* [5] *School of Veterinary Medicine, Murdoch University, Perth, WA, Australia*

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***Correspondence:**
Nuvee Prapasarakul
nuvee.p@chula.ac.th

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Colistin-resistant bacteria harboring plasmid-mediated *mcr* genes are of concern as they may be a cause of serious nosocomial infections. It is hypothesized that cessation of colistin use as a feed additive for pigs will reduce the occurrence and distribution of *mcr* genes in farms. The aim of this study was to investigate this hypothesis by longitudinal monitoring and characterizing of *mcr* positive *Escherichia coli* (MCRPE) isolates after colistin was withdrawn on a central Thailand pig farm that previously had a high frequency of MCRPE. Colistin use ceased at the beginning of 2017, and subsequently 170 samples were collected from farrowing sows and suckling piglets ($n = 70$), wastewater ($n = 50$) and farm workers ($n = 50$) over a 3.5-year period. Bacteria were identified by MALDI-TOF mass spectrometry and minimal inhibitory concentrations were determined by broth microdilution. The antibiogram of *mcr* positive *E. coli* isolates was determined using the Vitek2 automated susceptibility machine, and multiplex and simplex PCRs were performed for *mcr*-1–8 genes. MCRPE containing either *mcr*-1 or *mcr*-3 were isolated from pigs throughout the investigation period, but with a declining trend, whereas MCRPE isolates were recovered from humans only in 2017. MCRPE were still being recovered from wastewater in 2020. Most MCRPE isolates possessed the virulence genes *Stap, Stb*, or *Stx2e*, reflecting pathogenic potential in pigs, and showed high rates of resistance to ampicillin, gentamicin and tetracycline. Pulsed-field gel electrophoresis and multi-locus sequence typing showed that diverse MCRPE clones were distributed on the farm. The study identified a decline of pathogenic MCRPE following withdrawal of colistin, with pigs being the primary source, followed by wastewater. However, short-term therapeutic usage of other antibiotics could enhance the re-occurrence of *mcr*-carrying bacteria. Factors including the environment, management, and gene adaptations that allow maintenance of colistin resistance require further investigation, and longer-term studies are needed.

Keywords: colistin resistance, *Escherichia coli*, *mcr* genes, longitudinal monitoring, pigs

INTRODUCTION

Colistin (polymyxin E) is one of the World Health Organization's highest priority antimicrobials: it is regarded as a last resort antibiotic, and is the treatment of choice for multidrug-resistant *Enterobacteriaceae* infections (1). Unfortunately, the emergence of mobile colistin resistance genes of the *mcr* gene family has jeopardized the efficacy of colistin. The plasmid mediated colistin resistance gene (*mcr*-1) was firstly identified in *E. coli* of porcine origin from China (2). Subsequently, other *mcr* variants including *mcr*-2 to *mcr*-10 were discovered mainly from members of the *Enterobacteriaceae* family (3–6) from different geographical areas (7, 8). The *mcr* genes encode phosphoethanolamine transferases enzymes which change the lipid A portion of the lipopolysaccharides (LPS), suppressing colistin binding (9). The *mcr* genes have been reported not only from various livestock origins (pigs, poultry, bovine) (10, 11) and food products (12) but also from the environment as well as from humans (13). Since the extensive usage of colistin in livestock farms played a major role in the occurrence of colistin resistant *mcr* genes, controlling the dissemination of these resistant genes from farms to the environment has become a critical concern (14). Moreover, *mcr* genes could be co-located with other important antibiotic resistance genes such as Extended spectrum beta-lactamase (ESBL) and carbapenemase genes (15, 16). These reports raised awareness of colistin usage and the challenge to clinical medicine.

In the swine industry, colistin had been applied therapeutically and/or prophylactically in several countries (17). Because of the importance of colistin usage in clinical infections, many countries have restricted the prophylactic usage of colistin in pig productions (18). Following the first identification of *mcr*-1 during nation-wide surveillance in Thailand (19), from the start of 2017 the Department of Livestock and Development (DLD) has prohibited prophylactic use of colistin sulfate in pig farms. Data regarding colistin resistance in bacteria from livestock in Thailand is still limited, although *mcr* positive *E. coli* have been detected in pig slaughterhouses from the Thailand border areas even after implementation of the colistin withdraw policy (20). Therefore, currently it is still debatable how this withdrawal of colistin usage may have influenced the emergence and spread of *mcr* genes in pigs and in the farm environment (21). It is thought to be unlikely that resistance plasmids can be entirely eliminated from bacterial populations (22). Previous studies have found withdrawal it was likely to be beneficial in controlling the emergence of *mcr*-1 in pigs once the selective pressure is removed (21, 23). However, the variations in duration of *mcr* gene persistence after cessation of colistin usage and the rate of dissemination from pigs to the farm environment are still concerning. Moreover, resistant bacteria from livestock can potentially spread to farmers or the environment resulting in the occurrence of antibiotic resistance genes (ARG). Furthermore, some studies have shown that even after drastic reductions of antibiotic use on farms, antibiotic resistant bacteria could be maintained in the farm by various factors (24–26).

The aim of this study was to determine the occurrence and extent of persistence of MCRPE following the cessation of colistin sulfate use on a representative pig farm which had a history of a high prevalence of MCRPE (19). Representative *E. coli* isolates that contained *mcr* genes were obtained from pigs, wastewater and farm workers over the study period and were characterized for antimicrobial susceptibility patterns, virulence factors, plasmid replicons, and clonal relationships.

MATERIALS AND METHODS

Study Area and Farm Selection

A typical industrial pig farm with more than 1,000 breeder sows located in the central area of Thailand was selected for use in this study. Prior to 2017, colistin sulfate had been administered routinely to all suckling piglets from birth to weaning to prevent and control diarrhea. It was given via the water at a dose of 10 mg/kg body weight. The farm withdrew prophylactic colistin use in piglets from the beginning of 2017, following the guidance of the DLD. The farm management systems were not otherwise altered, and they continued to follow the recommendations of the Thai standard livestock farm criteria. Piglets with diarrhea were separated from healthy piglets by placing them in separate pens until they recovered. In cases of diarrhea in breeding sows and piglets, antibiotic injections including gentamicin, ceftriaxone, and/or penicillin/streptomycin combinations were used for treatment of individual animals.

Sample Collection and Processing

The number and types of samples (from pigs, wastewater and humans) that were obtained are summarized in **Table 1**. Sample sizes were calculated based on the prevalence of *mcr* genes detected in the pig farm from our previous study (19) by using Epitools program http://epitools.ausvet.com.au. Samples were collected at five-time points spanning a 3.5 year period from cessation of colistin use: June 2017, September 2018, March 2019, April 2019, and June 2020.

Approximately 25 g of fecal samples were collected from parity 1–6 farrowing sows, aged between 1 to 3 years ($n = 50$). Rectal swab samples from 21-day old suckling piglets belonging to the sows that were sampled were also collected ($n = 20$). Each farrowing sow with their respective litters were kept in farrowing pens, and at each visit one or two sows were sampled from each zone of the farrowing house. The same pens were visited at each sampling time, although the same sows were not necessarily sampled because of animal movements. In September 2018, only fecal samples from sows were collected since at the time of sampling the newly weaned piglets had been moved to another farm.

Wastewater samples ($n = 50$) from the wastewater tanks on the farm were collected, with 10 samples obtained at each visit. The wastewater was composed of pig manure along with the water used to clean the pig housing. Approximately 500 ml volumes were collected from wastewater tanks located before and after-biogas treatment, which were sited close to the sampled pig pens. The biogas process involves anaerobic fermentation by fermentative, acetogenic, and methanogenic bacteria to produce methane, carbon dioxide, hydrogen, and hydrogen sulfide gases. In addition, at the request of the company, at

TABLE 1 | Details of the sample types and numbers collected at five different sampling times between 2017 and 2020, and numbers of samples found positive for MCRPE.

Year	Type of sample	Sampling time	Number	Age of pigs at time of sampling	Samples positive for MCRPE
2017	Farrowing sows	1	10	1–3 years	9
	Suckling piglets	1	5	21 days	0
	Wastewater (Before-biogas treatment)	1	5	–	2
	Wastewater (After biogas treatment)	1	5	–	0
	Farm workers	1	10	–	4
2018	Farrowing sows	2	10	1–3 years	5
	Suckling piglets	2	0	–	0
	Wastewater (Before-biogas treatment)	2	5	–	1
	Wastewater (After biogas treatment)	2	5	–	0
	Farm workers	2	10	–	0
2019 March	Farrowing sows	3	10	1–3 years	0
	Suckling piglets	3	5	21 days	0
	Wastewater (Before-biogas treatment)	3	5	–	0
	Wastewater (After biogas treatment)	3	5	–	0
	Farm workers	3	10	–	0
2019 April	Farrowing sows	4	10	1–3 years	1
	Suckling piglets	4	5	21 days	0
	Wastewater (Before-biogas treatment)	4	5	–	0
	Wastewater (After biogas treatment)	4	5	–	0
	Farm workers	4	10	–	0
2020	Farrowing sows	5	10	1–3 years	0
	Suckling piglets	5	5	21 days	5
	Wastewater (Before-biogas treatment)	5	5	–	5
	Wastewater (After biogas treatment)	5	5	-	1
	Farm workers	5	10	-	0

each sample collection time the farm submitted rectal swab samples from the same 10 farm workers for routine diagnostic purposes ($n = 50$).

Sampling from the pigs and the wastewater was conducted by an authorized veterinarian for the farm. The biohazard execution control was approved by the Institutional Biosafety Committee of the Faculty of Veterinary Science, Chulalongkorn University (IBC 2031011). The wastewater sample collection protocol was applied according to HACH water analysis guidelines (27).

Bacterial Isolation and Identification

All samples were enriched in EC broth (Difco) containing 2 μg/ml colistin sulfate at a 1:9 ratio and incubated at 37°C overnight. The sample suspensions were grown on eosin-methylene blue (EMB) (Oxoid) agar containing 2 μg/ml colistin sulfate and were incubated overnight. One to three representative colonies with a characteristic metallic sheen on the EMB plates were randomly chosen and sub-cultured on tryptic soy agar (TSA) (Difco) from the samples from which growth was obtained. The colonies were identified as *E.*

coli using IMViC biochemical tests and Matrix-Assisted Laser Desorption Ionization combined with time of-flight analysis (MALDI Biotyper, Bruker, USA), according to the manufacturer's recommendations (28). For minimal inhibitory concentration (MIC) determinations, antibiotic susceptibility testing, and PCR detection for virulence genes and plasmid replicon types, a single representative isolate from each positive sample was used.

Antimicrobial Susceptibility Testing

The MIC for colistin was determined using the broth microdilution technique following CLSI guidelines (29). An MIC value of >2 μg/ml was considered to indicate colistin resistance (29). The antibiogram for *E. coli* isolates was determined using the AST-GN 38 test kit in a Vitek2 compact automated susceptibility level detection apparatus (BioMérieux, France). The antimicrobial groups that were included in Vitek2 were synchronized with veterinary guidelines (30). The 18 antimicrobials comprised amikacin (AK), amoxicillin (AMX), amoxicillin/clavulanic acid (AMC), ampicillin (AMP), cefalexin (CEX), cefpodoxime (CPD), cefovecin (INN), ceftiofur (XNL), chloramphenicol (C), enrofloxacin (ENR), gentamicin (GEN), imipenem (IMP), marbofloxacin (MBR), nitrofurantoin (NIT), piperacillin (PIP), tetracycline (TET), tobramycin (TOB), and trimethoprim/sulfamethoxazole (SXT). The MIC interpretations from the Vitek2 machine system (version-9) were made according to the Food and Drug Administration recommendations (31), CLSI guidelines (32) and EUCAST values (33). *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 25913 were used as the control strains.

Detection of Plasmid-Mediated Colistin Resistance Genes

Genomic DNA was extracted from all available MCRPE isolates using the Thermo Scientific GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific). Multiplex-PCR was used to detect *mcr*1-5 genes, following a previously published protocol (34). *E. coli* strain CUP13 (35) that is positive for *mcr*-1 and *mcr*-3 as confirmed by Sanger sequencing was used for the positive control, and *E. coli* ATCC25922 was the negative control. The PCR conditions for *mcr* 6, 7, and 8 were adjusted and performed according to a previous description (4).

Plasmid Replicon Typing

The 18 plasmid replicon types of *Enterobacteriaceae* were investigated by a set of multiplex and simplex PCRs. The primers used and the PCR conditions followed previously described methods (36). Briefly, PCR amplification, except the F-simplex, were conducted at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, 60°C for 30 s, 72°C for 1 min. The amplification was concluded with an extension program of 1 cycle at 72°C for 5 min. The PCR for F-simplex was performed in the same way except for annealing at 52°C.

Detection of Virulence Genes

The *mcr* positive *E. coli* were examined for virulence genes that are commonly present in enterotoxigenic *E. coli* (ETEC) and enterohaemorrhagic *E. coli* (EHEC) by using previously described PCRs (37). Previously sequenced ETEC and EHEC strain were used as positive controls (38). The PCR assays were performed with GoTaq® green master mix (Promega, USA) with the thermocycler conditions being an initial denaturation at 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 30 s, and annealing at 55°C for 45 s. Extension was at 72°C for 1.5 min increased by 3 s each cycle, followed by a final extension at 72°C for 10 min.

Conjugation Assay

To determine whether *mcr* genes were located on transmissible plasmids, and their transferability rate, conjugation assays were performed by the broth mating technique (39). All the *mcr* positive strains detected by PCR were designated as donors, and *E. coli* J53, resistant to sodium azide, was used as the recipient strain. This recipient *E. coli* J53 strain is negative for fertility factors, is resistant to sodium azide (MIC >512 μg/ml) and is sensitive to colistin (MIC <2 μg/ml). Briefly, an overnight culture of bacterial colonies was diluted in Lysogeny broth (LB) and adjusted to OD600 value 1. A 1:1 ratio of donor and recipient then was mixed to obtain a final volume of 2 ml which was incubated overnight. Ten-fold serial dilutions of the overnight mixture were plated on LB agar (Oxoid) plates containing colistin (2 μg/ml) and sodium azide (100 μg/ml). The plates were incubated at 37°C for 2 days, and the transconjugant colonies were counted. The rate of conjugal transfer frequencies was calculated by dividing the number of transconjugant colonies by the number of donor colonies (40). Phenotypic colistin susceptibility testing, PCR detection of *mcr* genes and replicon type detections were repeated on the transconjugants.

Pulsed-Field Gel Electrophoresis (PFGE)

To investigate clonal relatedness, PFGE was performed on all 65 available MCRPE isolates from the 33 positive samples (one to three isolates per sample), following the Centers for Disease Control and Prevention standard protocol (41). Briefly, overnight cultures of *E. coli* isolates were suspended in cell suspension buffer, and the cells were treated with proteinase K and mixed with the agarose gel solution. The gel plugs then were treated with lysis solution, and DNA in the plugs was digested with restriction enzyme *Xba*I (Thermo Scientific). Gel electrophoresis was undertaken using a Bio-Rad CHEF-DRIII system, with a 200 V field at an angle of 120° run for 17–20 h, incorporating *Salmonella* serovar Braenderup H9812 DNA as a standard. Dendrograms were created using the GeneTool program (Syngene, India) and analyzed by the GeneDirectory program (Syngene, India).

Multi-Locus Sequence Typing (MLST)

A representative isolate from each of the 34 PFGE pulsotypes that were identified was randomly selected and included for MLST typing. A simplex PCR was performed for each of the 7 housekeeping genes of *E. coli* used in the Achtman MLST

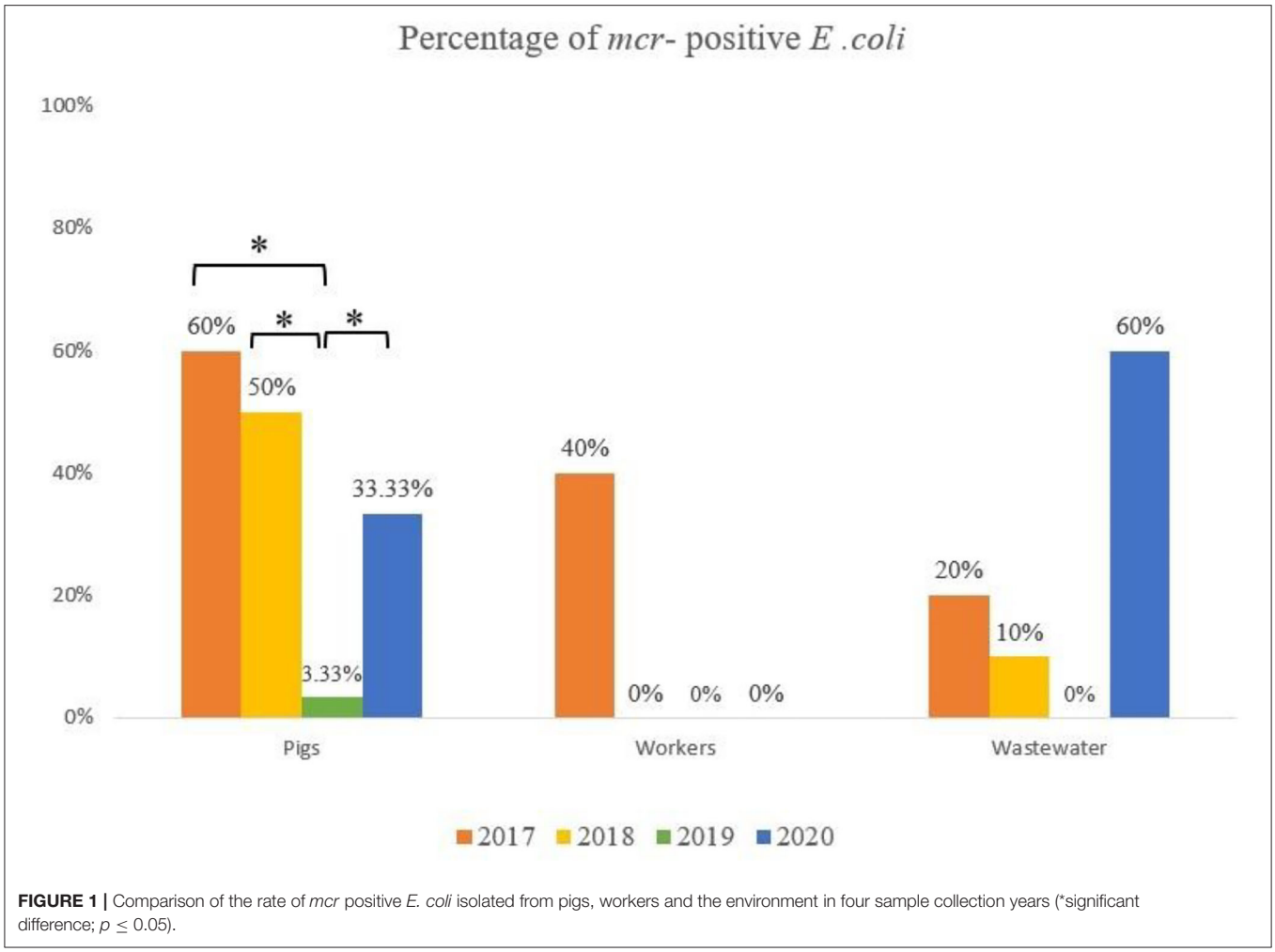

FIGURE 1 | Comparison of the rate of *mcr* positive *E. coli* isolated from pigs, workers and the environment in four sample collection years (*significant difference; $p \leq 0.05$).

scheme (42). These genes encoded isocitrate/isopropyl malate dehydrogenase (*icd*), ATP/GTP binding motif (*recA*), adenylate kinase (*adk*), DNA gyrase (*gyrB*), malate dehydrogenase (*mdh*), adenyl succinate dehydrogenase (*purA*) and fumarate hydratase (*fumc*). The sequences were obtained using the Sanger sequencing platform. The *E. coli* MLST database at http://mlst.warwick.ac.uk/mlst/dbs/Ecoli was used to determine allele and sequence types (STs).

Data Analysis

The colistin-resistance rates and virulence gene profiles for the representative isolates were described as percentages compared to different sources in each sample collection. The *mcr* positive rates among the samples and the association between each sample collection time were analyzed using Fischer's Exact Test ($p \leq 0.05$).

RESULTS

Detection of Colistin Resistant *E. coli*

A total of 33 of the 170 samples (20.6%) yielded colistin-resistant *E. coli*, and their MICs to colistin varied from 4 to 8 μg/ml. These positive samples were isolated from pigs (n = 20/70, 28.6%), wastewater (n = 9/50, 18%) and humans (n = 4/50, 8%). A comparison of the prevalence of MCRPE isolates for each sample type over the 3.5 years since colistin cessation is shown in **Figure 1**, and detailed information about the isolates is presented in **Table 2**. In pigs the high prevalence found in 2017 (60%) and 2018 (50%) was followed by only a single isolate recovered in 2019 (3.3%), and then another increase in 2020 (33.3%). In humans, resistant isolates were only found in 2017 (40%), while a comparatively low rate of positivity in wastewater in 2017 (20%) and 2018 (10%) was followed by none in 2019, and a high prevalence in 2020 (60%). The majority (8/50: 16%) of MCRPE isolates recovered from wastewater were obtained from samples taken before biogas treatment, with only one isolate recovered in 2020 being from a sample taken after the biogas treatment plant (**Table 1**).

Identification of Plasmid-Mediated Colistin Resistance Genes

Of the colistin resistant isolates obtained in 2017, the *mcr*-1 gene was detected in eight of the pig isolates, while *mcr*-1 and *mcr*-3

TABLE 2 | Characterization of 33 colistin-resistant *mcr* positive *E. coli* isolates from different years and sources.

Collection date	Source and number sampled	Number of resistant isolates obtained	*mcr* genes in resistant isolates	Virulence genes in *mcr* positive isolates
2017	Pigs ($n = 15$)	9/15 (60%)	*mcr*-1 (8/15, 53.3%) *mcr*-3 (1/15, 20%)	*StaP-Stb* (5/9, 55.6%) *StaP-Stb-Stx2e* (1/9, 11.1%) Non-pathogenic (3/9, 33.3%)
2017	Humans ($n = 10$)	4/10 (40%)	*mcr*-1 (4/10, 40%)	*StaP-Stb* (2/4, 50%) Non-pathogenic (2/4, 50%)
2017	Wastewater ($n = 10$)	2/10 (20%)	*mcr*-1 (2/10, 20%)	*Stb* (2/2, 100%) Non-pathogenic (0%)
2018	Pigs ($n = 10$)	5/10 (50%)	*mcr*-1 (3/10, 30%) *mcr*-3 (2/10, 20%)	*Stb* (2/5, 40%) Non-pathogenic (3/5, 60%)
2018	Wastewater ($n = 10$)	1/10 (10%)	*mcr*-1 (1/10, 10%)	*Stb* (1/1, 100%) Non-pathogenic (0%)
2019	Pigs ($n = 30$)	1/30 (3.33%)	*mcr*-1 (1/30, 3.33%)	Non-pathogenic (100%)
2020	Pigs ($n = 15$)	5/15 (33.3%)	*mcr*-1 (5/15, 33.3%)	*Stb* (4/5, 80%) Non-pathogenic (1/5, 20%)
2020	Wastewater ($n = 10$)	6/10 (60%)	*mcr*-1 (6/10, 60%)	*Stb* (2/6, 33.3%) Non-pathogenic (4/6, 66.7%)

were detected together in two of these, and *mcr*-3 alone in one pig isolate. At the same time, *mcr*-1 was detected in all four of the isolates from workers and in both the isolates from wastewater samples (**Table 2**). In 2018, after colistin withdrawal for one and a half years, *mcr*-1 was detected in three and *mcr*-3 in two of the five resistant isolates from pigs, and *mcr*-1 was found in the single resistant isolate from wastewater. In 2019 the single isolate from a breeder pig contained *mcr*-1. In 2020 *mcr*-1 positive *E. coli* isolates were found in all 5 piglets that had recent diarrhea symptoms and in wastewater samples (6/10).

Antimicrobial Susceptibility Determination

The antimicrobial resistance (AMR) profiles detected in the MCRPE isolates are shown in **Figure 2**. ESBL-producing *E. coli* were identified, and most MCRPE isolates from the first and second samplings were found to demonstrate extreme pan-drug resistance. Interestingly, besides colistin, the isolate from the positive pig sample in 2019 was phenotypically resistant only to ampicillin. On the other hand, the MCRPE isolates from the last sample collection in 2020 were resistant to aminoglycosides, ampicillin, and ceftiofur, and those antibiotics were used for individual treatments on the farm. The antibiogram results comparing isolates between the 4 sampling years are presented in **Supplementary Table 1**. High rates of resistance to ampicillin, gentamicin, and tetracycline were detected in almost all MCRPE isolates at each sampling time.

Various plasmid replicon types were detected among the MCRPE isolates (**Table 3**). All the *mcr* positive isolates from different sources contained more than one replicon type. The incompatibility group IncFIB and IncI type plasmids were found most commonly. Although a variety of plasmid types were detected in pigs in 2017 and 2018, there was a decrease in varieties of plasmid types in later sample collection years. For the conjugation assay, the donor *E. coli* transferred *mcr*-1 and *mcr*-3 genes (as confirmed by PCR) to recipient J53 strains with a frequency of $1.7–2 \times 10^{-4}$. Phenotypic colistin resistance of transconjugants identified by broth microdilution showed MIC values of $>4\,\mu g/ml$ (**Supplementary Table 2**). The IncI, IncX and IncF plasmid types were predominantly detected in *mcr*-1 transconjugants, while the IncHI2 and IncF plasmid types were detected on *mcr*-3 transconjugants (data not showed).

Virulence Gene Detection

Virulence gene detection was performed on all the 33 *mcr* positive *E. coli* isolates. Most of the isolates from pigs contained genes associated with ETEC strains (enterotoxin genes), with *StaP* and *Stb* being the most frequent pathotype found in 2017 (**Table 2**). One strain from a pig in 2017 showed a hybrid ETEC–EHEC genotype. Two of the four colistin-resistant *E. coli* recovered from farm workers in 2017 contained a combination of *StaP* and *Stb* genes. In contrast, the wastewater samples and the piglets' samples obtained after 2017 only contained the *Stb* enterotoxin gene.

Molecular Genotypic Characterizations

Thirty-four diverse PFGE patterns were obtained for the 65 MCRPE isolates from different sources (**Figure 3**). No dominant pulsotypes were responsible for *mcr* gene clonal carriage.

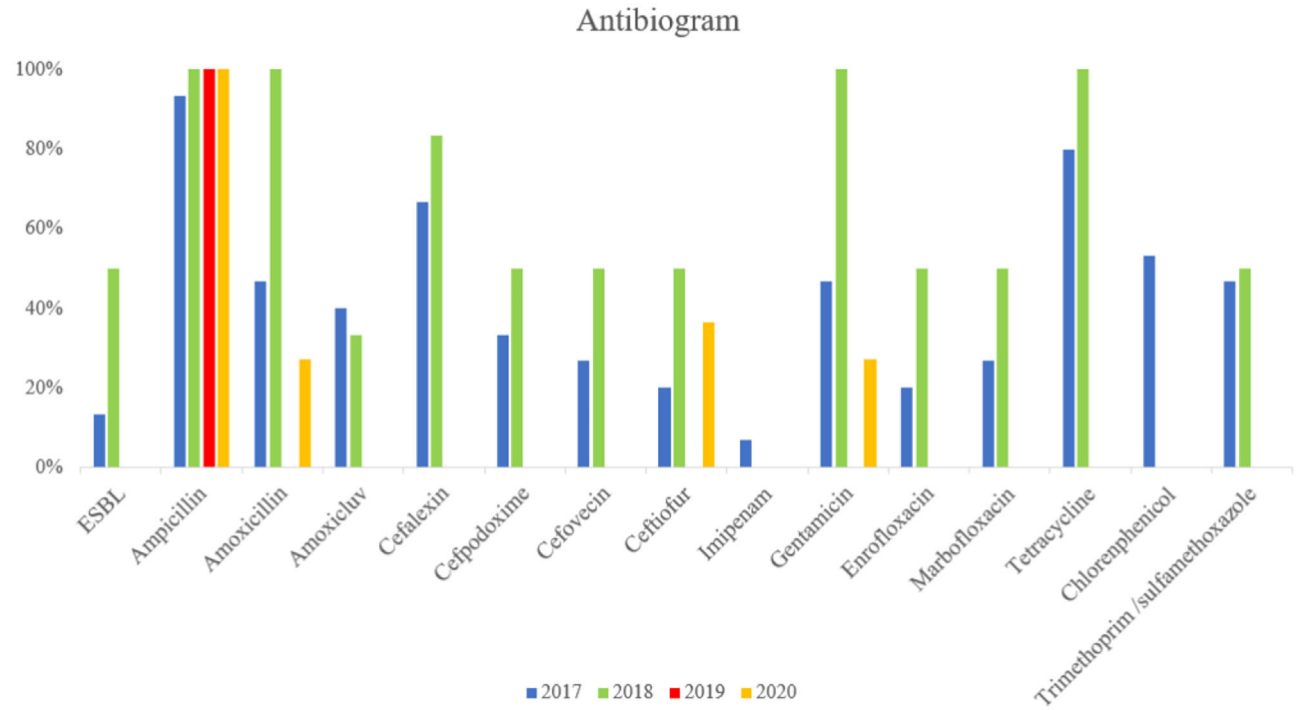

FIGURE 2 | Comparison between resistance rates against 18 antimicrobials and extended-spectrum beta-lactamase (ESBL) in MCRPE isolates at four different sample collection times.

TABLE 3 | Plasmid replicon types detected in 33 colistin-resistant *E. coli* among the three categories of samples at each sample collection time.

Trait	Pigs				Workers	Wastewater		
	2017	2018	2019	2020	2017	2017	2018	2020
	n = 9	*n* = 5	*n* = 1	*n* = 5	*n* = 4	*n* = 2	*n* = 1	*n* = 6
I1-Ir	+	+	−	+	−	−	−	+
HI1	+	+	−	−	−	−	−	−
HI2	+	−	−	+	−	−	−	−
N	+	+	−	−	−	−	−	−
X	+	−	−	−	+	−	+	−
FIB	+	+	+	+	+	+	−	+
FIA	+	+	+	−	−	−	−	−
FIC	−	+	+	−	+	−	+	−
P	+	+	−	−	−	−	−	−
Y	+	+	−	+	+	+	−	+
A/C	+	−	−	+	+	−	−	−
I	−	+	+	+	−	+	+	−

+, *detected*; −, *not detected*.

Moreover, most of the strains from each sample collection time were dispersed on different branches of the dendrogram and were not closely related genetically. The pulsotypes of the MCRPE from humans were not clonally related to any of those from pigs or wastewater. Strains with high similarity (>80%) occurred rarely and were found mainly in the same set of pig or human samples from the same sampling year. Only the MCRPE strains from piglets and wastewater samples in 2020 showed high clonal relatedness, suggesting that MCRPE strains from the piglets with diarrhea had contaminated the wastewater.

MLST gave similar results to PFGE, with most isolates belonging to different STs (**Supplementary Table 3**). Isolates of the common *E. coli* clonal complex ST10 were detected in 2 pigs and one human sample on the first sampling, and in one wastewater sample on the last sample collection. Isolates

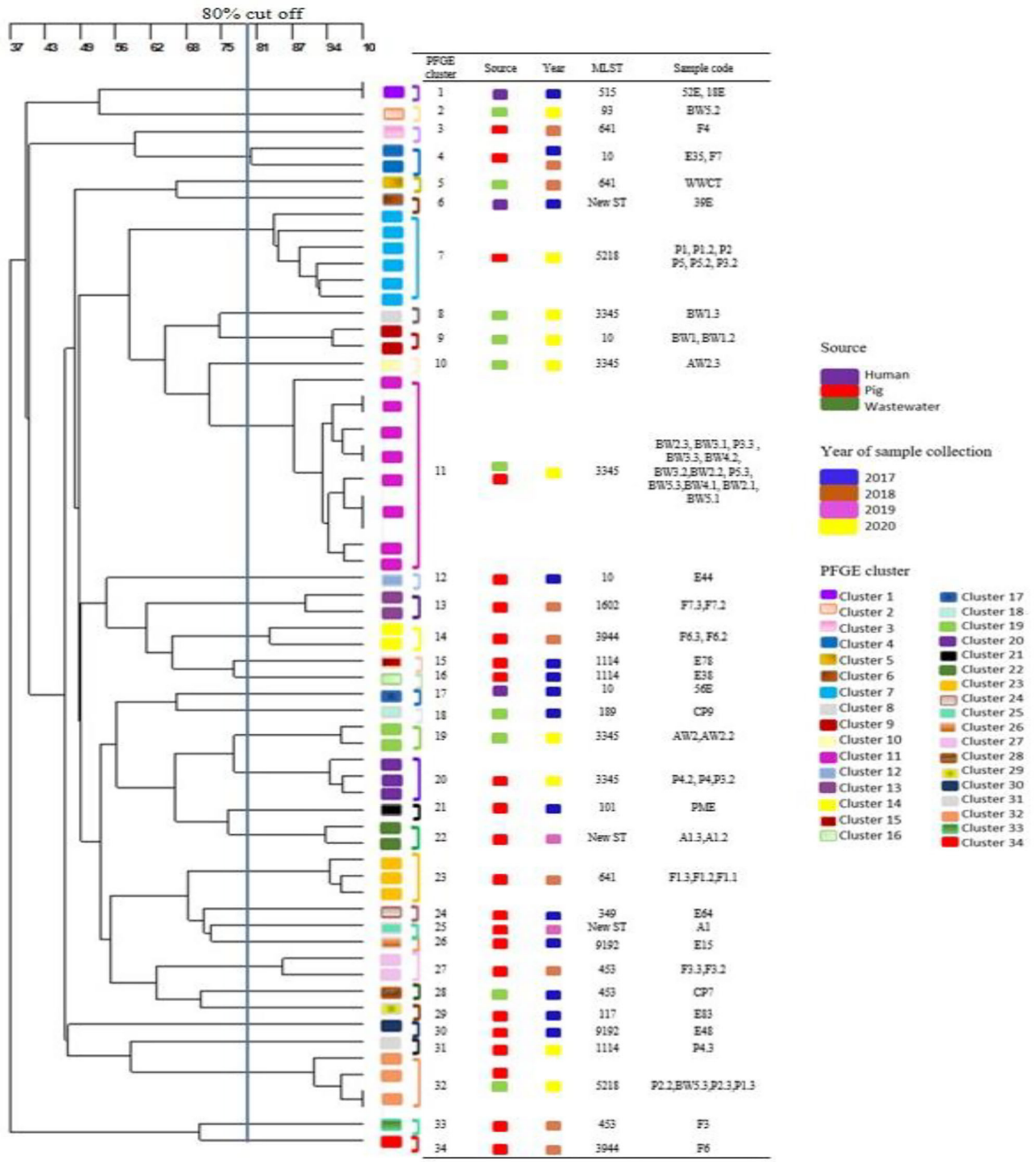

FIGURE 3 | Dendrogram generated from pulsed field gel electrophoresis analysis demonstrating the genetic relatedness among 65 *mcr* positive *Escherichia coli* strains (one to three isolates per positive sample) that were obtained from different sources at each sampling time.

belonging to ST 641 were detected in 2 pigs and one wastewater sample in 2018. In 2020, MCRPE isolates belonging to ST3345 ($n = 3$ in wastewater, $n = 2$ in pigs) and ST 5218 ($n = 2$ in pigs) were commonly detected.

DISCUSSION

The geographical distribution and characterization of colistin-resistant *E. coli* on large-scale pig farms across Thailand has

been reported previously (19). The current longitudinal study investigated the persistence and diversity of *mcr* positive *E. coli* on a selected commercial Thai pig farm following withdrawal of prophylactic colistin usage. According to the farm history, batches of piglets previously were consistently prescribed colistin up until the time that it was withdrawn from use at the start of 2017. In searching for potential changes in resistance to colistin after its withdrawal, this study focused on examining colistin resistance in *E. coli* from young sows and their suckling pigs, as well as from wastewater. Prior to 2017 colistin was mainly used for controlling *E. coli* in suckling pigs in their first 3 weeks of life, so it seemed logical to target this bacterial species and this age group when looking for ongoing resistance. In addition, sows were examined since piglets become colonized by oral exposure from the fecal microbiota of their mothers. The sows were exposed to colistin prior to 2017 and might be persistently colonized and hence transfer resistant bacteria to their piglets. The piglets themselves were destined for slaughter by around 5–6 months of age, and so by definition could not be involved in direct transmission in following years. Accordingly, more sows than piglets were sampled to determine whether they still represented a potential long-term reservoir of MCRPE infection. Wastewater also was sampled, as wastewater tanks on pig farms serve as hotspots for accumulating resistant bacteria since they are composed of pooled fecal discharge from large numbers of pigs housed in the same area. Inclusion of this material in the study increased the likelihood of detecting MCRPE. Although relatively small numbers of samples were examined at each sampling time, they were sufficient to confirm the presence of MCRPE throughout the study.

Even following colistin withdrawal for 21 months, MCRPE that were carrying *mcr* genes were still quite commonly found in pigs, indicating that this period is insufficient to have a significant effect on reducing the presence of colistin resistant bacteria after the drug's withdrawal. The presence of *mcr* positive *E. coli* in the feces of farm workers in mid-2017 is a matter of considerable concern. Bacteria from animals can be transmitted to humans either directly or through food or the environment, and then may transfer resistance genes to pathogenic bacteria that infect humans (43). Farm and food chain workers are likely to be exposed to resistant bacteria throughout the pig production cycle (44). Moreover, *Stb* and *StaP* virulence genes were found in MCRPE isolates from pigs and in two of the workers. These enterotoxin genes are linked to neonatal or postweaning diarrhea in pigs, but bacteria carrying the genes also can be shed in feces from healthy animals (45). The *Stb* enterotoxin is commonly found in *E. coli* strains from pigs but is rarely found in humans and is not associated with diarrhea in humans (46, 47). Therefore, these findings suggested that subclinical ETEC carriers can be found at various stages in the pig production cycle and may represent a source of transmission to humans. Even though isolates of identical genetic types were not found in humans and pigs, our results highlight possible transmission of *mcr* genes from bacteria infecting livestock to isolates that are present in humans and in the environment. The failure to recover MCRPE from human samples after 2017 may be associated with reduced exposure to colistin and/or to MRCPE from pigs and the environment. One possibility is that following identification of MCRPE in the workers in 2017 these individuals took greater care of their hygiene to reduce their exposure to MCRPE of pig origin.

Most pigs were still colonized with colistin-resistant *E. coli* when sampled 21 months after colistin withdrawal; however, by the third year there was a sharp decline in carriage by pigs and neither workers nor wastewater samples were positive for MCRPE. Therefore, the national ban on prophylactic use of colistin was highly likely to have been beneficial for controlling the emergence and dissemination of *mcr*-1 on this and on other pig farms. Nevertheless, some pigs and wastewater still contained MRCPE when samples 3.5 years after the withdrawal of colistin. Pigs reared for meat production are only kept for around 5–6 months before slaughter, although breeder pigs are retained for up to 3–4 years. Presumably transmission cycles of MCRPE between batches of pigs that are selected for meat production or breeding, and/or exposure to contaminated environments allowed them to remain for at least 3.5 years. A more extended study is required to determine for how long this carriage my persist. The long duration of persistence that was identified contrasted with a previous report from Britain, where *mcr*-1 was undetectable in isolates from pigs after the cessation of colistin use for approximately 20 months (7). The reason for the re-occurrence and increase in numbers of pigs shedding MCRPE and in isolates recovered from wastewater in the last year of the current study is unclear. These colonized pigs had shown diarrhea symptoms prior to sampling and had been given therapeutic antimicrobial treatments, unlike the situation in previous batches sampled in earlier years. A possible explanation for the re-occurrence without selective pressure applied from colistin exposure may be the existence of cross-resistance between colistin and other therapeutic antibiotics used in the piglets. A similar phenomenon was reported in previous studies where colistin resistance was found when other antimicrobials such as quinolones or cephalosporins were used in livestock farms (48, 49). However, more complete genomic characterization of the MCRPE isolates involved is required to investigate possible reason for this correlation. Nevertheless, these results are of concern because short-term β-lactam (ceftiofur) or gentamicin use in animals may select for *mcr*-1 in *E. coli* and maintain persistence on farms.

From the antibiotic susceptibility testing, some of the *mcr* positive *E. coli* isolates were found to be ESBL producers and showed extreme pan-drug resistance. A larger number of *E. coli* isolates with ESBL were observed in the samples from 2018 compared to the first sampling time. In Thailand, the application of antimicrobials in pig farms varies according to the management system and geographical area. In the central area of Thailand, the antimicrobials that are mainly used are colistin, cephalosporins, tiamulin, amoxicillin, tilmicosin, aminoglycosides (gentamycin), and oxytetracycline (50). The use of other antimicrobials during the production cycle of pigs could co-select for colistin resistance (51, 52). Resistances to other potential agents like heavy metals or biocides that may be linked with antibiotics resistance genes also are a matter for concern.

In the conjugation experiment, MCRPE recovered from pigs without selective pressures from colistin use showed a high transfer frequency. Moreover, various replicon types were found in the colistin-resistant *E. coli* isolates. According to previous reports, *mcr*-1 and *mcr*-3 genes have been found on IncI, IncHI2, and IncX4 plasmids (53). Likewise, *mcr*-1 was predominantly harbored on the IncX4 plasmid in isolates from healthy human beings in China (54). Different AMR genes can be located on the same plasmid or on different plasmids within the same bacterial host, and these represent multidrug resistant clones. Plasmids encoding the *mcr* genes, which co-exist with other antimicrobial resistance genes, are a problem for public health. To date, the majority of *mcr* genes have been identified in various plasmid types and are able to locate and/or transfer with other resistance genes by conjugation (55).

A large number of PFGE pulsotypes were observed among the *mcr* positive isolates. Therefore, no epidemic strains were dominant on the farm over time, and the *mcr* genes found in *E. coli* isolates were mainly plasmid-borne. A high diversity of MCRPE isolates from different hosts also was observed in a study from China (56). Similarly, in a Dutch study where ESBL positive *E. coli* from animals and humans were examined, ESBL transmission did not involve strain transfer but rather plasmid transfer by identical plasmids of the IncI and IncK types (57). Nevertheless, in our study some clonal relatedness was found in MCRPE from piglets and wastewater samples at the last sampling. In this case the resistant bacteria from pigs were likely to be the primary source of *mcr* genes contaminating wastewater. Thus, despite moderate persistence of *mcr* genes in pigs and low-level environmental dissemination in tested wastewater, the distribution of diverse strains with virulence potential from different niches across years is worrisome. Genes from these *mcr*-1 and *mcr*-3 positive isolates might be transferred to other sources and/or other pathogens.

In this study, *E. coli* carrying *mcr* genes were recovered, but with a gradual decline over 3.5 years following cessation of colistin use. Hence, banning colistin for prophylaxis use was effective for reducing the emergence and dissemination of *mcr*-1 in pigs and the pig farm environment. However, even in the absence of selective pressure exerted by colistin use, the application of other antimicrobials during the production cycle might co-select indirectly for the *mcr* genes and favor their spread. This study provides an initial insight into the reduction in dissemination of colistin resistant *E. coli* from pigs and the farm environment. Further long-term genomic investigations are necessary to improve understanding and control of MCRPE and colistin-resistance in the pig industry.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because All fecal samples were submitted from veterinarians in pig industrial field to the veterinary diagnostic laboratory as the annual surveillance. However, the biohazard execution control was approved by the Institutional Biosafety Committee of the Faculty of Veterinary Science, Chulalongkorn University (IBC 2031011).

AUTHOR CONTRIBUTIONS

NK contributed to conception and design, critical revision, analysis and interpretation of data, and drafting of manuscript. KL contributed to conception and design, analysis, and interpretation of data. WN performed analysis and interpretation of data. TP organized acquisition of data. DH contributed to critical revision, analysis and interpretation of data, and drafting of manuscript. NP organized study conception and design and contributed to critical revision, analysis, and interpretation of data. All authors contributed to manuscript revision, read, and approved the submitted version.

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ORIGINAL RESEARCH
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Effects of Anemoside B4 on Diarrhea Incidence, Serum Indices, and Fecal Microbial of Suckling Calves

Meng Lu[1,2], *Fengming Hu*[1,2], *Yanliang Bi*[1,2], *Tao Ma*[1,2], *Qiyu Diao*[1,2], *Linshu Jiang*[2] *and Yan Tu*[1,2*]

[1] *Key Laboratory of Feed Biotechnology of Ministry of Agriculture and Rural Affairs, Institute of Feed Research of Chinese Academy of Agricultural Sciences, Beijing, China,* [2] *Beijing Key Laboratory for Dairy Cow Nutrition, Beijing University of Agriculture, Beijing, China*

The study was conducted to evaluate the effects of Anemoside B4 on diarrhea incidence, serum indices, and fecal microbial of suckling calves. Sixty newborn Chinese Holstein calves with similar body weight (43.7 ± 3.9 kg) were randomly divided into four groups with 15 calves each, fed the diet which was supplied 0 (CON), 15 (A1), 30 (A2), and 45 (A3) mg/day of Anemoside B4, respectively. The trial period is 56 days. The blood and fecal samples were collected at 28 and 56 days of age. Results show that during the whole trial period, the diarrhea incidence in Group A1, A2, and A3 was significantly lower than that in Group CON ($p < 0.05$). Compared with the Group CON, Anemoside B4 supplementation significantly decreased the contents of serum D-lactic acid and diamine oxidase at 28-day-old ($p < 0.05$). At 56-day-old, the content of serum D-lactic acid in Group A3 tended to be higher ($0.05 < p < 0.01$), and the content of serum diamine oxidase in Group A3 increased significantly, in comparison with Group CON ($p < 0.05$). Group A3 increased the level of Chao1 and Simpson indices at 28-day-old ($0.05 < p < 0.01$), and Chao1, Observed_species, Shannon, and Simpson indices at 56-day-old ($p < 0.05$), in comparison to Group CON. Compared with Group CON, 45 mg / day Anemoside B4 supplementation significantly increased the contents of *Bacteroidota* (at the phylum level), *Prevotella* (at the genus level) at 28-day-old ($p < 0.05$), and the content of *Sutterella* (at the genus level) at 56-day-old ($p < 0.05$), promoted the processes of energy metabolism, glycan biosynthesis and metabolism, metabolism of cofactors and vitamins ($p < 0.05$). A positive correlation was observed between *Prevotella* and metabolism of cofactors and vitamins, energy metabolism, and glycan biosynthesis and metabolism. A positive correlation was observed between *Sutterella* and energy metabolism. In conclusion, Anemoside B4 could effectively alleviate calf diarrhea, protect the integrity of intestinal mucosa, and change the structure of intestinal microbiota, indicating the potential value of Anemoside B4 in regulating intestinal microbiota and the prevention of intestinal diseases.

Keywords: Anemoside B4, suckling calves, diarrhea, serum indices, fecal microbial

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Ashok Kumar Pattanaik, ICAR-Indian Veterinary Research Institute, India
Ramazan Yildiz, Mehmet Akif Ersoy University, Turkey

***Correspondence:**
Yan Tu
tuyan@caas.cn

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INTRODUCTION

Calve breeding is related to the quality and production level of the herd, which in turn affects the production efficiency of the entire cattle industry (1). Newborn calves are susceptible to disease, and diarrhea is the main cause of death in suckling calves, which has extremely high morbidity and mortality (2). According to the statistics of the U.S. National Animal Health Monitoring System study, published in 2018, 39% of calf mortality was caused by diarrhea in the first 3 weeks after birth (3), which is mainly caused by intestinal injury and incomplete establishment of intestinal flora in calves (2, 4, 5). Among them, *Escherichia coli* (*E.coli*), *Bovine viral diarrhea virus*, *B. coronavirus*, and *B. rotavirus* are the main pathogens that cause diarrhea in calves (6). The growth of pathogens in calves reduces the number of beneficial bacteria, which greatly increases the risk of disease (7). In addition, damage to the intestine will cause the increase of intestinal permeability, the invasion of the pathogen, the destruction of the intestinal immune barrier, the inflammatory reaction of the intestine tract, the decrease of calf immunity, and the diarrhea of calves (8).

Some plants contain a variety of active ingredients such as saponins, polysaccharides, and essential oils, which play important roles in inhibiting the growth of pathogens, improving immunity, and promoting intestinal health without toxic and side effects (9). Plant extracts are secondary metabolites of plants (10), which have the functions of antimicrobial (11), and anti-inflammatory (12), and have been proposed as substitutes for chemical feed additives with the banning of some ionophore feed additives in many countries (13).

Pulsatilla Radix, derived from the dry roots of Pulsatilla Chinensis Regel in Ranunculaceae, has a history of thousands of years growing in China and plays an important role in antibacterial, anti-inflammatory, and immune enhancement (14, 15). Saponins are a class of complex glycoside compounds, which are the basis for many plants to exert their pharmacological effects. Anemoside is the main component of Pulsatillae Radix and the basis for their anti-inflammatory and anti-tumor biological activities. The Anemoside in Pulsatillae Radix mainly includes Anemoside B4, Anemoside A3, and 23-hydroxy betulinic acid. Among them, Anemoside B4, a natural triterpenoid glycoside isolated, is the monomer component with the highest content in Pulsatillae Radix, which has antibacterial and anti-inflammatory effects (16). *In vitro* antibacterial test reveals that Anemoside B4 can inhibit the growth of pathogenic bacteria such as *E. coli* (17). Meanwhile, Anemoside B4 can inhibit the expression of pro-inflammatory factors such as IL-1β, IL-6, and IL-8, and enhance the anti-inflammatory ability of the body (18). In addition, a study proved that Anemoside B4 at 30 ml/q. d. I. M. in brachiocephalic muscle for 4–6 days can provide a largely side-effect-free cure for cows with clinical mastitis (19).

A few studies have demonstrated the antibacterial activity and therapeutic potential of triterpenoid saponin Anemoside B4, but its application in calves has not been reported. Thus, this study was conducted to study the effect of Anemoside B4 on diarrhea incidence, serum intestinal permeability indicators, and fecal microbes of suckling calves by adding Anemoside B4 into milk replacer, to provides the theoretical basis for the development of feed additives.

MATERIALS AND METHODS

Experimental Design, Animal Management, and Diet

The feeding trial was carried out at the Nankou Base of the Institute of Feed Research, Chinese Academy of Agricultural Science (IFRCAAS) (Beijing, China) from March to May 2021. The experimental procedures were approved by the Animal Ethics Committee of IFRCAAS (AEC-IFR-CAAS, Beijing, China).

Sixty healthy newborn Holstein bull calves (body weight = 43.7 ± 3.9 kg; the serum total protein concentration was >55g/L) from Shou Nong Group Co., Ltd. (Beijing, China) were selected for the experiment. The calves were removed from their dams shortly after birth and fed colostrum in a transfer room. Each calf consumed a total of 6 L colostrum, with 4 L fed within 2 h of life and the remaining 2 L fed 8 h after the first feeding. Then all calves were moved to a naturally ventilated hutch and kept in individual pens (4.5 × 1.5 m). The experiment lasted 56 days with 14 days for the preliminary feeding period and 42 days for a formal trial period. All calves were fed with commercial MR (Beijing Precision Animal Nutrition Research Center, Beijing, China) for the transition from 7 to 14 days of age, and then randomly assigned to one of the four groups (15 calves per group) at 14 days of age based on body weight and age.

The milk replacer is fed at 1.25% (dry matter basis) of body weight (at 07:00, 13:00, and 19:00) and adjusted fortnightly. The MR was mixed with boiling water and cooled to 50–60°C to form an emulsion at a ratio of 1:7 (weight/volume) and then fed to calves when its temperature was lowered to 38°C. Anemoside B4 was added to the milk replacer during morning feeding. The calves of the four groups were fed the diet with 0 (CON), 15 (A1), 30 (A2), and 45 (A3) mg/day per head Anemoside B4, respectively. The starter feed and fresh water were provided *ad libitum* throughout the study.

The milk replacer powder, configured in accordance with the national invention patent CN 02128844.5, was provided by Beijing Precision Animal Nutrition Center (Beijing, China), and the nutrient levels are shown in **Supplementary Table 1**. The starter feed was provided by Sanyuan Hefeng Animal Husbandry Co., Ltd. (Beijing, China), and the ingredients and compositions are shown in **Supplementary Table 2**. The Anemoside B4 (content 67.41%) was purchased from Nanjing Chunqiu Biological Engineering Company., Ltd. (Jiangsu, China).

Diarrhea Incidence

Observe the health of the calves every day and score the fecal by the four-point system. The fecal scoring standard is shown in **Table 1** (20). Whenever an animal presented a fecal score ≥3 for 2 consecutive days, they were considered diarrheic, and diarrhea incidence was calculated as follow:

Diarrhea incidence (%) = total number of diarrhea calves×100 / (number of calves in the group× trial days).

TABLE 1 | Fecal score standard.

Items	Outward	Dry matter (%)	Score
Normal	Bars or granules	>30	1
Mild	Soft manure, can be formed	25–30	2
Moderate	Thick, shapeless, no separation of manure, and water	20–25	3
Serious	Liquid, not shaped, separation of manure, and water	<20	4

TABLE 2 | Effects of Anemoside B4 on diarrhea incidence of suckling calves (%).

Items	Groups				χ2	*p-value*
	CON	A1	A2	A3		
7 to 56 d	27.38^{a}	20.62^{b}	18.77^{bc}	15.69^{c}	29.18	<0.01
7 to 14 d	60.58^{a}	43.27^{b}	40.38^{b}	35.58^{b}	14.95	0.002
15 to 28 d	31.32^{a}	20.88^{b}	15.38^{b}	14.84^{b}	19.51	<0.01
29 to 42 d	28.02^{a}	14.84^{b}	17.58^{b}	12.64^{b}	16.96	0.001
43 to 56 d	8.24	7.14	7.69	4.40	2.49	0.477

CON, the dosage of B4 is 0 mg / d; A1, the dosage of B4 is 15 mg / day; A2, the dosage of B4 is 30 mg / day; A3, the dosage of B4 is 45 mg / day; SEM, Standard error of the mean.
^{abc}Denotes a diversity trait with significant difference ($p < 0.05$).

Sample Collection

At 28 and 56 days of age, 2 h after morning feeding, six healthy calves in each group whose body weight was close to the average body weight of the group, were selected. Blood samples were collected from the jugular vein and centrifuged at 2,000 *g* for 10 min. The collected serum was placed in a 1.5 ml centrifuge tube and frozen at −20°C for further analysis. The concentrations of D-lactic acid (D-LA) and diamine oxidase (DAO) in serum samples were analyzed with commercial kits from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China) according to the enzyme-labeler (ST-360). In addition, fecal samples were collected by rectal fecal collection method, divided into 1.5 ml cryopreservation tubes, and quickly placed in liquid nitrogen for preservation, and then brought back to the laboratory for cryopreservation at −80°C for DNA extraction and identification of fecal microbial.

DNA Extraction and 16S rRNA Pyrosequencing

DNA was extracted using the kit, and the V3-V4 region of 16S rDNA was amplified with specific primers with barcode, primer sequence was 338F: ACTCCTACGGGAGGCAGCAG; 806 r: GGACTACHVGGGTWTCTAAT. PCR products were detected by 1% agarose gel electrophoresis and purified with Agencourt AMPure XP nucleic acid purification kit. The purified amplified products were mixed in equal quantities and connected to the "Y" shaped connector. The self-connecting segments of the connector were removed by magnetic bead screening. The library template was enriched by PCR amplification to generate single-stranded DNA fragments, and the MiSeq library was constructed and sequenced by MiSeq.

TABLE 3 | Effects of Anemoside B4 on serum indices of suckling calves.

Items	Groups				SEM	*p*-Valve
	C	A1	A2	A3		
28 days of age						
D-LA, nmol / L	11.87^{a}	11.08^{b}	10.77^{b}	9.92^{c}	0.176	<0.01
DAO, ng / mL	1.21^{a}	1.06^{b}	0.96^{b}	0.84^{c}	0.034	<0.01
56 days of age						
D-LA, nmol / L	12.81	12.46	12.47	12.23	0.110	0.08
DAO, ng / mL	1.41^{a}	1.39^{ab}	1.34^{ab}	1.27^{b}	0.024	0.04

CON, the dosage of B4 is 0 mg / d; A1, the dosage of B4 is 15 mg / day; A2, the dosage of B4 is 30 mg / day; A3, the dosage of B4 is 45 mg / day; SEM, Standard error of the mean; D-LA, D-lactic acid; DAO, diamine oxidase.
^{abc}Denotes a diversity trait with significant difference ($p < 0.05$).

Data Processing

The original data contains certain low-quality data, which easily affects the subsequent data analysis. To ensure the reliability and biological validity of the subsequent analysis, the original data needs to be preprocessed. Firstly, Trimmomatic (V 0.36) and Pear (V 0.9.6) were used to quality control the original data and obtain the Fasta sequence. Uchime method was used to compare and remove the chimera of the Fasta sequence according to the known database, while denovo method was used to remove the chimera of the Fasta sequence for the unknown database. At the same time, the unqualified short sequences were removed to obtain the high-quality sequence clean_tags. Qiime software (Version 1.8.0) was used to conduct statistical analysis of bioinformatics on OTU at a 97% similar level. Richness estimates and diversity indices including Chao 1, Observed_species, Goods_coverage, PD_whole_tree, Shannon, and Simpson were calculated using the QIIME V1.8. To obtain the species classification information corresponding to each OTU, BLAST (2.6.0+) was used for comparative analysis of OTU representative sequences, and the specific information of the community was annotated at various levels. linear discriminant analysis (LDA) effect size analysis was used to find species with significant differences in abundance between each group (21). First, the ANOVA test was used to detect species with significant differences in abundance between different groups. Then, the Wilcoxon rank-sum test was used to analyze the differences between different groups. Finally, LDA was used to reduce the data and assess the impact of species with significant differences, and the algebraic linear discriminant analysis score was set to ≥3.0. A PCoA based on the weighted UniFrac distances was conducted to compare all samples, and a distance-based matrices analysis was performed to evaluate differences among samples.

Microbial Function Prediction

Use phylogenetic investigation of communities by reconstruction of unobserved states version 2.0.0 to predict the function of the fecal microbial community (PICRUSt v 2.0.0; http://galaxy.morganlangille.com) (22). The information of KO, Pathway, EC was obtained according to the Kyoto Encyclopedia of Genes and

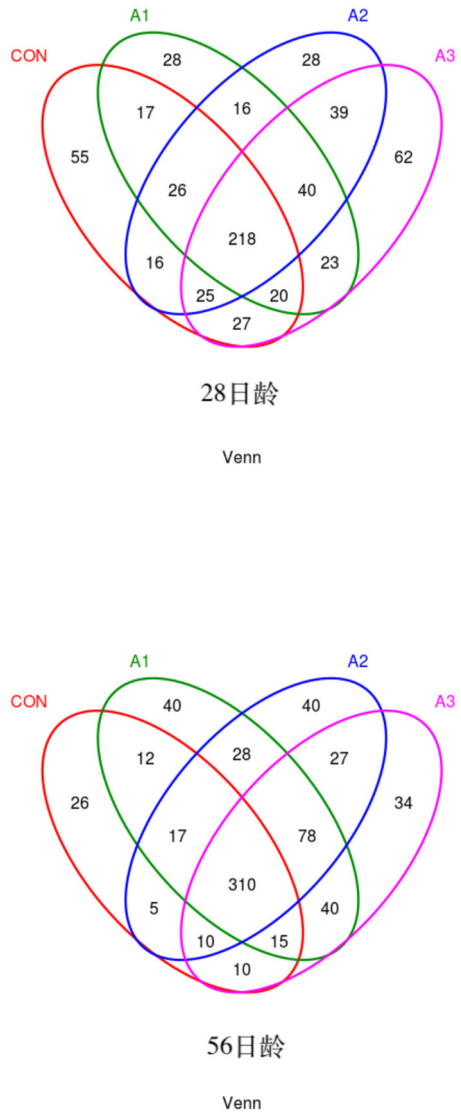

FIGURE 1 | Venn diagram illustrating overlap of microbial OTUs at 3% dissimilarity level among treatments. Venn diagram of bacteria at 28 days of age (left) or 56 days of age (right). CON, the dosage of B4 is 0 mg / day; A1, the dosage of B4 is 15 mg / day; A2, the dosage of B4 is 30 mg / day; A3, the dosage of B4 is 45 mg / day.

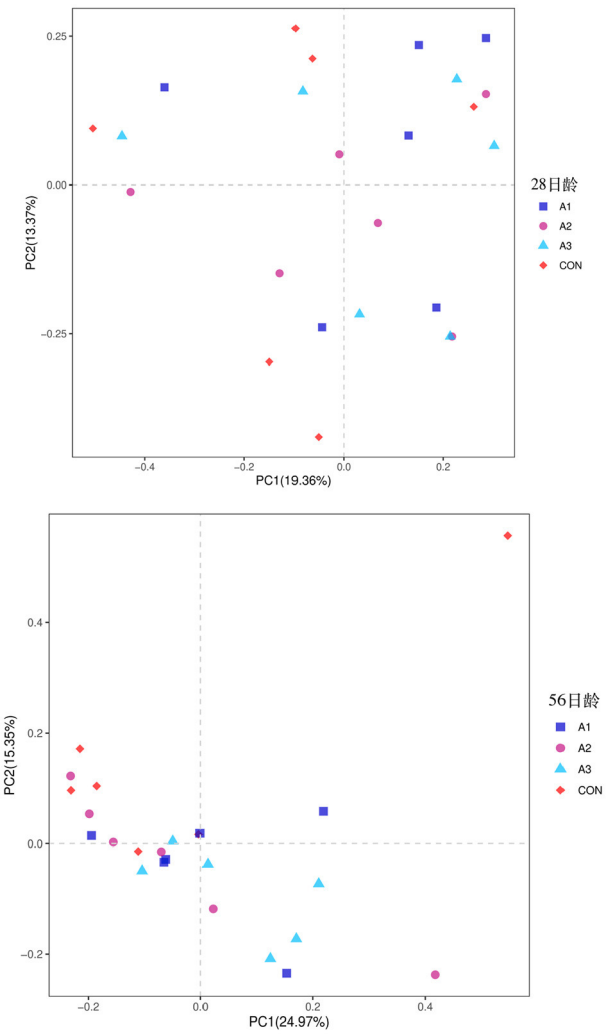

FIGURE 2 | Principal Coordinate Analysis (PCoA) of fecal bacterial community structures of suckling calves in the four groups at 28 days of age (left) and 56 days of age (right). CON, the dosage of B4 is 0 mg / day; A1, the dosage of B4 is 15 mg / day; A2, the dosage of B4 is 30 mg / day; A3, the dosage of B4 is 45 mg / day.

Genomes analysis module and calculated the abundance of each functional category.

Statistical Analysis

The $\chi 2$ procedure was used to compare the incidence of diarrhea between treatment groups. The fecal score was conducted using GraphPad Prism Version 9 (GraphPad Software Inc. CA, USA). The data of serum indices, alpha diversity indices, the relative abundance of microbial flora, and metabolism level were analyzed by the independent sample *t*-test, and all statistical analyses were performed by using SAS software (version 9.4, SAS Institute Inc., Cary, NC, USA). The 'ggcor' package GitHub of R was used to analyze the relationship between fecal microbial and metabolism. Treatment differences with $p < 0.05$ were considered statistically significant and $0.05 < p < 0.10$ was designated as a tendency. Some or all data, models, or code generated or used during the study are available from the corresponding author by request.

RESULTS

Diarrhea Incidence

As shown in **Table 2**, compared with Group CON, Anemoside B4 significantly decreased the diarrhea incidence of calves throughout the experiment ($p < 0.05$), and that in Group A3 decreased significantly in comparison to Group A1($p < 0.05$). From 7 to 42 days of age, the incidence of diarrhea in Group

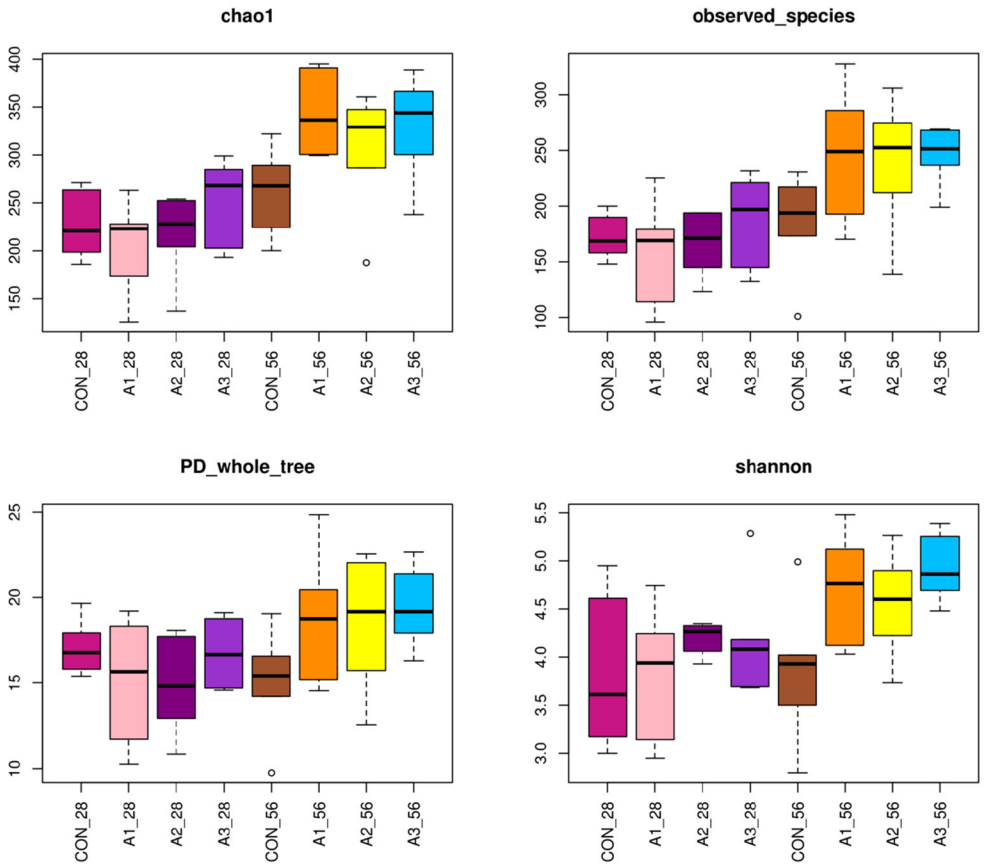

FIGURE 3 | Richness estimates and diversity indices for bacteria with four groups. The top and bottom boundaries of each box represent the 75th and 25th quartile values, respectively. The horizontal lines inside each box represent the median values. CON, the dosage of B4 is 0 mg / day; A1, the dosage of B4 is 15 mg / day; A2, the dosage of B4 is 30 mg / day; A3, the dosage of B4 is 45 mg / day.

A1, A2, and A3 was significantly lower compared to Group CON ($p < 0.05$). Moreover, the diarrhea incidence of calves in Group A1 and A3 was significantly lower at 29 to 42 days of age in comparison with that in Group CON ($p < 0.05$). There was no significant difference among the four groups at 43–56 days ($p > 0.05$).

Serum Profiles

Table 3 shows that Anemoside B4 increased the content of serum D-lactic acid (D-LA) and diamine oxidase (DAO) at 28 days of age ($p < 0.05$), and D-LA and DAO levels gradually increased with the addition of Anemoside B4. At 56 days of age, D-LA in Group A3 tended to be higher than that in Group CON ($0.05 < p < 0.10$), and the Group A3 had a higher level of DAO than Group CON ($p < 0.05$).

Sequencing Depth and Index of Microbial Community

The fecal microbes of calves in the four groups were sequenced based on 16S rDNA technology. After the original data were further removed from the chimera and short sequences, a total of 1,383,096 and 1,007,665 high-quality sequences were obtained at 28 and 56 days of age, accounting for 90.00 % and 93.43% of the original data, respectively. As shown in **Figure 1**, a total of 1,654 OUTs were identified, of which a total of 55, 28, 28, and 62 OUTs were unique in Group CON, A1, A2, and A3, respectively, at 28 days of age, and a total of 1,984 OUTs were identified, of which 405, 540, 515, and 524 OUTs were identified in Group CON, A1, A2, and A3 at 56 days of age, respectively. Based on the weighted UniFrac and bray-Curtis distance matrix, principal coordinate analysis (PCoA) was further used to analyze the structure of the microbial community in calf fecal as shown in **Figure 2**. Through the distance of the samples, the differences between individuals or groups could be observed. The greater the distance between the samples, the greater the difference in the composition of the microbial community between the samples. PCoA plots of bacterial 16S rRNA showed that the data points of the calf stool sample at 28 days of age were relatively scattered, and the different treatments were not well-clustered, indicating that there was a serious microbial imbalance in the intestinal tract of calves with diarrhea. At 56 days of age, the sample points were well-clustered, and the four groups could be largely separated as shown in **Figure 2**. In addition, the Chao1 indices of fecal microbial in Group A3 tended to be higher in comparison

TABLE 4 | Effects of Anemoside B4 on the comparison of dominant phylum of suckling calves at 28 days of age (%).

Phylum	Diets				SEM	*p*-value
	CON	A1	A2	A3		
Firmicutes	78.93	73.63	71.90	62.55	0.042	0.60
Proteobacteria	5.15	16.47	7.80	5.87	0.028	0.47
Actinobacteriota	5.99	5.42	4.33	2.25	0.008	0.44
Verrucomicrobia	0.31	0.00	0.00	0.01	0.001	0.42
Bacteroidetes	1.78^{b}	4.35^{b}	12.57^{ab}	23.17^{a}	0.032	0.02
Fusobacteriota	7.75	0.02	3.32	5.97	0.023	0.70
Desulfobacterota	0.01	0.03	0.02	0.11	0.000	0.09
Euryarchaeota	0.05	0.00	0.02	0.00	0.000	0.06
Campilobacterota	0.02	0.01	0.01	0.01	0.000	0.86
Synergistota	0.01	0.01	0.01	0.03	0.000	0.54
Patescibacteria	0.00	0.02	0.02	0.01	0.000	0.72
Spirochaetota	0.00	0.00	0.00	0.01	0.000	0.42
Cyanobacteria	0.00	0.04	0.00	0.00	0.000	0.43
Acidobacteriota	0.00	0.00	0.00	0.00	0.000	0.41

CON, the dosage of B4 is 0 mg / d; A1, the dosage of B4 is 15 mg / d; A2, the dosage of B4 is 30 mg / d; A3, the dosage of B4 is 45 mg / d; SEM, Standard error of the mean.
^{ab}Denotes a diversity trait with significant difference ($p < 0.05$).

TABLE 5 | Effects of Anemoside B4 on the comparison of dominant phylum of suckling calves at 56 days of age (%).

Phylum	Diets				SEM	*p*-value
	CON	A1	A2	A3		
Bacteroidota	53.08	35.32	45.44	37.88	0.034	0.07
Firmicutes	43.99	59.20	49.97	52.08	0.030	0.09
Proteobacteria	1.68	1.81	2.42	4.47	0.005	0.06
Actinobacteriota	1.18	3.26	2.02	5.15	0.008	0.37
Desulfobacterota	0.05	0.30	0.05	0.28	0.000	0.07
Synergistota	0.01	0.02	0.01	0.03	0.000	0.38
Cyanobacteria	0.00	0.04	0.03	0.02	0.000	0.37
Campilobacterota	0.00	0.00	0.00	0.00	0.000	0.41
Verrucomicrobia	0.00	0.00	0.00	0.01	0.000	0.39
Euryarchaeota	0.00	0.00	0.00	0.00	0.000	0.41
Spirochaetota	0.00	0.02	0.01	0.03	0.000	0.64
Patescibacteria	0.00	0.04	0.01	0.03	0.000	0.36
Fusobacteriota	0.00	0.00	0.03	0.03	0.000	0.60

CON, the dosage of B4 is 0 mg / d; A1, the dosage of B4 is 15 mg / d; A2, the dosage of B4 is 30 mg / d; A3, the dosage of B4 is 45 mg / day; SEM, Standard error of the mean.

with that in Group A1 at 28 days of age ($0.05 < p < 0.10$), which in Group CON decreased by 11.44% in **Figure 3**. The PD_whole_Tree and Shannon indices showed no marked differences among the four groups ($p > 0.05$), while Simpson indices in Group A2 showed a higher trend than that in Group CON ($0.05 < p < 0.10$). At 56 days of age, the alpha diversity indices in Group A3, and the Shannon and Simpson indices in Group A2 increased significantly compared with Group CON ($p < 0.05$).

Bacterial Composition of Fecal Microbial Flora Across Different Treatments

There were 14 phylum and 203 genera were identified in fecal microbes. As shown in **Tables 4**, **5**, the main bacteria at the phylum level among four groups were *Firmicutes*, *Bacteroidota*, followed by *Fusobacteriota*, *Actinobacteriota*, and *Proteobacteria*. *Megasphaera* and *Prevotella* were dominant bacteria at the genus level in **Tables 6**, **7**. At the age of 28 days, the abundance of *Bacteroidota* at phylum level in Group A3 (23.17%) was significantly higher than that in Group CON (1.78%) and Group A1 (4.35%). Compared with Group CON, the *Euryarchaeota* abundance in Group A1 (0.05%) and *Desulfobacterota* abundance in Group A3 (0.01%) tended to increase in **Table 4** ($0.05 < p < 0.10$). At the genus level, *Prevotella* abundance in Group CON (0.91%) and A1 (1.91%) ($p < 0.05$) decreased significantly compared with Group A3 (12.21%), and there is a trend of significant difference of *Sutterella* abundance among Group A3 (1.72%), Group A1 (0.19%), and A2 (0.25%) ($0.05 < p < 0.10$). The *Mitsuokella* abundance of the calf fecal microflora in Group A2 (4.49%) tended to be higher in comparison to Group CON (0.74%) in **Table 5** ($0.05 < p < 0.10$). As shown in **Table 6**, at 56 days of age, no significant differences were observed in fecal microbial flora community and relative abundance among four groups ($p > 0.05$), among which the abundance of *Bacteroidota* in Group CON tended to be higher than that in Group A1 ($0.05 < p < 0.10$), while *Firmicutes* abundance tended to be lower ($0.05 < p < 0.10$). Besides, the abundance of *Proteobacteria* and *Desulfobacterota* in Group A3 was higher than those in Group CON ($0.05 < p < 0.10$). At the genus level, there were no significant differences among the four groups except for *Prevotella*, *Faecalibacterium*, *Acidaminococcus*, *Dialister*, and *Sutterella* as shown in **Table 7**. Compared with Group CON, *Prevotella* in Group A1 and A3 tended to decrease ($0.05 < p < 0.10$), *Faecalibacterium* and *Sutterella* in Group A3 and *Acidaminococcus* in Group A1 increased significantly ($p < 0.05$), and *Dialister* in Group A2 showed a trend to increase ($0.05 < p < 0.10$).

The potential biomarkers of different groups are presented by LEfSe as shown in **Figure 4**. The microbial with the LDA score > 3 is a specific microbial, which distinguishes one group from other groups. At 28 days of age, a total of 33 specific microbial genera (LDA score >3) were found in Group A3 and CON, in which the *c_Bacilli*, *o_Lactobacillales*, *f_Lactobacillaceae* were enriched in Group CON, and *c_Bacteroidia*, *p_Bacteroidota*, *o_Bacteroidales* were enriched in Group A3. At 56 days of age, a total of 24 specific microbial genera (LDA score >3) were found in Group A1, A2, and A3, among which the *c_Clostridia*, *o_Osicillospirales*, and *f_Lachnospiraceae* were enriched in Group A3. These genera had a significant impact on the sample Grouping.

Predictive Analysis of Bacterial Community Function

Before 28 days of age, the diarrhea incidence of calves in each group was significantly different. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States two

TABLE 6 | Effects of Anemoside B4 on the comparison of dominant genus of suckling calves at 28 days of age (%).

Genus	Diets				SEM	*p*-value
	CON	A1	A2	A3		
Megasphaera	15.62	22.44	13.97	20.18	0.032	0.79
Prevotella	0.91^{b}	1.91^{b}	8.44^{ab}	12.21^{a}	0.016	0.03
Parasutterella	0.90	0.60	2.42	3.28	0.006	0.40
Dialister	3.42	2.69	6.12	2.88	0.011	0.67
Romboutsia	2.46	0.05	0.23	2.25	0.007	0.44
Faecalibacterium	10.66	5.27	5.52	7.51	0.026	0.89
Mitsuokella	0.74	3.92	4.49	2.11	0.007	0.08
Megamonas	1.13	7.46	9.14	6.40	0.022	0.62
Bacteroides	0.11	0.48	2.04	4.34	0.010	0.49
Fusobacterium	7.75	0.02	3.32	5.97	0.023	0.70
Clostridium_sensu_stricto_1	2.88	1.25	0.93	3.41	0.008	0.67
Olsenella	2.18	0.57	1.65	1.69	0.005	0.68
Lachnospiraceae_NK3A20_Group	3.08	2.41	0.77	2.06	0.008	0.77
Sutterella	0.53^{ab}	0.19^{b}	0.25^{b}	1.72^{a}	0.003	0.04
Alloprevotella	0.34	1.62	1.82	5.04	0.011	0.50

CON, the dosage of B4 is 0 mg / d; A1, the dosage of B4 is 15 mg / d; A2, the dosage of B4 is 30 mg / d; A3, the dosage of B4 is 45 mg / d; SEM, Standard error of the mean.
^{ab}Denotes a diversity trait with significant difference ($p < 0.05$).

TABLE 7 | Effects of Anemoside B4 on the comparison of dominant genus of suckling calves at 56 days of age (%).

Genus	Diets				SEM	*p*-value
	CON	A1	A2	A3		
Prevotella	50.65	30.97	36.4	30.68	0.037	0.06
Megasphaera	15.79	16.92	20.05	14.54	0.020	0.82
Faecalibacterium	2.06^{b}	3.58^{ab}	4.72^{ab}	7.84^{a}	0.010	0.04
Acidaminococcus	4.53^{b}	13.39^{a}	6.7^{ab}	4.94^{b}	0.014	0.02
Bifidobacterium	0.35	1.77	1.09	3.47	0.007	0.44
Dialister	1.64	1.88	4.58	3.04	0.005	0.06
Bacteroides	0.46	1.05	2.33	2.84	0.007	0.64
Subdoligranulum	0.58	3.15	2.32	2.63	0.006	0.43
Megamonas	0.79	1.59	0.09	2.11	0.006	0.64
Sutterella	0.28^{b}	0.42^{b}	0.46^{b}	2.05^{a}	0.003	0.02
Mitsuokella	2.3	2.27	3.56	1.8	0.004	0.52
uncultured	1.09	1.32	1.95	1.59	0.003	0.72
Alloprevotella	0.93	1.31	3.18	1.58	0.007	0.72
Escherichia-Shigella	0.58	0.45	0.79	1.47	0.003	0.57
Phascolarctobacterium	0.5	0.61	0.4	1.41	0.002	0.47

CON, the dosage of B4 is 0 mg / d; A1, the dosage of B4 is 15 mg / d; A2, the dosage of B4 is 30 mg / d; A3, the dosage of B4 is 45 mg / day; SEM, Standard error of the mean.
^{ab}Denotes a diversity trait with significant difference ($p < 0.05$).

was used to predict the function of marker genes in the four treatments groups at 28 days of age, indicating that it was mainly concentrated in metabolism, genetic information processing, environmental information processing, and cellular processes and organic systems. The relative abundance of metabolism and genetic information processing is relatively high. The genetic information processing was similar among the four groups ($p > 0.05$), while metabolism showed a great difference ($p < 0.05$). Among these, carbohydrate metabolism in Group A1 and A2 was significantly higher than that in Group A3 ($p < 0.05$), while no obvious differences were found between Group CON and other groups ($p > 0.05$). The lipid metabolism and xenobiotics biodegradation and metabolism in Group CON were significantly higher than those in Group A2 and A3 ($p < 0.05$), while the metabolism of cofactors and vitamins and energy metabolism showed the opposite. In addition, Group A3 had a higher abundance of glycan biosynthesis and metabolism than other groups ($p < 0.05$) in **Table 8**.

Correlation Between Fecal Microbial and Metabolism

As shown in **Figure 5**, the relationships between the dominant microbial and metabolism were evaluated in this study. Carbohydrate metabolism was negatively correlated with *Parasutterella* and *Dialister* ($p < 0.05$). The lipid metabolism was negatively correlated with *Prevotella*, *Mitsuokella*, and *Megamonas* ($p < 0.05$), while positively correlated with *Olsenella* ($p < 0.01$). The Metabolism of cofactors and vitamins was positively correlated with *Megasphaera*, *Prevotella*, *Mitsuokella*, and *Megamonas* ($p < 0.05$), and negatively correlated with *Olsenella* ($p < 0.01$). Energy metabolism has a positive correlation with *Prevotella*, *Bacteroides*, and *Sutterella* ($p < 0.05$), and a negative correlation with *Clostridium_sensu_stricto_1* ($p < 0.05$). Xenobiotics biodegradation and metabolism was negatively correlated with *Prevotella* ($p < 0.05$), and positively correlated with *Clostridium_sensu_stricto_1*, *Olsenella*, and *Lachnospiraceae_NK3A20_group* ($p < 0.05$). Glycan biosynthesis and metabolism were positively correlated with *Prevotella* and *Bacteroides* ($p < 0.05$), and negatively correlated with *Olsenella* ($p < 0.05$), respectively.

DISCUSSION

In vitro antibacterial test showed that the extract of Pulsatillae Radix has a certain inhibitory effect on *Staphylococcus aureus*, *Shigella dysenteriae*, and *Salmonella typhi* (23). In this study, feeding 45 mg/day Anemoside B4 could reduce the diarrhea incidence of calves from 7 to 56-day-old, especially before 42 days of age, indicated that Anemoside B4 might have a direct inhibitory effect on the pathogenic bacteria of diarrhea, which is consistent with the results of the previous study (24). After 42 days of age, there was no significant difference in the incidence of diarrhea among the groups. This was mainly due to the gradual improvement of the calf's own immune system and the gradual stabilization of the intestinal flora, which can effectively resist the invasion of external pathogenic bacteria. The effect of Anemoside B4 on diarrhea gradually decreases with the age of calves increasing, which also indicates that the prevention and treatment effect of Anemoside B4 on the diarrhea of calves are mainly effective before 42 days of age.

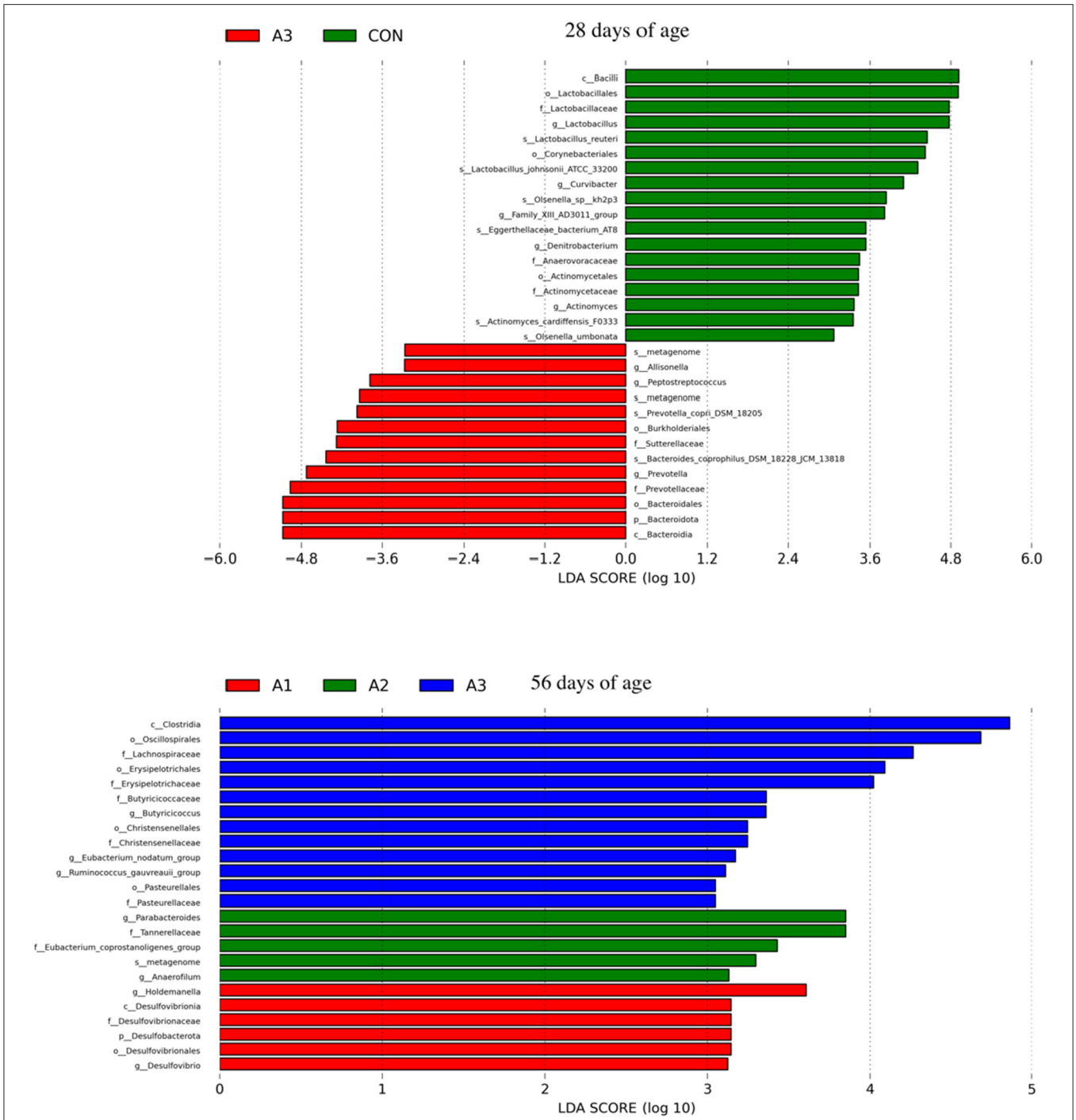

FIGURE 4 | The potential biomarkers were defined by LEfSe at 28 and 56 days of age. The significantly different taxa are signified by different colors representing the four groups. Histogram of the LDA scores for differentially abundant features among groups. The threshold on the logarithmic LDA score for discriminative features was set to 3.0. CON, the dosage of B4 is 0 mg / day; A1, the dosage of B4 is 15 mg / day; A2, the dosage of B4 is 30 mg / day; A3, the dosage of B4 is 45 mg / day.

The occurrence of diarrhea of suckling calves is mainly related to the immune status, and newborn calves with low immunity are prone to be affected by external pathogens (25). In a previous study, Anemoside B4 could significantly increase the content of IgG in the serum of suckling calves, indicating that Anemoside B4 could improve the immune ability of calves (26). In addition, diarrhea is associated with intestinal injury and disturbance of intestinal flora in calves (3, 4). The increase of intestinal

TABLE 8 | Effects of Anemoside B4 on metabolism of suckling calves at 28 days of age (%).

Genus	Diets				SEM	*p*-value
	CON	A1	A2	A3		
Carbohydrate metabolism	14.87^{ab}	15.65^{a}	15.33^{a}	14.30^{b}	0.182	<0.01
Lipid metabolism	5.85^{a}	5.05^{b}	4.31^{b}	4.18^{b}	0.229	0.02
Metabolism of cofactors and vitamins	12.30^{b}	12.97^{ab}	13.75^{a}	14.17^{a}	0.260	0.04
Energy metabolism	4.97^{b}	5.07^{ab}	5.23^{a}	5.30^{a}	0.049	0.02
Nucleotide metabolism	2.22	2.14	2.15	2.20	0.031	0.80
Amino acid metabolism	12.44	12.59	13.31	13.17	0.203	0.36
Metabolism of terpenoids and polyketides	10.26	9.36	9.78	10.13	0.187	0.34
Biosynthesis of other secondary metabolites	2.11	2.01	1.80	2.00	0.082	0.62
Xenobiotics biodegradation and metabolism	2.96^{a}	2.70^{ab}	1.55^{b}	1.17^{b}	0.272	0.02
Metabolism of other amino acids	7.48	7.61	7.33	7.34	0.098	0.73
Glycan biosynthesis and metabolism	3.48^{b}	3.82^{ab}	4.37^{ab}	4.92^{a}	0.239	0.03

CON, the dosage of B4 is 0 mg / d; A1, the dosage of B4 is 15 mg / day; A2, the dosage of B4 is 30 mg / d; A3, the dosage of B4 is 45 mg / d; SEM, Standard error of the mean.
^{abc}Denotes a diversity trait with significant difference ($P < 0.05$).

permeability and the disorder of intestinal microbiota are the key factors leading to diarrhea caused by pathogens (27). DAO is a highly active cytoplasmic enzyme found in mammalian intestinal epithelial cells, and serum D-LA is a metabolite of gastrointestinal flora. Serum D-LA and DAO levels are negatively correlated with the integrity and maturity of the intestinal mucosa, so its level can indirectly reflect the changes in intestinal mucosal permeability and the degree of damage to the intestinal mechanical barrier, and it is also an important indicator for evaluating the integrity of the intestinal mucosa (28). When a disease occurs in the body, the intestinal permeability increases and a large amount of DAO and D-LA enter the blood circulation with the increase in intestinal permeability, resulting in an increase in serum DAO and D-LA levels. In this study, at 28 days of age, the serum D-LA and DAO levels of the calves without Anemoside B4 treatment are significantly higher than others, indicating that they are most likely to have intestinal mucosa diseases, such as injury and increased permeability. These also proved that Anemoside B4 has the effect of protecting the intestinal mucosa of suckling calves, and it is mainly achieved by reducing the permeability of the intestinal mucosa and improving the integrity of the intestinal mucosa (8). Therefore, Anemoside B4 can not only directly inhibit the reproduction of diarrhea-causing bacteria, but also can effectively reduce the permeability and damage of the intestinal mucosa of calves.

Anemoside B4 had notable anti-inflammation activity, and it could inhibit the expression of pro-inflammatory cytokines and enhance immunity (18). Newborn calves have low immunity, they need to obtain nutrients from the external environment to improve their own immunity for achieving the effect of active immunity (29). But it is also susceptible to the stimulation of various external environments to the disturbance of the calf intestinal flora, which leads to the occurrence of diarrhea. The complex microbiome plays a very important role in the barrier function of the intestinal mucosa and the growth and development of the body (30), and its composition is one of the important factors that determine whether a calf has diarrhea or not. The more diversified microorganisms are, the more beneficial it is to the health of the body. It is also considered to be a sign of the maturity of the intestinal microbial community (31), but the diversified microflora is not necessarily beneficial to the health of the body. The structure of the microbial community will have an adverse effect on the body's development (32). In this study, Anemoside B4 can improve the abundance and diversity of fecal microbial, mainly because low dose saponins can promote the penetration of cell membranes, make bacterial cells absorb more nutrients, promote the growth of bacterial (33), and reduce the number of protozoa (34, 35).

A healthy intestinal microbial can regulate host nutrition and intestinal development, digest nutrients that are difficult for the host to digest and absorb, produce metabolites required for host growth and development, enhance the host immune system by enhancing the interaction between antigens and immune cells, and prevent the colonization of foreign pathogens (27, 36). Therefore, microbial can also be regarded as a metabolic organ and play an important role (37). It is found that the dominant bacteria in the animal gastrointestinal tract are Bacteroides and Firmicutes (38–40), which is consistent with the results of this study. Those indicate that Anemoside B4 supplementation would promote the stability of the intestinal flora of calves and have no adverse effect. In this study, the main phyla in the low diarrhea incidence group were *Firmicute* (62.55%), *Bacteroidetes* (23.17%), *Fusobacteriota* (5.97%), and *Proteobacteria* (5.87%), and the main phyla in the high diarrhea incidence group were *Firmicute* (78.93%), *Fusobacteriota* (7.75%), *Actinobacteriota* (5.99%), and *Proteobacteria* (5.15%) at 28 days of age. In which, there was no significant difference except for *Bacteroidetes*. *Firmicutes* are mainly involved in the hydrolysis of carbohydrates and proteins, and *Bacteroidetes* mainly act on steroids, polysaccharides, and bile acids (41, 42), indicating that Anemoside B4 reduced calf diarrhea incidence mainly by regulating the relative abundance of *Bacteroidetes* in the intestinal flora of calves and promoting the degradation of non-cellulose substances.

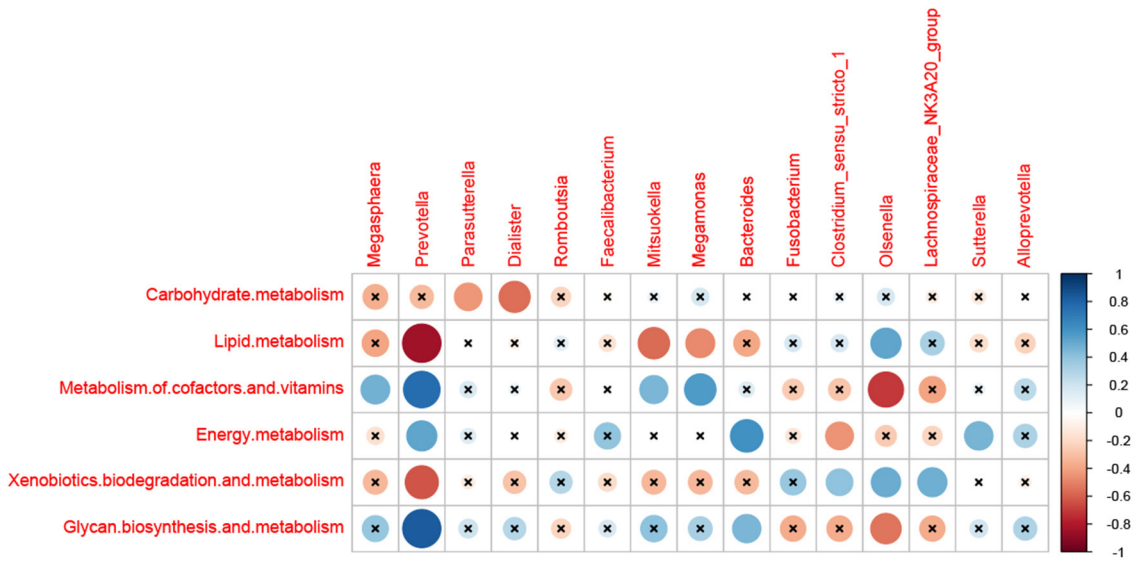

FIGURE 5 | Correlation matrix between the metabolism and the dominant microbial at 28 days of age. The abscissa is the differential flora, and the ordinate is the differential metabolisms. Blue is positive and red is negative.

Prevotella is a genus of dominant bacteria that widely exists in the rumen and gastrointestinal tract of herbivores and omnivores. It plays an important role in protein decomposition. At the same time, it can also use the succinic acid pathway to ferment starch, monosaccharides, and other non-cellulosic polysaccharides to produce propylene acid, and further, maintain the glucose homeostasis in animals through the gluconeogenesis pathway (43). In this study, 45 mg/day Anemoside B4 significantly increased the relative abundance of *Prevotella* in the fecal microorganisms of calves, indicating that the addition of Anemoside B4 helps calves to digest and absorb nutrients and reduce the feed conversion rate. In addition, the relative abundance of *Sutterella* in the fecal microbial of calves fed 45 mg/day Anemoside B4 was the highest while the incidence of diarrhea was the lowest. The study found that *Sutterella* and *Bacteroides* may be closely related to the occurrence and development of antibiotic-associated diarrhea (AAD) in rats (44). The relative abundance of *Sutterella* is negatively correlated with the administration of Shen Ling Bai Zhu San (SLBZS), indicating that *Sutterella* is the main bacterium causing diarrhea after antibiotic treatment. Our result is contrary to the above results, which may be caused by differences in animal species. Another explanation may be different causes of calf diarrhea. A study showed that in the context of other inflammatory bowel diseases (IBDs), the detrimental effects of *Sutterella* seem to be specific to ulcerative colitis (UC), as the low abundance of *Sutterella* in patients with Crohn's disease (CD) (45). At present, there are few studies on the relationship between *Sutterella* and calf diarrhea, and specific reasons need to be further studied. The *Faecalibacterium prausnitzii* is the only bacterial species in the genus *Faecalibacterium*, and it is also one of the most important symbiotic bacteria in human intestinal microbes (46). Studies have shown that *F. prausnitzii* can metabolize unabsorbed carbohydrates in the intestine to produce butyrate and exert the body's anti-inflammatory effect by upregulating the expression of the Dact3 gene (47). In this study, Anemoside B4 increased the relative abundance of *Faecalibacterium* in the fecal flora of calves, of which 45 mg/day achieved the best effect. The above results indicate that Anemoside B4 can regulate the intestinal flora of calves and enhance the immunity of calves by increasing the relative abundance of *Faecalibacterium* in the intestines of calves and reducing the incidence of calf diarrhea. Thus, the improvement of calf diarrhea may be due to the intestinal microbial changes induced by Anemoside B4, including the increase of the relative abundance of *Prevotella*, *Sutterella*, and *Faecalibacterium*.

Gene function prediction results show that Anemoside B4 has an important influence on the metabolism of calves. Among them, the addition of Anemoside B4 in the calf can promote the processes of energy metabolism, glycan biosynthesis and metabolism, metabolism of cofactors and vitamins, and inhibit lipid metabolism, xenobiotics biodegradation, and metabolism. It has been found that intestinal microbes are closely related to the metabolism of the body, and intestinal flora can influence metabolic activity by improving the energy output of food, regulating the diet, or changing the host's metabolic pathway (48). Energy metabolism is closely related to the growth and development of the body. When the energy level cannot meet the needs of the body, it will decrease feed conversion rate and productivity. A certain promotion effect reflects the enhancement of anabolism in the anabolism, which is beneficial to the growth of calves, and there is a positive correlation between energy metabolism and the relative abundance of *Prevotella*, *Bacteroides*, and *Sutterella* in the intestine. *Prevotella* can degrade starch, monosaccharide, and other non-cellulose polysaccharides to produce volatile fatty acid (VFA) to provide energy for the body and promote rumen development (49), and *Bacteroides* is a bacterium that produces short-chain fatty

acids. In this study, 45 mg/day Anemoside B4 increased the relative abundance of *Bacteroides*, which can promote fiber degradation to generate energy, inhibit opportunistic pathogens, protect the host from inflammation and colonic diseases, and reduce the diarrhea incidence (50, 51). Besides, *Prevotella* was positively correlated with the metabolism of cofactors and vitamins and glycan biosynthesis and metabolism. The study also found that with the increase of piglet age, the function of the intestinal flora gradually matures, glycan biosynthesis and metabolism, vitamin B biosynthesis significantly increased (52). In this study, glycan biosynthesis and metabolism, metabolism of cofactors and vitamins gradually increased with the addition of Anemoside B4, indicating that Anemoside B4 contributes to the maturation and stability of calf intestinal flora, thus significantly reducing the diarrhea incidence of calves. Soy isoflavone can affect drugs and exogenous metabolism by regulating the expression and activity of phase I cytochrome P450 (CYPs) enzymes and phase I detoxification enzymes, and have anticancer, antiobesity, and antioxidant activities (53). In this study, lipid metabolism and xenobiotics biodegradation and metabolism gradually decreased with the addition of Anemoside B4, indicating that Anemoside B4 contributes to fat deposition in calves. Lipid metabolism and xenobiotics biodegradation and metabolism were positively correlated with *Olsenella*, and negatively correlated with *Prevotella*. Thus, this is mainly due to B4 decreasing *Olsenella* and increasing the relative abundance of *Prevotella*, and the specific reasons need to be further studied.

CONCLUSION

This study demonstrated that adding Anemoside B4 (45 mg/day) to the milk replacer significantly reduced the diarrhea incidence of suckling calves before 42 days of age. It also highlights that Anemoside B4 could promote the integrity and maturity of the intestinal mucosa. Based on 16S rRNA gene sequencing results, this study indicated that the addition of Anemoside B4 increased the relative abundance of *Bacteroidota* and decrease the relative abundance of *Firmicutes* in the fecal microbial of calves at 28 days of age. It was also discovered that 45 mg/day Anemoside B4 significantly increased the relative abundance of *Prevotella* at 28 days of age and *Sutterella* at 56 days of age. According to the function prediction of PICRUSt 2, Anemoside B4 could promote the processes of metabolism. The above results indicated that Anemoside B4 could protect the integrity of intestinal mucosa, change the structure of intestinal microbiota of calves, indicating the potential value of Anemoside B4 on regulating intestinal microbiota and treating intestinal diseases, alleviate diarrhea effectively.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethics Committee of IFRCAAS (AEC-IFR-CAAS, Beijing, China). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

ML carried out this study and then did the sampling and laboratory works, and YT critically reviewed the manuscript. FH participated in the whole experiment. YB and TM did the sampling. YT, QD, and LJ designed and approved the study plan. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.851865/full#supplementary-material

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A Small Multihost Plasmid Carrying *erm*(T) Identified in *Enterococcus faecalis*

Xing-Yun Li†, Rui Yu†, Chunyan Xu, Yanhong Shang, Dexi Li and Xiang-Dang Du**

Department of Basic Veterinary Medicine, College of Veterinary Medicine, Henan Agricultural University, Zhengzhou, China

The aim of this study was to determine the mobile genetic elements involved in the horizontal transfer of *erm*(T) in *Enterococcus faecalis*, and its transmission ability in heterologous hosts. A total of 159 erythromycin-resistant enterococci isolates were screened for the presence of macrolide resistance genes by PCR. Whole genome sequencing for *erm*(T)-carrying *E. faecalis* E165 was performed. The transmission ability in heterologous hosts was explored by conjugation, transformation, and fitness cost. The *erm*(T) gene was detected only in an *E. faecalis* isolate E165 (1/159), which was located on a 4,244-bp small plasmid, designed pE165. Using *E. faecalis* OG1RF as the recipient strain, pE165 is transferable. Natural transformation experiments using *Streptococcus suis* P1/7 and *Streptococcus mutans* UA159 as the recipients indicated it is transmissible, which was also observed by electrotransformation using *Staphylococcus aureus* RN4220 as a recipient. The *erm*(T)-carrying pE165 can replicate in the heterologous host including *E. faecalis* OG1RF, *S. suis* P1/7, *S. mutans* UA159, and *S. aureus* RN4220 and conferred resistance to erythromycin and clindamycin to all hosts. Although there is no disadvantage of pE165 in the recipient strains in growth curve experiments, all the pE165-carrying recipients had a fitness cost compared to the corresponding original recipients in growth competition experiments. In brief, an *erm*(T)-carrying plasmid was for the first time described in *E. faecalis* and as transmissible to heterologous hosts.

Keywords: *Enterococcus faecalis*, erythromycin, clindamycin, resistance, fitness cost, transmission

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Kristina Kadlec, Independent Researcher, Wunstorf, Germany

****Correspondence:***
Xiang-Dang Du
xddu@henau.edu.cn
Dexi Li
lidexi2006@126.com

†These authors have contributed equally to this work

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INTRODUCTION

Macrolides are a class of important natural or semisynthetic antibiotics that bind to the 50S ribosomal subunit and inhibit protein synthesis (1, 2). They have antimicrobial activity against Gram-positive and selected Gram-negative organisms (3, 4). The frequent use of macrolides in medical clinics and animal husbandry is accompanied by increased macrolide resistance, which may result in a failure of the treatment (5).

There are three ways to acquire macrolide resistance: modification of the target site, efflux pump, and drug inactivation (2, 5). Modification of the target site is mediated by 23S rRNA methylation enzymes, encoded by *erm* genes that confer resistance to macrolides; in the case of constitutive expression, they can also confer resistance to lincosamides and streptogramin B (6–11). Among the different *erm* genes currently known to occur in the different species of bacteria, *erm*(A) and *erm*(B) are most frequent (12, 13), mainly carried by a plasmid, transposons, translocatable units (TUs), and

integrative and conjugative elements (ICEs) (13). Ribosomal protection gene *msr* codes for ABC-F protein confers macrolide and streptogramin B resistance. The *mef* gene codes for an efflux pump confers macrolide resistance only. Drug inactivation enzymes including phosphotransferases and esterases, which are encoded by *mph* genes and *ere* genes, respectively, confer macrolide resistance (13, 14).

Since *erm*(T) had been detected in *Lactobacillus*, it has also been described in isolates of the bacterial species *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus gallolyticus, Staphylococcus aureus*, *Erysipelothrix rhusiopathiae*, *Haemophilus parasuis,* and *Enterococcus faecium* and *Streptococcus suis* (15–22), which revealed its widespread presence. However, whether it is transmissible and whether the *erm*(T)-carrying mobile genetic elements can replicate in a heterologous host had not been fully explored. So, in this study, the presence of *erm*(T) in enterococci was investigated and the associated mobile genetic elements involved in the horizontal transfer of *erm*(T) were explored. In addition, the transmission ability and maintenance of *erm*(T)-carrying plasmid in the heterologous host was elucidated using conjugation, transformation, and fitness cost experiments.

MATERIALS AND METHODS

Bacterial Strains and Antimicrobial Susceptibility Testing

During the routine survey for the presence of *erm*(T) in enterocci of swine origin, a total of 159 non-duplicate enterococci isolates with erythromycin MICs of no <8 mg/L were collected in July and September 2018 from anal swabs of healthy pigs at two farms in Henan Province, China.

Antimicrobial susceptibility testing (AST) was carried out by broth microdilution according to recommendations given in document M100 (Twenty-Eighth Edition) issued by CLSI (21). *S. aureus* ATCC 29213 served as the quality control strain.

E. faecalis OG1RF, *S. aureus* RN4220, *Streptococcus suis* P1/7, and *Streptococcus mutans* UA159 served as recipients in conjugation and transformation experiments.

PCR Analysis

The aforementioned erythromycin-resistant enterococci strains were detected for the presence of macrolide resistance gene *erm*(T) and other resistance genes by PCR using the primers listed in **Table 1** (15, 23–26). PCR products for *erm*(T) in *E. faecalis* E165 and its transconjugants and transformants were subjected to Sanger sequencing.

Whole Genome Sequencing (WGS) and Analysis

Whole genome DNA of *E. faecalis* E165 was sequenced by the PacBio RS and Illumina MiSeq platforms (Shanghai Personal Biotechnology Co., Ltd, China). The PacBio sequence reads were assembled with HGAP4 and CANU (Version 1.6), and corrected by Illumina MiSeq with pilon (Version 1.22). The prediction of ORFs and their annotation was performed using Glimmer 3.0.

Intraspecies Transformation and Interspecies Transformation

To investigate whether the *erm*(T)-carrying plasmid could be transferred into bacteria of the same and other species, plasmid DNA extracted from *E. faecalis* E165 was obtained by using Qiagen Mini-prep kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

To determine the intraspecies transmissibility of the *erm*(T)-carrying plasmid pE165, conjugation experiments were performed using *E. faecalis* E165 as the donor and *E. faecalis* OG1RF as the recipient as previously described (27). Transconjugants were screened on brain heart infusion (BHI, Oxoid, British) agar supplemented with 32 mg/L rifampicin, 32 mg/L fucidin acid, and 8 mg/L erythromycin. The corresponding transconjugant was designated *E. faecalis* OG1RF-Tc.

Natural transformation experiment using the recipient *S. suis* P1/7 was performed as described to investigate the interspecies transmissibility of *erm*(T)-carrying plasmid pE165 (28). The peptide (GNWGTWVEE) was dissolved in Milli-Q water at a final concentration of 5 mM and was used as a pheromone for the transformation. The erythromycin-susceptible recipient strain *S. suis* P1/7 was grown overnight in THY broth (3 g Todd-Hewitt Broth and 2 g yeast for 100 ml, Oxoid, British) at 37°C under 5% CO_2. The overnight culture was diluted 1:40 into pre-warmed THY broth, and grown at 37°C under 5% CO_2 without shaking. Plasmid DNA (1.2 μg) and stock peptide (5 μl) were added when the recipient culture reached an OD_{600} between 0.035 and 0.058, and then incubated for 2h at 37°C under 5% CO_2. The samples were diluted and plated on THA (Todd-Hewitt Broth with 1.3% agar powder, Oxoid, British) containing 8 mg/L erythromycin. The corresponding transformant was designated *S. suis* P1/7-Tm.

Natural transformation experiment with the recipient *S. mutans* UA159 was performed following procedures as previously described (29). For the screening of the transformants, THA was supplemented with 8 mg/L erythromycin. The corresponding transformant was designated *S. mutans* UA159-Tm.

The electrotransformation experiment with recipient strain *S. aureus* RN4220 was performed as described in a previous study (30). The corresponding transformant was designated *S. aureus* RN4220-Tm.

All colonies from the selective plates after incubation for 24–48 h at 37°C were further confirmed by *erm*(A), *erm*(B), and *erm*(T) gene detection (15, 23), 16S rRNA sequencing, AST, and multilocus sequence typing (MLST) following the harmonized protocols (http://www.mlst.net/). The plasmids obtained from *E. faecalis* E165, *E. faecalis* OG1RF-Tc, *S. suis* P1/7-Tm, *S. mutans* UA159-Tm, and *S. aureus* RN4220-Tm were digested by Sac I and Pac I (New England Biolabs, Inc., USA), and then southern bolt using *erm*(T) gene as the probe was performed.

Growth Curve and Growth Competition Experiments

Growth kinetics were determined for *E. faecalis* OG1RF and *E. faecalis* OG1RF-Tc, *S. aureus* RN4220 and *S. aureus* RN4220-Tm, *S. suis* P1/7 and *S. suis* P1/7-Tm, and *S. mutans* UA159

TABLE 1 | Primer pairs used in this study.

Gene(s)	Primer	Sequence(5′-3′)	Product size	References
erm(A)	*erm*(A) forward	GCATGACATAAACCTTCA	208bp	(23)
	erm(A) reverse	AGGTTATAATGAAACAGA		
erm(B)	*erm*(B) forward	GAAAAGGTACTCAACCAAATA	639bp	(23)
	erm(B) reverse	AGTAACGGTACTTAAATTGTTTAC		
erm(C)	*erm*(C) forward	AATCGTCAATTCCTGCATAT	299bp	(24)
	erm(C) reverse	TAATCCTGGAATACGGGTTTG		
erm(F)	*erm*(F) forward	TAGATATTGGGGCAGGCAAG	178bp	(25)
	erm(F) reverse	GGAAATTTCGGAACTGCAAA		
erm(G)	*erm*(G) forward	ATAGGTGCAGGGAAAGGTCA	177bp	(25)
	erm(G) reverse	TGGATTGTGGCTAGGAAATGT		
erm(X)	*erm*(X) forward	TGACGCTGTACTCCTCATGC	410bp	(26)
	erm(X) reverse	GAGGAACCAGTCACCTGGAA		
erm(T)	*erm*(T) forward	CCGCCATTGAAATAGATCCT	478bp	(15)
	erm(T) reverse	GCTTGATAAAATTGGTTTTTGGA		

and *S. mutans* UA159-Tm (19, 31). Volumes of 30 ml BHI broth were inoculated independently with 10^7 CFU of OG1RF, OG1RF-Tc, RN4220, and RN4220-Tm; cultures were grown for 12 h at 200 rpm and 37°C. Volumes of 30 ml Todd-Hewitt Broth (THB, Oxoid, British) supplemented with 5% fetal calf serum were inoculated independently with 10^7 CFU of P1/7, P1/7-Tm, UA159, and UA159-Tm; cultures were grown for 12 h at 200 rpm and 37°C. The absorbance at 600 nm was measured every hour.

The fitness cost of pE165 was determined between *E. faecalis* OG1RF and *E. faecalis* OG1RF-Tc, *S. aureus* RN4220 and *S. aureus* RN4220-Tm, *S. suis* P1/7 and *S. suis* P1/7-Tm, and *S. mutans* UA159 and *S. mutans* UA159-Tm, as previously described (31, 32), with the following modifications. OG1RF and OG1RF-Tc, RN4220, and RN4220-Tm, were cultured in BHI broth for 24h at 37°C and 200 rpm. P1/7 and P1/7-Tm, UA159 and UA159-Tm, were cultured in THB (supplemented with 5% fetal calf serum) for 24 h at 37°C and 200 rpm. Then 1×10^8 CFU of recipient strain was mixed with 1×10^8 CFU of corresponding transconjugant/ transformant in 30 ml antibiotic-free BHI broth/THB (supplemented with 5% fetal calf serum). The mixtures were grown at 37°C and 200 rpm and diluted at 1:100 to fresh BHI broth/THB (supplemented with 5% fetal calf serum) every 24 h. For each sample, aliquots were plated onto non-selective and erythromycin-containing BHI agar/THA (supplemented with 5% fetal calf serum) plates. The proportion of pE165-carrying strains was calculated by the number of colonies on the selective plate divided by the number of colonies on the non-selective plate.

RESULTS

Identification and Characterization of *erm*(T)-Carrying Plasmid in *E. faecalis*

All erythromycin-resistant strains were investigated for the presence of the macrolide resistance genes by PCR. Of the 159 erythromycin-resistant enterococci isolates, 24(15.1%) contained solely *erm*(A), 33 (20.8%) contained solely *erm*(B), and 102 (64.2%) were positive for both *erm*(A) and *erm*(B). No strain was *erm*(C)/*erm*(F)/*erm*(G)/*erm*(X)-positive. A single *E. faecalis* strain E165 was positive for *erm*(A), *erm*(B), and *erm*(T). This is the first description of the *erm*(T) gene in *E. faecalis*.

Whole genome sequencing, assembly, and analysis for *E. faecalis* E165 showed a 4,244-bp small plasmid (designated pE165) harbored the *erm*(T) gene, with an average GC content of 33.0%. A total of three open reading frames (ORFs) encoding proteins of >100 amino acids were identified. The *erm*(T) gene coded for a 244-amino-acid (aa) protein identical to *erm*(T) of pKKS25 in *S. aureus* 25 (CAY48681.1) (18), and was highly similar (99.2% amino acid identity, 99.7% DNA sequence identity) to that of pGT633 from *Lactobacillus reuteri* 100-63 (NG_047838) (**Figure 1**) (32). The plasmid mobilization protein encoded by the *mob* gene from pE165 showed a high level of homology (identity≥90.6%) to that encoded by *mob* genes from *Lactococcus garvieae* (WP_207144600), *E. faecium* (HAR1670775.1), *S. suis* (WP_105139626), *S. aureus* (CCQ43999), and *Escherichia coli* (EFG1049274). The replication protein encoded by the *rep* gene from pE165 exhibited ≥99.5% identities to that encoded by *rep* genes from *E. faecalis* (HBI2052878), *Listeria monocytogenes* (HAB0665403), *S. suis* (NQK16007), *S. agalactiae* (WP_228308086), *E. faecium* (HAZ0989061), *Bacillus paranthracis* (AHN52261), *E. coli* (EFG1049261), *Lactimicrobium massiliense* (WP_108775117), and *Amylolactobacillus amylophilus* (WP_054746480), which revealed how widespread the *erm*(T)-carrying pE165-like plasmid is.

In addition, *erm*(A) gene carried by Tn*6674* was located on the chromosome. Three copies of *erm*(B) gene were located on a 65,052 bp conjugative plasmid (designated pE165-2) and the conjugative region from pE165-2 exhibits 99% DNA identity to pL15 described in an *E. faecalis* isolated from swine in Brazil (CP042214). pE165-2 also includes *tet*(M) and *tet*(L) conferring resistance to tetracyclines, *dfrG* conferring resistance to trimethoprim, *aacA-aphD* conferring

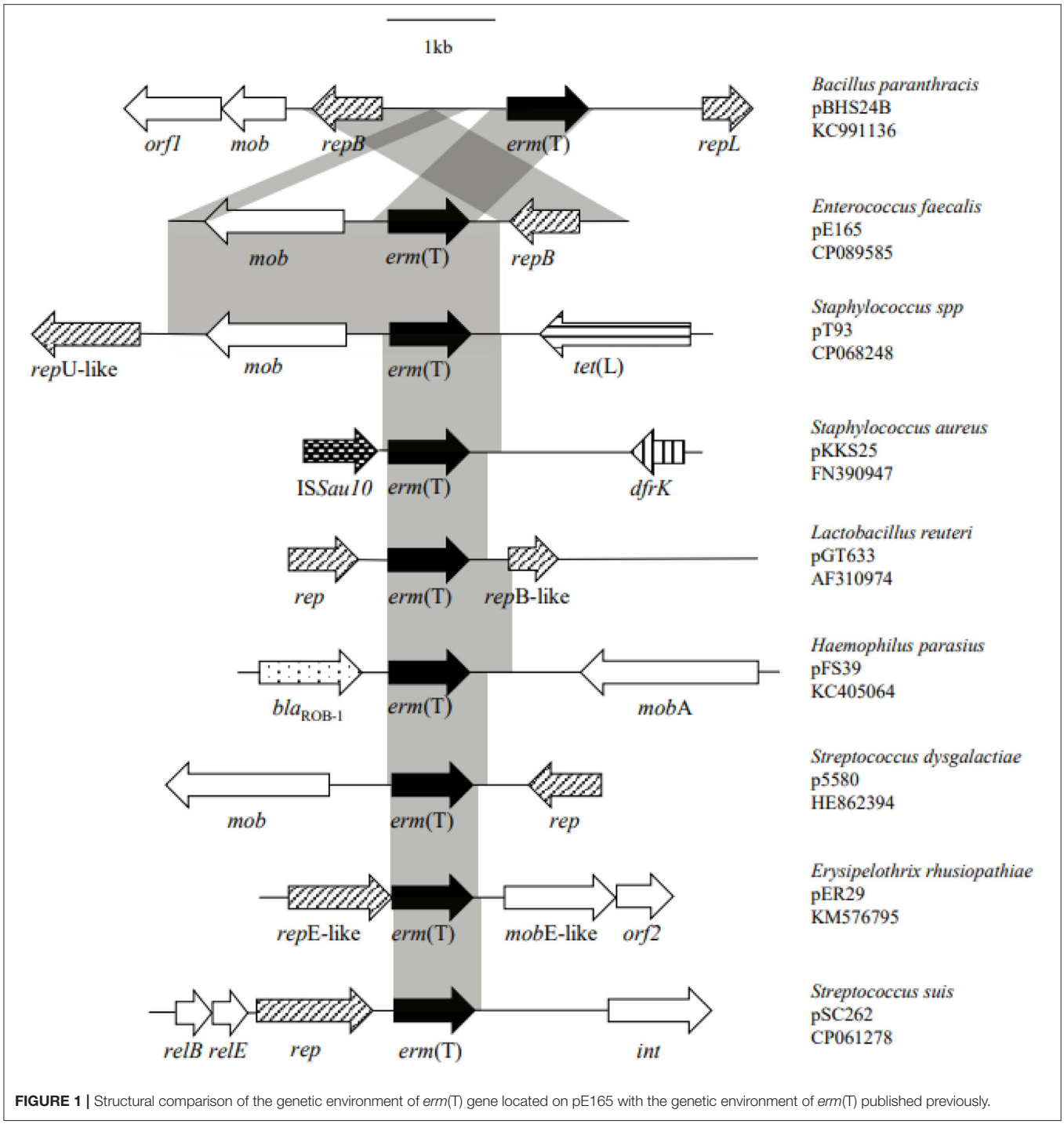

FIGURE 1 | Structural comparison of the genetic environment of *erm*(T) gene located on pE165 with the genetic environment of *erm*(T) published previously.

resistance to aminoglycosides, and *cat* encoding chloramphenicol acetyltransferase. A 2,836 bp small plasmid that did not carry any resistance gene was also detected in *E. faecalis* E165 (designated pE165-3).

Previous studies identified a complete translational attenuator immediately upstream of the *erm*(T) gene which consisted of two pairs of inverted-repeat sequences of 12 bp each and a reading frame for a regulatory peptide of 19 aa (15, 17, 33). Inducible *erm* gene expression often required an intact translational attenuator, while deletions or duplications that appeared in the regulatory region would cause constitutive *erm* gene expression (34). A comparison of the *erm*(T) regulatory region of pE165 with that of plasmids pRW35 (EU192194) revealed that the *erm*(T) regulatory region of pE165 had four bp point mutations and one bp deletion in the regulatory peptide ORF. This single nucleotide deletion resulted in a frame shift mutation, which

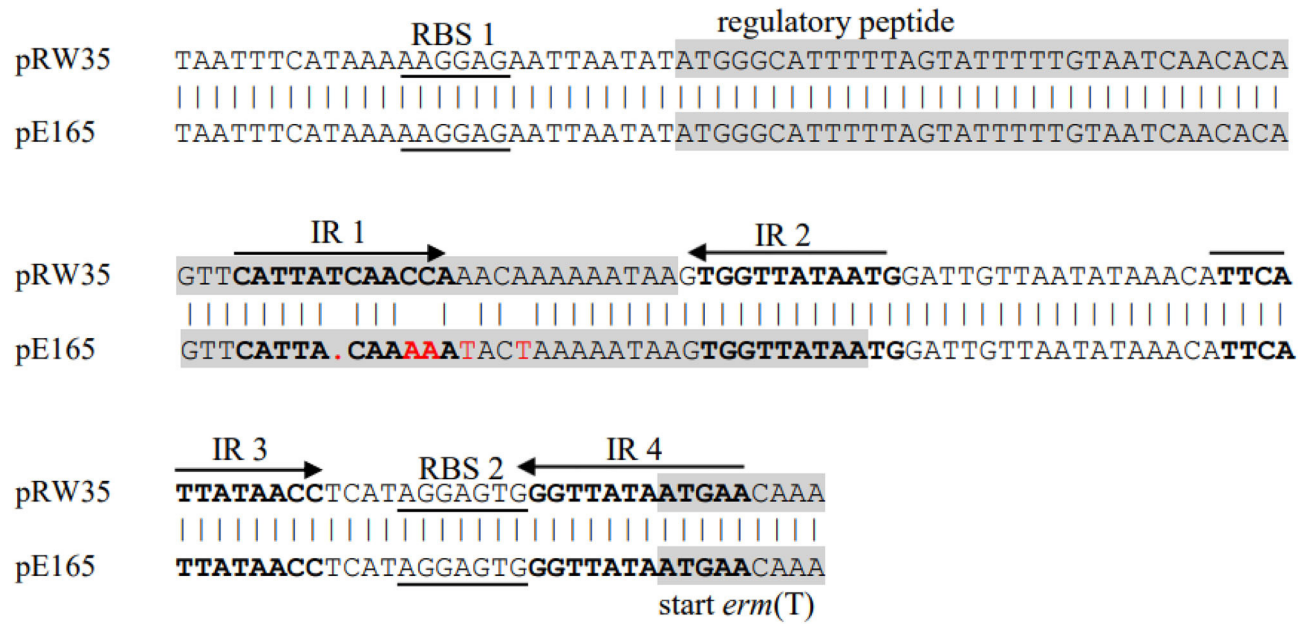

FIGURE 2 | Alignment of *erm*(T) regulatory region of pRW35.seq (upper line) and *erm*(T) regulatory region of pE165.seq (lower line). Identity = 97.40%(150/154), Gap = 0.65%(1/155).

TABLE 2 | MICs of *erm*(T)-carrying *E. faecalis* E165, recipient strains *E. faecalis* OG1RF, *S. aureus* RN4220, *S. suis* P1/7, *S. mutans* UA159, and corresponding transconjugants/transformants.

Strains	MICs (mg/L)[a]							
	ERY	LIN	CLI	CHL	FFC	TET	LND	CIP
E165	>512	>512	512	128	64	64	4	32
OG1RF	1	32	16	4	2	<1	0.5	2
OG1RF-Tc	>512	512	256	4	2	<1	0.5	2
RN4220	<1	<1	<1	2	2	<1	0.25	<1
RN4220-Tm	>512	512	128	2	2	<1	0.25	<1
P1/7	<1	<1	<1	1	1	<1	0.25	1
P1/7-Tm	512	64	32	1	1	<1	0.25	1
UA159	<1	<1	<1	2	1	<1	0.25	1
UA159-Tm	>512	512	256	2	1	<1	0.25	1

[a]*ERY, erythromycin; LIN, lincomycin; CLI, clindamycin; CHL, chloramphenicol; FFC, florfenicol; TET, tetracycline; LND, linezolid; CIP, ciprofloxacin.*

extended the reading frame for the regulatory peptide from 19 aa to 22 aa (**Figure 2**). In addition, *erm*(T) regulatory region of pE165 was compared to those of pSC262 and pUR2940 with mutations in previous reports (22, 35), and the results are shown in **Supplementary Figures S1**, **S2**.

The *erm*(T)-Carrying Plasmid Is Transmissible

Intraspecies transmissibility of pE165 was investigated by conjugation experiments using *E. faecalis* E165 as the donor and *E. faecalis* OG1RF as the recipient. The transconjugant OG1RF-Tc was successfully obtained on the selective plates with the transfer frequencies of 1.5×10^{-5}. PCR assay was used for the detection of *erm*(T), *erm*(A), and *erm*(B) in transconjugant, and the results revealed that only *erm*(T) gene was transferred into the recipient. Minimum inhibition concentrations (MICs) of *E. faecalis* E165, OG1RF, and OG1RF-Tc were determined and are shown in **Table 2**. Compared to *E. faecalis* OG1RF, OG1RF-Tc displayed a higher erythromycin MIC (>512 mg/L) and higher clindamycin MIC (256 mg/L).

Interspecies transmissibility of pE165 was investigated by natural transformation using *S. suis* P1/7 and *S. mutans* UA159 as the recipients and electrotransformation using *S. aureus* RN4220 as the recipient. Transformants P1/7-Tm, UA159-Tm, and RN4220-Tm were successfully obtained, and the transfer frequencies were 0.63×10^2 μg^{-1},2.1×10^2 μg^{-1} and 4.9×10^4 μg^{-1}. The transformants were confirmed by AST and sequencing of 16S rRNA. PCR assay also revealed that only *erm*(T)

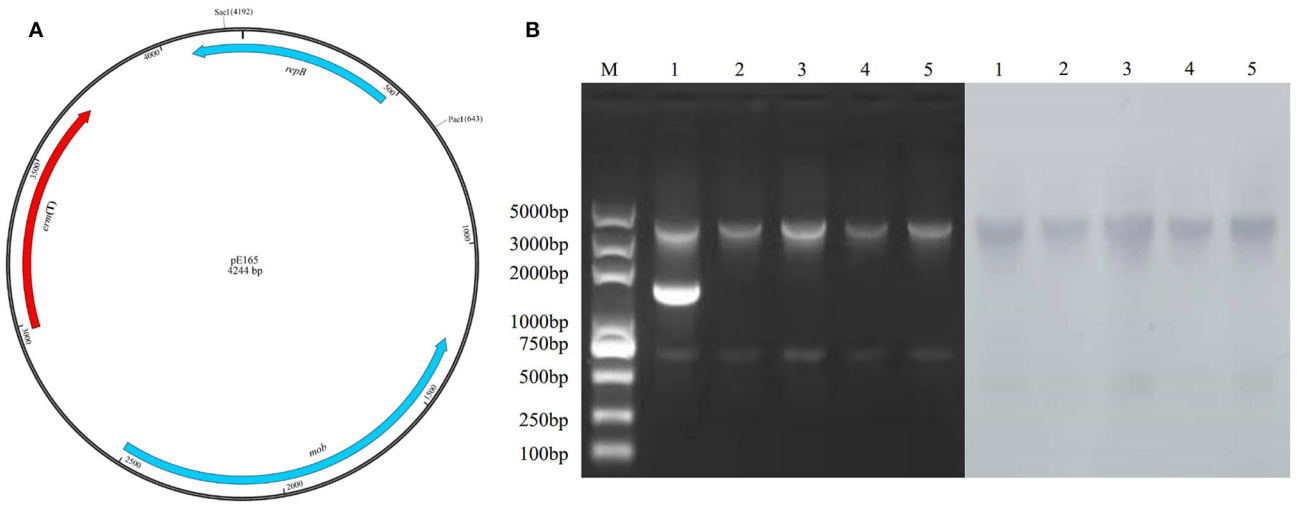

FIGURE 3 | (A) Restriction enzyme cutting sites of Sac I and Pac I on pE165. **(B)** Restriction enzyme-digested plasmid profiles treated by Sac I, Pac I, and southern blot using *erm*(T) gene as the probe. M: marker 1; *E. faecalis* E165, 2; *E. faecalis* OG1RF-Tc, 3; *S. mutans* UA159-Tm, 4; *S. suis* P1/7-Tm, 5; *S. aureus* RN4220-Tm. The upper and lower bands were the fragments of pE165 digested by Sac I and Pac I. The middle band in line 1 was supercoiled pE165-3.

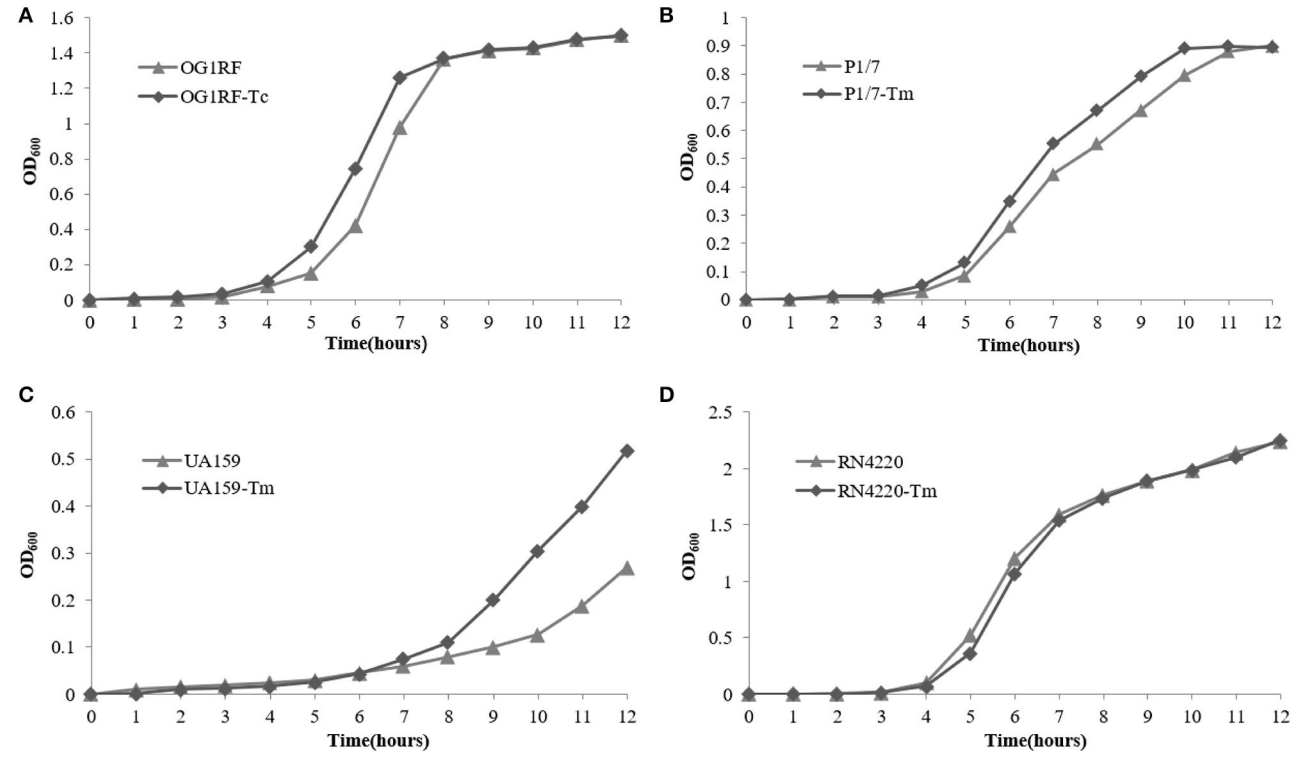

FIGURE 4 | Fitness cost of pE165. **(A–D)** Comparison of growth kinetics of *E. faecalis* OG1RF and *E. faecalis* OG1RF-Tc, *S. aureus* RN4220 and *S. aureus* RN4220-Tm, *S. suis* P1/7 and *S. suis* P1/7-Tm, *S. mutans* UA159 and *S. mutans* UA159-Tm in the absence of erythromycin.

could be detected in these transformants. MICs of *E. faecalis* E165, *S. suis* P1/7, *S. mutans* UA159, *S. aureus* RN4220, and their transformants are shown in **Table 2**. *S. suis* transformant P1/7-Tm displayed a higher erythromycin MIC (>512 mg/L) and a higher clindamycin MIC (32 mg/L) compared with *S. suis* P1/7. *S. mutans* transformant UA159-Tm displayed higher erythromycin MIC (>512 mg/L) and clindamycin MIC (256 mg/L) compared with *S. mutans* UA159. *S. aureus* transformant

RN4220-Tm displayed higher erythromycin MIC (>512 mg/L) and higher clindamycin MIC (128 mg/L) compared with *S. aureus* RN4220.

The physical map of pE165 and restriction enzyme-digested plasmid profiles of the plasmids from *E. faecalis* E165, transconjugant *E. faecalis* OG1RF-Tc, transformants *S. suis* P1/7-Tm, *S. mutans* UA159-Tm, and *S. aureus* RN4220-Tm are shown in **Figure 3**. The results indicated that only pE165 can transfer into the recipient strains and pE165 can replicate in heterogenous hosts. The result of the southern bolt revealed that *erm*(T) gene was located on pE165 in heterogenous hosts. The results of AST indicated that pE165 can constitutively express erythromycin- and clindamycin- resistance phenotype in heterogenous hosts.

Fitness Cost

The growth curve of *E. faecalis* E165, *S. suis* P1/7, *S. mutans* UA159, *S. aureus* RN4220, and their transconjugants and transformants in the absence of erythromycin are shown in **Figure 4**. The results showed that no significant fitness burden for pE165-carrying transconjugants and transformants was observed compared with the recipient strains in the absence of selective pressure.

Competition experiments can offer a more discriminative and precise measurement of fitness, and the competitive disadvantage of the fitness burden caused by pE165 can be reflected during all the phases of the growth cycle and in successive cycles. During the competition experiment between *E. faecalis* OG1RF and OG1RF-Tc, from day 1 on, a successive decrease in the proportion of *E. faecalis* OG1RF-Tc was observed, and all the colonies tested were pE165 free on day 14 (**Figure 5A**). From day 1 on, a fast and constant decrease in the proportion of *S. suis* P1/7-Tm was observed and all the strains were tested pE165 free on day 6 (**Figure 5B**). In the process of competition experiment between *S. mutans* UA159 and UA159-Tm, an

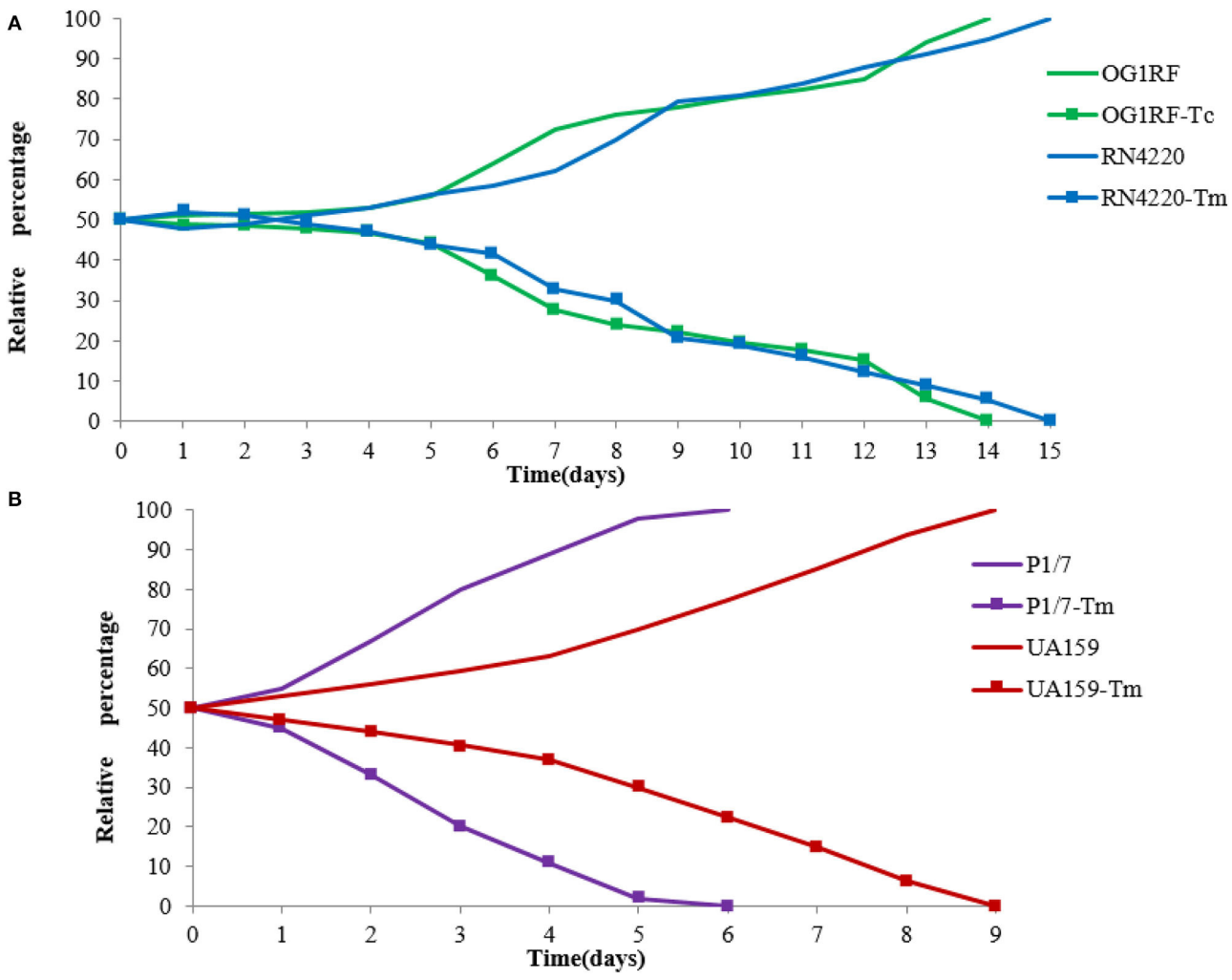

FIGURE 5 | (A) Growth competition experiments between *E. faecalis* OG1RF and *E. faecalis* OG1RF-Tc, *S. aureus* RN4220 and *S. aureus* RN4220-Tm. **(B)** Growth competition experiments between *S. suis* P1/7 and *S. suis* P1/7-Tm, *S. mutans* UA159 and *S. mutans* UA159-Tm. The initial ratio of the recipient strain carrying pE165 to the original recipient strain was 1:1. The results above were averagely calculated from four independent experiments.

obvious decrease in the proportion of UA159-Tm was observed. On day 9, all the colonies tested were pE165 free (**Figure 5B**). For the result of the competition experiment between *S. aureus* RN4220 and RN4220-Tm, it had an obvious decrease from day 3 on, and RN4220-Tm could not be detected on day 15 (**Figure 5A**). The above results suggested that all the pE165-carrying transconjugants and transformants had a fitness cost compared to the recipient strains without pE165, but the fitness cost among the different transconjugants and transformants differed.

DISCUSSION

Since *erm*(T) has been reported from *Lactobacillus reuteri* (33), it had been described in various genera: *Lactobacillus*, *Streptococcus* (*S. pyogenes*, *S. suis*), *S. aureus*, *E. faecium,* and other gram-positive bacteria including *E. rhusiopathiae* (KM576795.1), even in gram-negative bacteria such as *H. parasuis* (KC405064.1) and *Klebsiella pneumonia* (CP040837.1), revealing its widespread presence. Mobile genetic elements play a crucial role in the horizontal gene transfer of *erm*(T), including plasmids (15, 16, 18, 20, 35–37),transposons (38, 39), and insert sequence (17). *erm*(T)-positive plasmids of *S. agalactiae* could efficiently be transferred into group B Streptococcus and in the *E. faecalis* recipient strain (16).

In this study, *erm*(T)-positive plasmid pE165 was identified in a *E. faecalis* strain E165. Whole-genomic sequencing for E165 was performed. The genetic environment of *erm*(T) in this study was then analyzed by comparing it with similar *erm*(T) genetic environments published previously (19, 20, 33, 35, 39, 40). The homology analysis of the *rep* and *mob* genes located on pE165 suggested that this plasmid had the potential ability to transfer into enterococci and other species. Transformation experiments confirmed that pE165 was successfully transferred into *Enterococcus*, *Streptococcus,* and *Staphylococcus*. Elevated MICs of erythromycin and clindamycin were conferred by *erm*(T) in the recipient strains. According to recommendations given in CLSI, inducible expression of *erm*(T) cannot produce clindamycin resistance unless it is induced by erythromycin (41). In this study, the inducible clindamycin resistance tests indicated that the transconjugant *E. faecalis* OG1RF-Tc, transformants P1/7-Tm, UA159-Tm, and RN4220-Tm were resistant to both erythromycin and clindamycin (**Table 2**), which revealed the expression of *erm*(T) in these strains was constitutive. This is also in agreement with the observation that deletions or duplications appeared in the regulatory region of *erm*(T) in these strains.

It can be found that the proportion of pE165-carrying strains constantly decreased until undetectable in the competition experiments between transformants and original recipients performed by successive culturing in the absence of antibiotic pressure. Although plasmids can mediate the horizontal transmission of resistance genes between bacteria and facilitate their adaptation to the pressure of antibiotics, they also entail a metabolic burden that reduces the competitiveness of the plasmid-carrying clone in the absence of selection (42). Acquisition and maintenance of a plasmid are directly associated with fitness effects on the recipient strain. The constitutive expression of *erm*(T) will produce a burden (fitness cost) in the recipient strain, and the low prevalence of *erm*(T) gene in many genera of bacteria may be explained in this way.

CONCLUSIONS

The *erm*(T) gene was first reported in an *E. faecalis* strain. A 4244 bp *erm*(T)-positive plasmid pE165 was characterized. The transmissibility of pE165 was investigated between intra- and inter-species. The presence of pE165 greatly elevated the MICs of erythromycin and clindamycin which indicated the expression of *erm*(T) in the recipient strains was constitutive. Although the fitness cost showed us this plasmid reduced the competitiveness of the host strain, the potential possibility of dissemination of *erm*(T) among species of bacteria should not be ignored.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below https://www.ncbi.nlm.nih.gov/, CP089585.

AUTHOR CONTRIBUTIONS

X-DD and DL designed the research and supervised the study. X-YL, RY, CX, and YS performed the experiments and analyzed the data. X-YL, RY, and X-DD wrote the manuscript. All authors revised the manuscript and approved the final version for submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.850466/full#supplementary-material

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Nationwide Monitoring of Antimicrobial-Resistant *Escherichia coli* and *Enterococcus* spp. Isolated From Diseased and Healthy Dogs and Cats in Japan

Yukari Furuya, Mari Matsuda, Saki Harada, Mio Kumakawa, Takahiro Shirakawa, Mariko Uchiyama, Ryoko Akama, Manao Ozawa, Michiko Kawanishi, Yoko Shimazaki and Hideto Sekiguchi*

National Veterinary Assay Laboratory, Ministry of Agriculture, Forestry and Fisheries, Tokyo, Japan

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Xin Zhao,
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Longping Li,
Shaanxi Provincial Engineering & Technology Research Center of Cashmere Goats, China

***Correspondence:**
Yukari Furuya
yukari_furuya970@maff.go.jp

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The Japanese Veterinary Antimicrobial Resistance Monitoring System (JVARM) was established for nationwide monitoring of antimicrobial-resistant bacteria isolated from animals. Here, antimicrobial resistance of *Escherichia coli* and *Enterococcus* spp. isolates from diseased and healthy dogs and cats was investigated. Isolates were collected from diseased dogs and cats and from healthy dogs and cats in 2018 to 2020. Minimum inhibitory concentrations were determined for 1873 *E. coli* and 1383 *Enterococcus* spp. isolates. *E. coli* isolates were most commonly resistant to nalidixic acid [diseased dog (DD), 62.1%; diseased cat (DC), 59.9%; healthy dog (HD), 23.5%; healthy cat (HC, 24.0%] and ampicillin (DD, 54.4%; DC, 64.1%; HD, 28.4%; HC, 25.2%), followed by ciprofloxacin (DD, 45.0%; DC, 44.0%; HD, 12.9%; HC, 10.4%). *Enterococcus* spp. isolates were most resistant to tetracycline (DD, 66.9%; DC, 67.8%; HD, 47.0%; HC, 52.0%), followed by erythromycin (DD, 43.2%; DC, 46.6%; HD, 27.8%; HC, 34.0%) and ciprofloxacin (DD, 27.9%; DC, 43.7%; HD, 9.7%; HC 12.9%). Only a few *E. coli* isolates were resistant to colistin and none were resistant to meropenem. Also, none of the *Enterococcus* spp. isolates we have tested were resistant to vancomycin. The significantly higher resistance rates of *E. coli* and *Enterococcus* spp. isolates from diseased, as opposed to healthy, dogs and cats against most of the tested antimicrobials indicates that the use of antimicrobials could select resistant *E. coli* and *Enterococcus* spp.

Keywords: ***Escherichia coli*, *Enterococcus* spp., monitoring, antimicrobial-resistant, companion animals**

INTRODUCTION

The emergence and spread of antimicrobial-resistant (AMR) bacteria is widely recognized as a global health threat (1). The concept of "One Health" is crucial to address this issue, because humans, animals, foods, and environments are potential reservoirs of AMR bacteria and genes (2). In the veterinary field, globally, livestock is the main target for monitoring and risk assessment of AMR bacteria. The Japanese Veterinary Antimicrobial Resistance Monitoring System (JVARM) was established in 1999 to monitor AMR bacteria isolated from livestock but not companion

animals (i.e., dogs and cats) as same as many countries (3). In some countries, including Sweden, France and Norway, monitor AMR bacteria in companion animals (4–6).

Although AMR is also major concern in companion animals, there are few monitoring results for risk assessment (7). Studies in Japan detected extended-spectrum beta-lactamase-producing *Escherichia coli* and bla_{CTX-M} type β-lactamase genes in samples collected from dogs and cats (8, 9). Additionally, greater rates of enrofloxacin-resistant *Enterococcus* spp. were confirmed in diseased, as opposed to healthy, dogs, and cats (10). These studies revealed potential treatment failure risks in dogs and cats and transmission risks of AMR bacteria from companion animals to humans and vice versa. Therefore, our group developed a national monitoring system for dogs and cats under the JVARM framework to assess the risks according to the strategy of the first national action plan on AMR in Japan (11).

Monitoring of targeted pathogens isolated from diseased dogs and cats was initiated from the veterinary medical perspective. In addition, as a fundamental data of AMR bacteria, *E. coli* and *Enterococcus* spp. from healthy animals is also important to monitor, since *E. coli* and *Enterococcus* spp. are globally known as a multi-sectoral indicator bacteria and often selected as target bacteria of AMR monitoring. Also, *E. coli* and *Enterococcus* spp. are commensal microorganisms causing opportunistic infections (12, 13). Therefore, we collected *E. coli* and *Enterococcus* spp. from both diseased and healthy dogs and cats for this monitoring.

Monitoring of both diseased and healthy dogs and cats is almost unprecedented; however, the aim of this study was to summarize the results of nationwide monitoring of AMR *E. coli* and *Enterococcus* spp. from diseased and healthy dogs and cats in Japan.

MATERIALS AND METHODS

Sampling

In order to determine an appropriate number of samples to avoid bias, all prefectures of Japan were divided into blocks and the numbers of samples were calculated based on the number of small animal clinics in each block. Hence, collection of only one *E. coli* and/or *Enterococcus* spp. isolate from each clinic, which was to be in total 200 isolates, suggest that each bacterial species should be collected from dogs and cats per year.

E. coli strains were isolated from clinical urine or genital tract samples and *Enterococcus* spp. strains were isolated from clinical urine or ear samples of diseased dogs and cats. All of *E. coli* and *Enterococcus* spp. isolated from diseased dogs and cats are isolated and identified in clinical laboratories from clinical samples.

In addition, *E. coli* and *Enterococcus* spp. were isolated from rectal swabs of healthy dogs and cats brought to small animal clinics for either medical checkups or vaccinations, but not treatment.

Informed Consent for Sampling

Clinical samples from diseased dogs and cats, submitted to clinical laboratories, were sourced from veterinarians and owners and used under the agreement of the use for research.

The owners were explained the purpose of the surveillance and requirement of isolates from healthy dogs and cats, and written informed consent was obtained prior to sample collection.

All of the isolates were anonymized.

Identification of *E. coli* and *Enterococcus* spp.

For diseased dogs and cats, clinical laboratories cultured suspected *E. coli* isolates on MacConkey agar or deoxycholate-hydrogen sulfide-lactose (DHL) agar and identified through the IMViC test, which is a combination of the indole, methyl red or Voges–Proskauer and citrate tests or using matrix assisted laser desorptionization-time of flight mass spectrophotometry (MALDI TOF MS) (Bruker Daltonics, Germany) or MicroScanWalkAway Plus System (Beckman Coulter, Inc., Japan) for automated identification. Suspected *Enterococcus* spp. were cultured on Trypticase soy agar with 5% sheep blood or phenylethyl alcohol sheep blood agar and identified using the catalase test and confirmed colonies on EF agar (Nissui Pharmaceutical Co., Ltd., Japan) or used MicroScanWalkAway Plus System or MALDI TOF MS. We confirmed *E. coli* by colored colonies on DHL agar, and *Enterococcus* spp. using the Rapid ID32 Strep kit (BioMerieux Vitek, Marcy-I'Etoile, France) and the oxidase test.

For healthy dogs and cats, suspected *E. coli* isolates were tested using DHL agar, then cultured on triple sugar iron agar, and subjected to the IMViC test. Suspected *Enterococcus* spp. isolates were smeared on enterococcosel agar and three colonies were randomly sampled and subjected to Gram staining, the catalase test and the pyrrolidonyl arylamidase test, and then cultured in heart infusion broth with NaCl. The species of the suspected *Enterococcus* spp. isolates were identified using the Rapid ID32 Strep kit.

Antimicrobial Susceptibility Testing

The tested antimicrobials were selected from two perspectives, in order to enable comparison with existing data of livestock in JVARM and to grasp resistant rates of antimicrobials frequently used in small animal clinical practices.

The *E. coli* isolates were tested for minimum inhibitory concentrations (MICs) of ampicillin, cefazolin, cephalexin, cefotaxime, meropenem, kanamycin, gentamicin, streptomycin, tetracycline, chloramphenicol, colistin, ciprofloxacin, nalidixic acid, and trimethoprim/sulfamethoxazole.

The *Enterococcus* spp. isolates were tested for MICs of ampicillin, gentamicin, erythromycin, azithromycin, ciprofloxacin, chloramphenicol, and tetracycline. MICs of vancomycin was assessed for isolates from diseased dogs and cats collected in 2019 and 2020, and all isolates from healthy dogs and cats.

MICs were calculated using a standardized microdilution method in accordance with the Clinical and Laboratory Standards Institute (CLSI) standard (14) using "Dry Plate 'Eiken' 192" bacterial drug sensitivity testing reagent (EIKEN Chemical Co., Ltd., Japan). The breakpoints as listed in CLSI document M100 (15) and VET01S (16) were applied. Considering the

purpose of nationwide continual monitoring, break points set in M100 were adopted instead of break points set by each samples' origins in VET01S. *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Enterococcus faecalis* ATCC29212 were used as control strains.

Statistics Analysis

The Fisher's exact test was used to identify differences in the resistance rates among diseased and healthy dogs and cats. A probability $p < 0.05$ was considered statistically significant.

RESULTS

In total, 1,873 *E. coli* and 1,383 *Enterococcus* spp. isolates were collected. Eight hundred ninety one *E. coli* isolates were collected from diseased dogs and cats and 982 from healthy dogs and cats, whereas 695 *Enterococcus* spp. isolates were collected from diseased dogs and cats and 688 from healthy dogs and cats. All of the isolates were collected in 2018, 2019, and 2020.

For *Enterococcus* spp. isolates, *E. faecalis* [diseased dog (DD), 74.7% (287/384); diseased cat (DC), 62.7% (195/311); healthy dog (HD), 74.5% (322/432); healthy cat (HC), 79.7% (204/256)], *E. faecium* [DD, 17.4% (67/384); DC, 29.3% (91/311); HD, 8.3% (36/432); HC, 4.3% (11/256)], *E. gallinarum* [DD, 2.9% (11/384); DC, 2.9% (9/311); HD, 6.0% (26/432); HC, 3.9% (10/256)], *E. casseliflavus* [DD, 1.0% (4/384); DC, 1.3% (4/311); HD, 2.3% (10/432); HC, 1.2% (3/256)], *E. hirae* [DD, 0.3% (1/384); DC, 0.6% (2/311); HD, 3.5% (15/432); HC, 3.5% (9/256)], *E. avium* [DD, 2.1% (8/384); DC, 1.0% (311); HD, 1.6% (7/432); HC, 3.9% (10/256)], *E. durans* [DD, 0.8% (3/384); DC, 0.6% (2/311); HD, 3.5% (15/432); HC, 3.1% (8/256)] and other *Enterococcus* spp. isolates were detected.

The MIC profiles of the *E. coli* isolates are shown in **Table 1**. More than 30% of the isolates from both diseased dogs and cats were resistant to nalidixic acid (DD, 62.1%; DC, 59.9%), ampicillin (DD, 54.4%; DC, 64.1%), ciprofloxacin (DD, 45.0%; DC, 44.0%), cefazolin (DD, 34.8%; DC, 36.4%), cephalexin (DD, 35.4%; DC, 37.8%) and cefotaxime (DD, 31.2%; DC, 31.7%). In contrast, <30% of the isolates from healthy dogs and cats were resistant to all antimicrobials. Resistance to ampicillin was most common (HD, 28.4%; HC, 25.2%), followed by nalidixic acid (HD, 23.5%; HC, 24.0%) and cefazolin (HD, 15.1%; HC, 12.2%). None of the *E. coli* isolates were resistant to meropenem and few were resistant to colistin (DD, 0.0%; HD, 0.4%; DC, 0.6%; HC, 0.2%). With the exception of meropenem and colistin, the resistance rates to all antimicrobials were significantly ($p < 0.01$) greater among the isolates from diseased, as opposed to healthy, dogs and cats.

The MICs of the *Enterococcus* spp. isolates are shown in **Table 2**. The isolates from diseased dogs and cats were most commonly resistant to tetracycline (DD, 66.9%; DC, 67.8%), followed by erythromycin (DD, 43.2%; DC, 46.6%) and ciprofloxacin (DD, 27.9%; DC, 43.7%). The isolates from healthy dogs and cats were also highly resistant to tetracycline (HD, 47.0%; HC, 52.0%), erythromycin (HD, 27.8%; HC, 34.0%) and ciprofloxacin (HD, 9.7%; HC, 12.9%). Isolates from diseased dogs and cats showed significantly higher resistance rates ($p < 0.01$) to all antimicrobials, except for chloramphenicol and vancomycin, than those from healthy dogs and cats.

Notably, 91.0% (61/67) and 92.1% (82/89) of the *E. faecium* from diseased dogs and cats, respectively, 0.3% (1/287) and none (0/195) of the *E. faecalis* isolates from diseased dogs and cats, respectively, were resistant to ampicillin, while 22.2% (8/36) and 9.1% (1/11) of the *E. faecium* and 0.3% (1/322) and none (0/204) of *E. faecalis* isolates from healthy dogs and cats were resistant to ampicillin. Even by each years, *E. faecium* [DD, 100% (15/15), 90.0% (27/30), 86.4% (19/22); DC, 100% (18/18), 94.3% (33/35), 81.6% (31/38); HD, 29.2% (7/24), 0.0% (0/3), 0.0% (0/9); HC, 14.3% (1/7), 0.0% (0/1), 0.0% (0/3), in 2018, in 2019, and in 2020, respectively] and *E. faecalis* [DD, 0.0% (0/52), 0.0% (0/100), 0.8% (1/130); DC, 0.0% (0/39), 0.0% (0/62), 0.0% (0/94); HD, 1.0% (1/100), 0.0% (0/123), 1.0% (1/9); HC, 14.3% (1/7), 0.0% (0/1), 0.0% (0/3), in 2018, in 2019, and in 2020, respectively], showed pattern of certain resistant rates to ampicillin.

The resistance rates of the *E. coli* and *Enterococcus* spp. isolates grouped by year are shown in **Figures 1**, **2**. In **Figure 1**, the resistant rates of *E. coli* isolates collected from diseased dogs and cats in 2018 were higher than those collected in 2019 and 2020, whereas the resistant rates of *E. coli* isolates from healthy dogs and cats collected in 2018 and 2019 were similar.

DISCUSSION

Overall, resistance to most of the tested antimicrobials was significantly higher in diseased, as opposed to healthy, dogs and cats. In this study, background information of the samples to trace antimicrobial use was limited; however, diseased dogs and cats are more likely to have been treated with antimicrobials. According to Nippon AMR One Health Report (17), the highest volume of veterinary antimicrobials estimated sales for dogs and cats were cephalosporins, especially the first generation cephalosporins followed by penicillins. Conversely, almost none carbapenems were sold. It is may be correlated with resistant rates of *E. coli* isolated from diseased dogs and cats against cefazolin, cefalexin, cefotaxime, and ampicillin, which were more than 30%, and none were resistant to meropenem. This results indicate that among diseased dogs and cats that were administered antimicrobials, the use of antimicrobials is may be responsible for the selective pressure of bacterial flora of *E. coli* and *Enterococcus* spp. In addition to the fact that there were differences in resistance rates among diseased and healthy dogs and cats, there were similarities in the types of antimicrobials they showed resistance to (e.g., *E. coli* resistance to nalidixic acid and ampicillin; *Enterococcus* spp. resistance to tetracycline, erythromycin, and ciprofloxacin).

According to data collected by JVARM in 2016 and 2017 (18), *E. coli* isolates from cattle, pigs and broilers in slaughterhouses were most resistant to streptomycin (19.0–51.3%) and tetracycline (21.0–56.7%), while isolates from diseased livestock were highly resistant to tetracycline (54.5–87.3%) followed by streptomycin (38.9–74.5%), ampicillin (33.3–74.5%) and chloramphenicol (11.1–69.6%). Resistance to cefotaxime and ciprofloxacin was generally lower in *E. coli*

TABLE 1 | MIC for *E. coli* isolated from diseased and healthy dogs and cats in Japan.

Antimicobial agent			Diseased dog ($n = 509$)			Healthy dog ($n = 490$)			Diseased cat ($n = 382$)			Healthy cat ($n = 492$)		
	Range	Breakpoint	MIC_{50}	MIC_{90}	Number of resistant isolates (%)	MIC_{50}	MIC_{90}	Number of resistant isolates (%)[a]	MIC_{50}	MIC_{90}	Number of resistant isolates (%)	MIC_{50}	MIC_{90}	Number of resistant isolates (%)[a]
	(mg/L)	(mg/L)	(mg/L)	(mg/L)		(mg/L)	(mg/L)		(mg/L)	(mg/L)		(mg/L)	(mg/L)	
Ampicillin	≤4–>128	32	>128	>128	277 (54.4%)	8	>128	139 (28.4%)**	16	>128	229 (64.1%)	≤4	>128	124 (25.2%)**
Cefazolin	≤2–>128	32	≤2	>128	177 (34.8%)	≤2	>128	74 (15.1%)**	≤2	>128	130 (36.4%)	≤2	64	60 (12.2%)**
Cefalexin	≤2–>128	32	8	>128	180 (35.4%)	8	>128	74 (15.1%)**	8	>128	135 (37.8%)	8	>128	67 (13.6%)**
Cefotaxime	≤0.5–>64	4	≤0.5	64	159 (31.2%)	≤0.5	8	56 (11.4%)**	≤0.5	64	113 (31.7%)	≤0.5	≤0.5	33 (6.7%)**
Meropenem	≤0.5–>8	4	≤0.5	≤0.5	0 (0.0%)	≤0.5	≤0.5	0 (0.0%)	≤0.5	≤0.5	0 (0.0%)	≤0.5	≤0.5	0 (0.0%)
Streptomycin	≤4–>128	–	8	>128	–	8	128	–	8	>128	–	≤4	32	–
Gentamicin	≤2–>64	8	≤2	32	77 (15.1%)	≤2	≤2	22 (4.5%)**	≤2	16	46 (12.9%)	≤2	≤2	18 (3.7%)**
Kanamycin	≤4–>128	64	≤4	16	31 (6.1%)	≤4	8	21 (4.3%)**	≤4	16	27 (7.6%)	≤4	≤4	14 (2.8%)**
Tetracycline	≤2–>64	16	4	>64	122 (24.0%)	≤2	64	68 (13.9%)**	≤2	>64	88 (24.6%)	≤2	4	48 (9.8%)**
Chloramphenicol	≤4–>128	32	8	32	60 (11.8%)	8	16	26 (5.3%)**	8	16	32 (9.0%)	8	8	11 (2.2%)**
Colistin	≤0.5–>16	4	≤0.5	≤0.5	0 (0.0%)	≤0.5	≤0.5	2 (0.4%)	≤0.5	≤0.5	2 (0.6%)	≤0.5	≤0.5	1 (0.2%)
Nalidixic acid	≤4–>128	32	>128	>128	316 (62.1%)	≤4	>128	115 (23.5%)**	>128	>128	214 (59.9%)	≤4	>128	118 (24.0%)**
Ciprofloxacin	≤0.06–>8	1	0.5	>8	229 (45.0%)	≤0.06	8	63 (12.9%)**	0.25	>8	157 (44.0%)	≤0.06	1	51 (10.4%)**
Sulfamethoxazole/ trimethoprim	≤9.5/0.5–>152/8	76/4	≤9.5/0.5	>152/8	108 (21.2%)	≤9.5/0.5	>152/8	52 (10.6%)**	≤9.5/0.5	>152/8	84 (22.0%)	≤9.5/0.5	≤9.5/0.5	45 (9.1%)**

[a]*p-values were determined by Fisher's exact test. **$p < 0.01$.*

TABLE 2 | MIC for *Enterococcus* spp. isolated from diseased and healthy dogs and cats in Japan.

Antimicobial agent	Diseased dog (n = 384/306[b])					Healthy dog (n = 432)			Diseased cat (n = 311/251[b])			Healthy cat (n = 256)		
	Range	Breakpoint	Number of samples	MIC_{50}	MIC_{90}	Number of resistant isolates (%)	MIC_{50}	MIC_{90}	Number of resistant isolates (%)[a]	MIC_{50}	MIC_{90}	Number of resistant isolates (%)	MIC_{50}	MIC_{90}	Number of resistant isolates (%)[a]
	(mg/L)	(mg/L)		(mg/L)	(mg/L)		(mg/L)	(mg/L)		(mg/L)	(mg/L)		(mg/L)	(mg/L)	
Ampicillin	≤0.5–>64	16	384	1	>64	68 (17.7%)	1	2	20 (4.6%)**	1	>64	94 (30.2%)	1	2	6 (2.3%)**
Vancomycin	≤0.12–>32	32	306[b]	1	2	0 (0.0%)	1	2	0 (0.0%)	1	2	0 (0.0%)	1	2	0 (0.0%)
Gentamicin	≤1–>64	–	384	8	>64	–	8	64	–	8	>64	–	8	>64	–
Erythromycin	≤0.25–>32	8	384	2	>32	166 (43.2%)	2	>32	120 (27.8%)**	4	>32	145 (46.6%)	2	>32	87 (34.0%)*
Azithromycin	≤0.25–>32	–	384	8	>32	–	4	>32	–	8	>32	–	4	>32	–
Ciprofloxacin	≤0.25–>32	4	384	1	>32	107 (27.9%)	1	2	42 (9.7%)**	2	>32	136 (43.7%)	1	16	33 (12.9%)**
Chloramphenicol	≤1–>64	32	384	8	64	61 (15.9%)	8	32	52 (12.0%)	8	64	45 (14.5%)	8	32	34 (13.3%)
Tetracycline	≤0.5–>64	16	384	64	>64	257 (66.9%)	1	64	203 (47.0%)**	64	64	211 (67.8%)	32	64	133 (52.0%)**

[a]*p-values were determined by Fisher's exact test. *$p < 0.05$; **$p < 0.01$.*

[b]*MIC for Vancomycin was tested for all isolates except diseased dogs and cats in 2018.*

isolates from healthy than diseased livestock (0.0–5.7 and 2.9–8.9 vs. 0.0–12.0 and 11.1–28.5%, respectively). Notably, <30% of all *E. coli* isolates from diseased and healthy cattle, pigs and broilers, but >30% of those from diseased dogs and cats were resistant to cefotaxime and ciprofloxacin. In contrast, *E. faecalis* and *E. faecium* isolates collected from healthy pigs and broilers in 2017 were highly resistant to oxytetracycline (31.8–84.6%) and erythromycin (27.3–61.5%) (18). Kimura et al. (19), also found similar trends for *Enterococcus* spp. isolated from diseased companion animals in Japan (non-susceptibility rates against doxycycline and minocycline of 40–56% and erythromycin of 40–93%).

Data collected by the Japan Nosocomial Infections Surveillance System (20) indicate that trends in *E. coli* in human resistance to penicillins, quinolones/fluoroquinolones, and cephalosporins are similar to those of dogs and cats, in addition to high resistance of *Enterococcus* spp. to tetracycline and erythromycin.

Although the trends in resistance of *Enterococcus* spp. concurred with those of all animal species considered in this study, there were notable differences in the AMR profiles of *E. coli* isolates from humans, dogs and cats vs. livestock, which may be due to differences in the antimicrobial classes used for treatment of infections.

Consistent with previous studies, most *E. faecium* and few *E. faecalis* isolates from diseased dogs and cats were resistant to ampicillin (21). However, interestingly, the resistance rates of *E. faecium* from healthy dogs and cats were significantly lower than from diseased dogs and cats (22.2 and 9.1 vs. 91.0 and 92.1%, respectively), even in each years. Jackson et al. reported that 47.4 and 51.6% of *E. faecium* isolates from healthy dogs and cats in the United States were resistant to penicillin (22), which are not exceptionally high resistance rates. Origins of the human *E. faecium* infection are broadly categorized as hospital or community-associated (23, 24). Most cases of hospital-associated *E. faecium* are resistant to ampicillin while community-associated cases are generally susceptible to ampicillin, suggesting the possibility of similar types of *E. faecium* infection of dogs and cats.

As shown in **Figures 1**, **2**, there were small annual fluctuations with regular, rather than random patterns. Despite collecting only 3 years of data, this monitoring system seemed to be useful to illustrate trends. Although there were differences in the resistant rates between diseased and healthy, resistant rates between animal species, dogs and cats, were similar in both *E. coli* and *Enterococcus* spp. isolates.

Few countries monitor AMR bacteria for dogs and cats. Swedish Veterinary Antimicrobial Resistance Monitoring (SVARM) program (4), refers to MICs of *E. coli* from clinical urine samples of dogs and cats. In the SVARM report, 13% of *E. coli* isolates from dogs and 16% from cats were resistant to ampicillin. In France, the national surveillance network for antimicrobial resistance in bacteria from diseased animals (RESAPATH) (5), collects data *via* the disc diffusion method of *E. coli* isolates from clinical samples of dogs and cats with various pathologies. According to the RESAPATH data, 70, 61, and 73% of *E. coli* isolates from kidney/urinary tract,

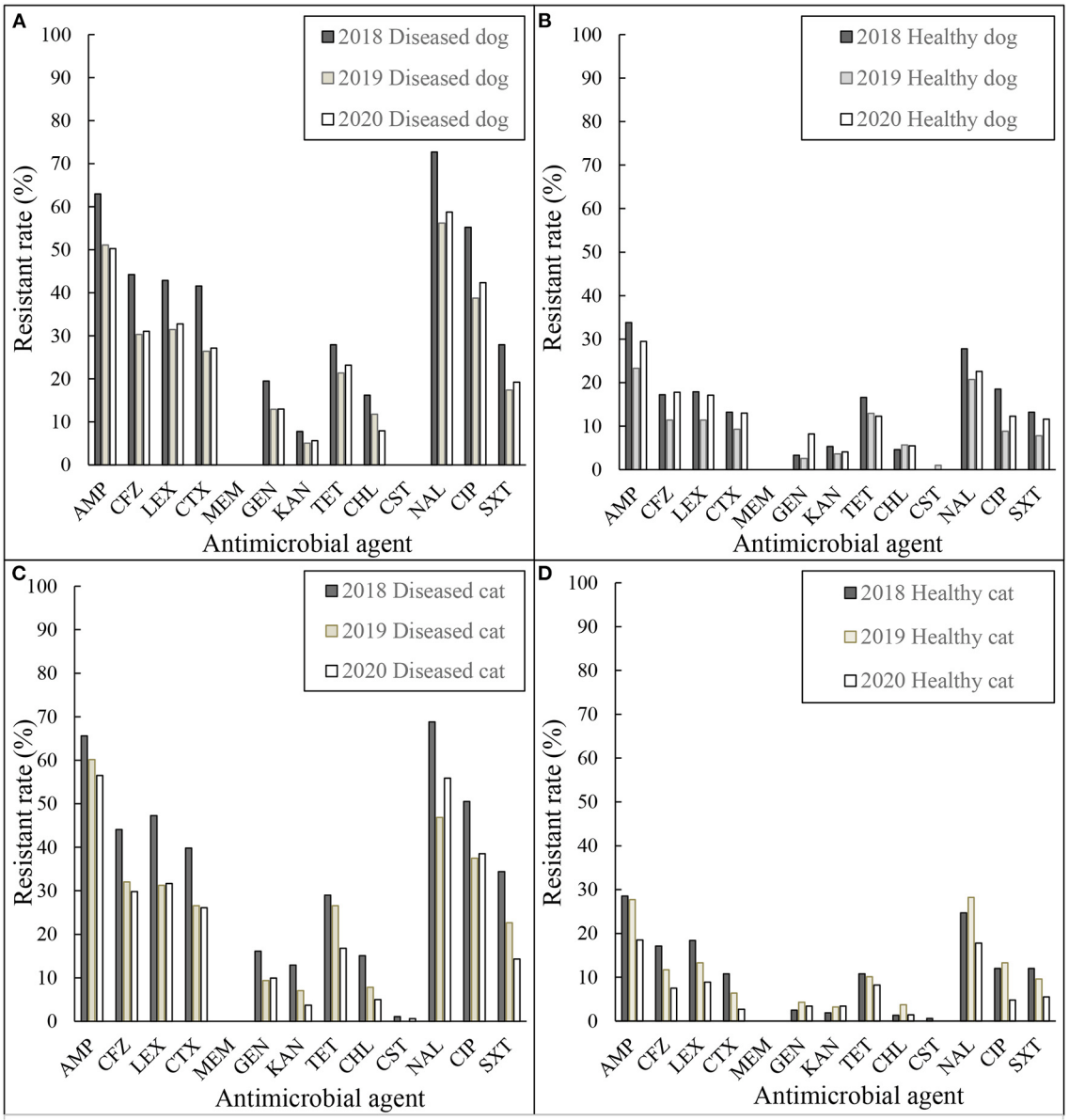

FIGURE 1 | Resistance rates of *E. coli* **(A)** diseased dog 2018–2020, **(B)** healthy dog 2018–2020, **(C)** diseased cat 2018–2020, and **(D)** healthy cat 2018–2020. AMP, ampicillin; CFZ, cefazolin; LEX, cephalexin; CTX, cefotaxime; MEM, meropenem; STR, streptomycin; GEN, gentamicin; KAN, kanamycin; TET, tetracycline; CHL, chloramphenicol; CST, colistin; NAL, nalidixic acid; CIP, ciprofloxacin; SXT, sulfamethoxazole/trimethoprim.

skin/soft tissue, and otitis samples, respectively, from dogs and 70% from cats with all pathologies were susceptible to amoxicillin. Furthermore, according to the national monitoring program data for antimicrobial resistance in the veterinary and food production sectors in Norway (NORM-VET) (6), 20.2 and 46.5% of *E. coli* isolates from urine of dogs with urinary tract infections and other infections, respectively, were resistant to ampicillin. It should be noted that the SVARM data includes ECOFFs as defined by the EUCAST, while the RESAPATH system uses the NF U47-107 standard of the Antibiogram Committee of the French Society of Microbiology, and the NORM-VET program uses the clinical breakpoints or ECOFFs.

Each of the cited surveillance systems adopted different breakpoints and there were differences in the numbers and types of tested antimicrobial agents as well as the sampling methods (passive sample collection method of the SVARM, RESAPATH and NORM-VET systems vs. an active sample collection method in the present study). Therefore, data comparisons were challenging. In our study, 54.4 and 64.1% of the *E. coli* isolates from diseased dogs and cats, respectively, were resistant to ampicillin, which were higher rates than reported in Sweden,

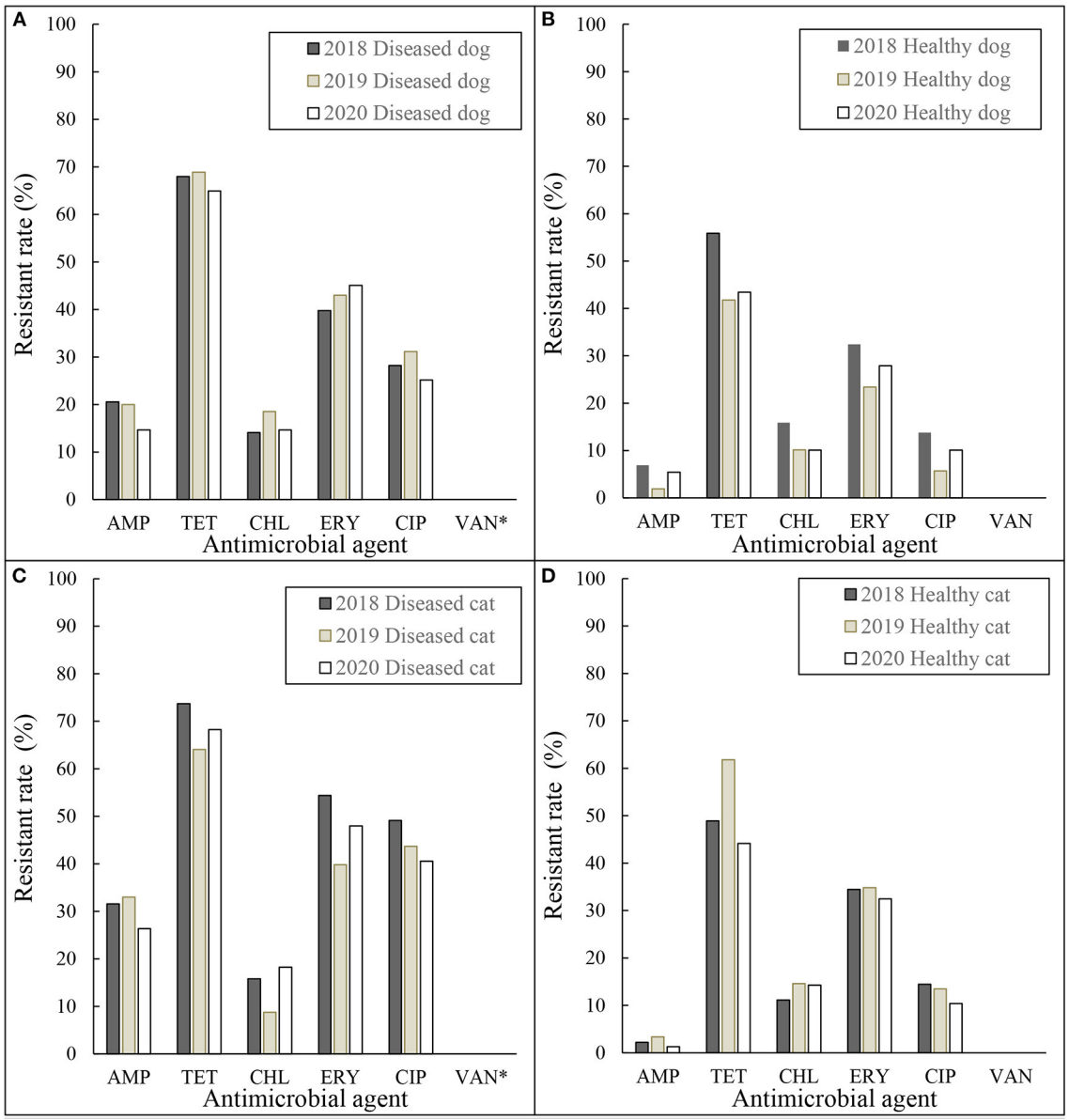

FIGURE 2 | Resistance rates of *Enterococcus* spp. **(A)** diseased dog 2018–2020, **(B)** healthy dog 2018–2020, **(C)** diseased cat 2018–2020, and **(D)** healthy cat 2018–2020. AMP, ampicillin; TET, tetracycline; CHL, chloramphenicol; ERY, erythromycin; CIP, ciprofloxacin; VAN, vancomycin. *MIC for Vancomycin was tested for all isolates except diseased dogs and cats in 2018.

France and Norway, where aggressive measures against AMR have been enacted.

In the present study, the resistance rates of *E. coli* and *Enterococcus* spp. isolates from diseased dogs and cats were significantly higher than those from healthy dogs and cats against most of the tested antimicrobials, indicating that use of antimicrobials could be selective pressure for resistant *E. coli* and *Enterococcus* spp.

The limitation of our study lies on difference of sample origins between diseased and healthy dogs and cats, urine/genital/ear sample origin vs. rectal swab origin due to collect enough numbers of strains. Also, there are lack of genetic data including serotype. However, our results are valuable to know AMR situation in dogs and cats in Japan and useful to consider AMR measures.

In conclusion, this is the huge step toward continued AMR monitoring of isolates from diseased and healthy dogs and cats. To the best of our knowledge, nationwide monitoring systems of AMR bacteria isolated from both diseased and healthy dogs and cats are rare. In 2020 (25), our group published guidelines for use of antimicrobials for companion animal veterinarians to avoid selection of AMR bacteria. The results of this study suggest that it is crucial to promote prudent use of antimicrobials in companion animals and to continue monitoring trends in AMR bacteria.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

YF, HS, YS, RA, MO, MM, SH, and MKa contributed to conception and design of the study. MU, MKu, and TS organized the sampling methods and approached to the related organizations. All authors contributed to manuscript revision, read, and approved the submitted version.

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projects/antimicrobial-resistance/good-practices-addressing-amr-in-asia-and-the-pacific-region/japan/

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EDITED BY
Patrick Rik Butaye,
Ghent University, Belgium

REVIEWED BY
Chengming Wang,
Auburn University, United States
Chong Chen,
Yangzhou University, China
Xinglong Wang,
Northwest University, China

*CORRESPONDENCE
Hong Yao
yaoh0913@henau.edu.cn
Xiang-Dang Du
xddu@henau.edu.cn

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Characterization of a genomic Island carrying the *tet*(X4) gene in porcine *Acinetobacter towneri* co-harboring plasmid-borne bla_{NDM-1} and bla_{OXA-58} genes

Aijuan Li[1], Runhao Yu[1], Wenbo Zhao[1], Stefan Schwarz[2,3], Chenglong Li[1], Hong Yao[1]* and Xiang-Dang Du[1]*

[1]College of Veterinary Medicine, Henan Agricultural University, Zhengzhou, China, [2]Department of Veterinary Medicine, Centre for Infection Medicine, Institute of Microbiology and Epizootics, Freie Universität Berlin, Berlin, Germany, [3]Veterinary Centre of Resistance Research (TZR), Freie Universität Berlin, Berlin, Germany

Tigecycline and carbapenems are last-resort antimicrobial agents to treat serious infections caused by multi-drug resistant bacterial pathogens. However, the co-occurrence of tigecycline and carbapenem resistance determinants challenges the clinical efficacy of these antimicrobial agents. In this study, we report the co-existence of *tet*(X4), bla_{NDM-1} and bla_{OXA-58} genes in the porcine *Acinetobacter towneri* isolate 19110F47. Sequence analysis revealed that *tet*(X4) gene, along with the florfenicol resistance gene *floR*, was flanked by three copies of IS*91*-like elements, which can form three different translocatable units (TUs), and were located in a 41,098-bp multidrug resistance region (MDRR) within a novel 100,354-bp genomic island (GI) region. TUs comprising *floR*-*vir*D2-IS*Vsa3*, *hp*-*abh*-*tet*(X4)-IS*Vsa3* and *virD2*-*floR*-IS*Vsa3*-*hp*-*abh*-*tet*(X4)-IS*Vsa3* can be looped out from the chromosomal DNA and facilitate the transfer of the TU-based resistance genes into other plasmidic or chromosomal sites. In addition, the carbapenemase genes bla_{NDM-1} and bla_{OXA-58} were found on different non-conjugative multiresistance plasmids in this isolate, with the genetic contexts IS*Aba125*-bla_{NDM-1}-ble_{MBL}-*tnpR* and ΔIS*Aba3*-bla_{OXA-58}-IS*Aba3*, respectively. The simultaneous occurrence of *tet*(X4), bla_{NDM-1} and bla_{OXA-58} in the same porcine *A. towneri* isolate emphasizes the importance of antimicrobial resistance surveillance in food-producing animals.

KEYWORDS

tigecycline, carbapenem, resistance, *tet*(X4), bla_{NDM-1}, bla_{OXA-58}, *Acinetobacter towneri*

Introduction

Antimicrobial resistance poses a significant threat to public health globally. The presence of extensively drug-resistant (XDR) Gram-negative bacteria, in particular carbapenem-resistant *Enterobacteriaceae* and *Acinetobacter* spp., compromises the efficacy of carbapenems. Moreover, the choices of effective antimicrobial agents against carbapenem-resistant bacteria are very limited (1). Tigecycline has been recognized as a last-resort antimicrobial agent to treat infections caused by XDR Gram-negative bacteria (2). However, a variety of plasmid-borne *tet*(X) variants genes, which confer high-level resistance to tigecycline, have been reported in *Acinetobacter* spp. and *Enterobacteriaceae* from China (3, 4). The gene *tet*(X4) was detected in bacteria from food-producing animals, meat for human consumption, migratory birds, humans and environmental samples (5–7).

Acinetobacter spp. are considered as ubiquitous in the nature and have emerged as a major cause of nosocomial infections globally in recent decades (8). In addition, *Acinetobacter* spp. are not only associated with hospital-acquired infections, but also responsible for community-acquired infections (9). XDR *Acinetobacter* isolates, especially when they are carbapenem-resistant, are recognized as one of the most troublesome pathogens worldwide (10). Food-producing animals have been regarded as a potential reservoir for *Acinetobacter* spp. in many countries (11). To date, the *tet*(X4) gene was mostly reported in *E. coli*, and sometimes described in other bacterial species, such as *Acinetobacter* spp. (4, 12, 13). Currently, the genetic basis for co-resistance against tigecycline and carbapenems has been investigated in *Acinetobacter* spp. The tigecycline resistance genes *tet*(X) have been reported with carbapenem resistance gene bla_{NDM} in *A. baumannii*, *A. indicus*, *A. schindleri*, *A. lwoffii*, and other *Acinetobacter* (14–16).

In this study, we investigated a tigecycline- and carbapenem-resistant *A. towneri* isolate collected from a pig in China for the tigecycline- and carbapenem resistance genes present, their association with mobile genetic elements and their transfer potential.

Materials and methods

Sample collection and bacterial isolation

A total of 1,146 non-duplicate anal swab samples were collected from three unrelated and geographically distant pig farms and one pig slaughterhouse located in the Henan Province of China in 2019. Brain heart infusion (BHI) broth was used as transport medium for the anal swabs. The swabs were streaked on BHI agar plates supplemented with tigecycline (2 mg/L) and meropenem (2 mg/L) and incubated at 37°C for 24 h. Bacteria growing on these double-selective plates were identified to the species level by 16S rRNA amplification and sequencing of the amplicons (17).

TABLE 1 Primers used in this study.

Primers	5′-3′
TU1-rv	ACGACGCCCGCTATGATCCAA
TU1-fw	AACGCGGCACGTATAGGAAG
TU2-rv	AGTCCAACGGGTCCACCAC
TU2-fw	TGCTCATTTGATGCCTCCTT
TU3-rv	ACTTAAGGGCTATCTTGTTG
TU3-fw	TCATGGGATTTCTCGACCAC

Antimicrobial susceptibility testing

The minimal inhibitory concentrations (MICs) of the *A. towneri* isolate to meropenem, tigecycline, ceftazidime, florfenicol, tetracycline, colistin and gentamicin were determined and evaluated using broth microdilution according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (18). *E. coli* ATCC25922 was used as quality control strain.

Whole-genome sequencing analysis

The *A. towneri* isolate 19110F47, was sequenced by using the PacBio RS and Illumina MiSeq platforms (Shanghai Personal Biotechnology Co., Ltd, China). The PacBio long reads were assembled with HGAP4 and CANU (Version 1.6) and corrected by the Illumina MiSeq short reads with pilon (Version 1.22). The prediction of ORFs and their annotations were performed using Glimmer 3.0. The blast software is used following the procedures at https://blast.ncbi.nlm.nih.gov.

PCR analysis

The presence of translocatable units (TUs) was detected by PCR using the primers shown in Table 1. All the PCR products were subjected to Sanger sequencing. The obtained sequences were analyzed by BLAST comparison with the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Transfer experiments

Conjugation assays were performed to assess the transferability of carbapenem and tigecycline resistance genes from *A. towneri* isolate to the azide-resistant recipient

E. coli J53 and the rifampicin-resistant recipient *E. coli* EC600 according to a previously described method with a minor modification (1). Briefly, the donor and recipient strains were mixed at a ratio of 1:4 and incubated on LB agar for 5 h. The mixtures were collected and then plated on LB agar containing two selective markers, including azide (128 μg/mL) and meropenem (2 μg/mL), rifampicin (64 μg/mL) and meropenem (2 μg/mL), azide (128 μg/mL) and tigecycline (2 μg/mL), rifampicin (64 μg/mL) and tigecycline (2 μg/mL), respectively. The transconjugants were confirmed by PCR analysis.

Results

Identification of a carbapenem- and tigecycline-resistant *A. towneri* isolate

An isolate conferring resistance to both meropenem and tigecycline, designated 19110F47, was identified from swine origin in 2019. 16S rRNA sequence analysis suggested that it was assigned to the species *A. towneri*, which is involved in nosocomial infections as described in a previous study (16). Antimicrobial susceptibility testing results revealed that it had an expanded resistance profile, including resistances to meropenem (MIC, 16 μg/mL), tigecycline (8 μg/mL), ceftazidime (256 μg/mL), tetracycline (32 μg/mL), gentamicin (64 μg/mL) and florfenicol (128 μg/mL). However, it was classified by the CLSI breakpoints as colistin-intermediate (<0.5 μg/mL). It is noteworthy that CLSI classifies all isolates with colistin MICs of ≤2 μg/mL as intermediate and does not provide a breakpoint for the category susceptible. Instead, when applying the breakpoints of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org), this isolate would have been classified as colistin-susceptible.

tet(X4) was located in the chromosomal DNA in *A. towneri* and is part of TUs

The *tet*(X4) gene was found to be a part of a multidrug resistance region (MDRR) located on a 100,354-bp genomic island (GI) that was present in the chromosomal DNA of *A. towneri* 19110F47. The complete GI shared only a low query coverage (16–37%) with the sequences deposited in GenBank. It was inserted between the DUF3375 and AAA family ATPase encoding genes in the chromosomal DNA of *A. towneri* 19110F47. Database searches identified a distinctly smaller GI without an MDR region being present at the same location in *A. towneri* CIP_107472_V1 (accession number GCA_000368785.1) from an activated sludge plant in Australia. A total of 94 ORFs were identified in the GI of the present study. These ORFs encoded proteins responsible for several functions, such as gene regulation, transfer, efflux and antimicrobial resistance (Figure 1). The 41,098-bp MDRR consists of 33 ORFs [from IS*26* to *aph(6)*-Ib] (Figure 1). Among them, a total of 12 resistance genes were identified, including *tet*(X4), the aminoglycoside resistance genes *aadA1*, *aadA2b*, *aph(3")-Ib*, *aph(3')-Ia* and *aph(6)-Id*, the sulfonamide resistance genes *sul1* and *sul2*, the phenicol resistance genes *cmlA1* and *floR*, the β-lactam resistance gene bla_{CARB-2} and the trimethoprim resistance gene *dfrA16* (Figure 1). In the close vicinity of *floR* and *tet*(X4), one truncated and two complete copies of IS*Vsa3*, all in the same orientation, were found (Figure 1), which was similar to the corresponding region on p47EC from a porcine *E. coli* (MK134376).

Three PCR assays were developed to detect IS*Vsa3*-mediated rolling-circle transposition of *tet*(X4) and/or *floR*. The results revealed that three TUs, including TU1 (*virD2-floR*-IS*Vsa3*, 4,274 bp), TU2 [*hp-abh-tet*(X4)-IS*Vsa3*, 4,608 bp] and TU3 [*virD2-floR*-IS*CR2-hp-abh-tet*(X4)-IS*Vsa3*, 8,882 bp] were formed in *A. towneri* 19110F47 (Figure 1).

bla_{NDM-1} and bla_{OXA-58} were located on novel non-conjugative plasmids

The bla_{NDM-1} gene was located on a plasmid with a size of 47,094 bp, designated p19110F47-1 (Figure 2A). BLAST analysis revealed that p19110F47-1 showed similiarities with two other plasmids, pAT232 (GN014838) and pGX7 (CP071772), present in the GenBank database. However, these similarities did neither include the regions covering the antimicrobial resistance genes, nor those with the plasmid replication gene. Overall, a low query coverage (with the highest of 48%) (Supplementary Figure 1) was detected, suggesting that p19110F47-1 is a novel carbapenem resistance plasmid. Further analysis of the flanking regions of bla_{NDM-1} revealed that the insertion sequence IS*Aba125* was located upstream of bla_{NDM-1}. Moreover, the bla_{NDM-1} gene was part of a region that contained also the resistance genes *aac(3)-IId*, *aphA6* and ble_{MBL} (Figure 2A).

The bla_{OXA-58}-carrying plasmid p19110F47-2 was 143,035 bp in size, also carried the aminoglycoside resistance genes *aac(3)-IV*, *aph(3')-Ia* and *aph(4)-Ia*, *floR*, *sul2*, the tetracycline resistance gene *tet*(M), the macrolide-lincosamide-streptogramin B resistance gene *erm*(B), and the macrolide resistance genes *mph*(E) and *msr*(E) (Figure 2B). GenBank database searches identified the complete sequences of seven plasmids from *Acinetobacter* spp. with p19110F47-2 query coverage ranges from 40 to 87% (Supplementary Figure 2), and most of these plasmids harbored both bla_{OXA-58} and *tet*(X) orthologs (19, 20).

Conjugation experiments were performed using *A. towneri* 19110F47 as donor and two different *E. coli* strains as recipients.

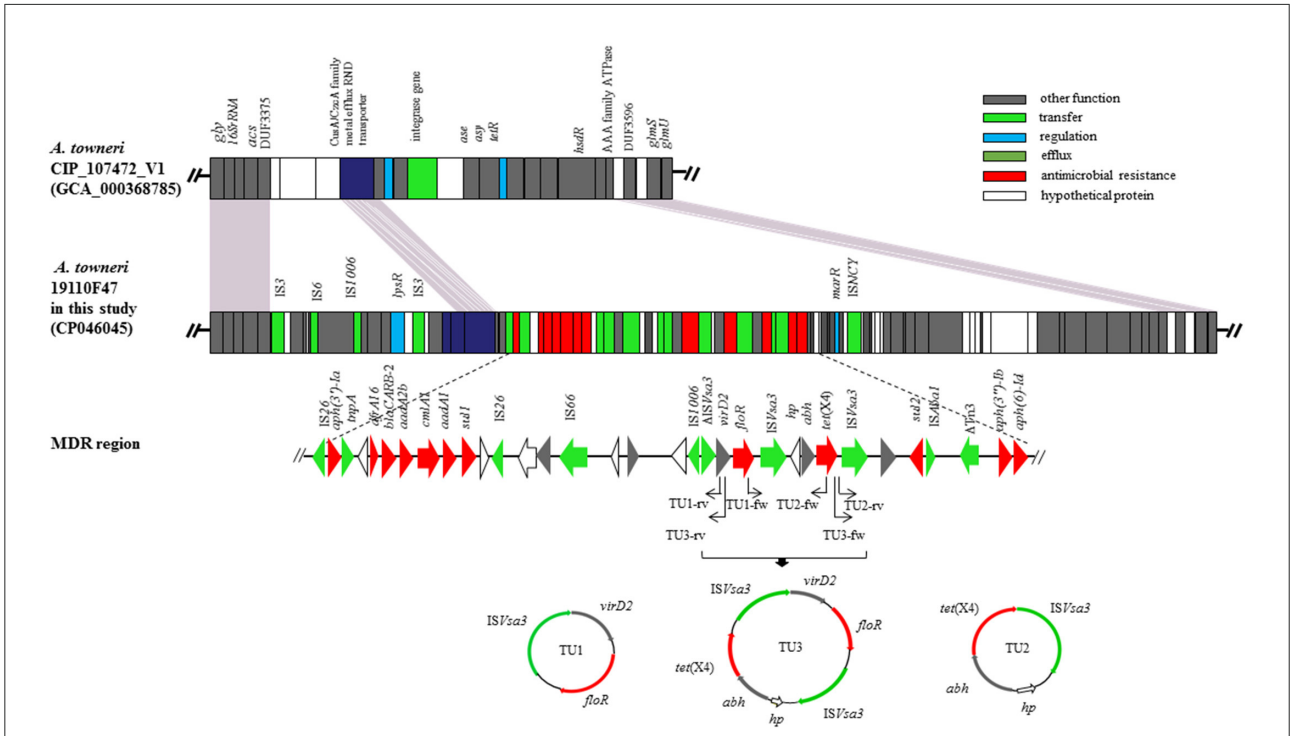

FIGURE 1
The genomic island carrying of the *tet*(X4) gene in the chromosomal DNA of *A. towneri* 19110F47 in this study. Genes are indicated by different colors. Δ indicates that the gene is truncated. The primers used for PCR were shown by arrows. Three TUs, including TU1 (*virD2-floR*-IS*Vsa3*, 4,274 bp), TU2 [*hp-abh-tet*(X4)-IS*Vsa3*, 4,608 bp] and TU3 [*virD2-floR*-IS*Vsa3-hp-abh-tet*(X4)-IS*Vsa3*, 8,882 bp] were formed.

No transconjugants were obtained, which might be explained by the fact that the bla_{NDM-1}-, but also the bla_{OXA-58}-carrying plasmids lack a conjugative transfer region (Figure 2).

Discussion

Acinetobacter spp. are ubiquitous in the natural environment, and have become important opportunistic pathogens, e.g., *A. towneri, A. baumannii, A. indicus, and A. lwoffii. Acinetobacter* spp. strains are widely distributed in a variety of environmental sources, including water, soil, foods, and livestock (15, 16, 21). In this study, we report, the identification of an *Acinetobacter* spp. strain co-harboring bla_{NDM-1}, bla_{OXA-58} and *tet*(X4) collected from food-producing animals in China. It belonged to *Acinetobacter towneri*, which is involved in nosocomial infections (16). Carbapenem-resistant *Acinetobacter* spp. is one of the most dangerous pathogens in the world (21). And *Acinetobacter* spp. was the major reservoir of tigecycline-resistant *tet*(X) genes (12). The co-location of *tet*(X) and carbapenem resistance gene bla_{NDM-1} was previously described in *Acinetobacter* isolates from animals and the environment (14, 22). The strain 19110F47 had an expanded resistance profile. More attention should be paid to multidrug resistant (MDR) *A. towneri* isolates, because they have been reported increasingly in recent years, especially in hospital sewage and from livestock (23–26).

The *tet*(X4) gene conferring resistance to tigecycline was found to be on the chromosome of 19110F47. The *floR* and *tet*(X4) were found to be flanked by one truncated and two complete copies of IS*Vsa3*. IS*Vsa3* is 977 bp in size and represents a member of the IS*91* family. This family of insertion sequences differs from others in that they transpose by rolling circle transposition (27). Members of the IS*91* family can mobilize genes, including resistance genes that are located in their close vicinity. For this, only a single copy of the IS*91* element—not two copies as with most other insertion sequences—is necessary (27). The *tet*(X4) is the part of active TUs in the chromosomal DNA, may excise from the chromosomal DNA and transfer to other plasmidic or chromosomal sites. Of three TUs identified in *A. towneri* 19110F47, a sequence indistinguishable from that of TU2 was also found in p47EC (MK134376) from *E. coli* in a previous study (3), suggesting its mobility across genus boundaries. Of note, TU3 contained both *tet*(X4) and *floR*, implying that tigecycline resistance can be co-selected by florfenicol, a veterinary antimicrobial agent commonly used in cattle, pigs, poultry and fish (28).

The bla_{NDM-1} located on the plasmid p19110F47-1. Insertion of bla_{NDM-1} together with IS*Aba125* have been found

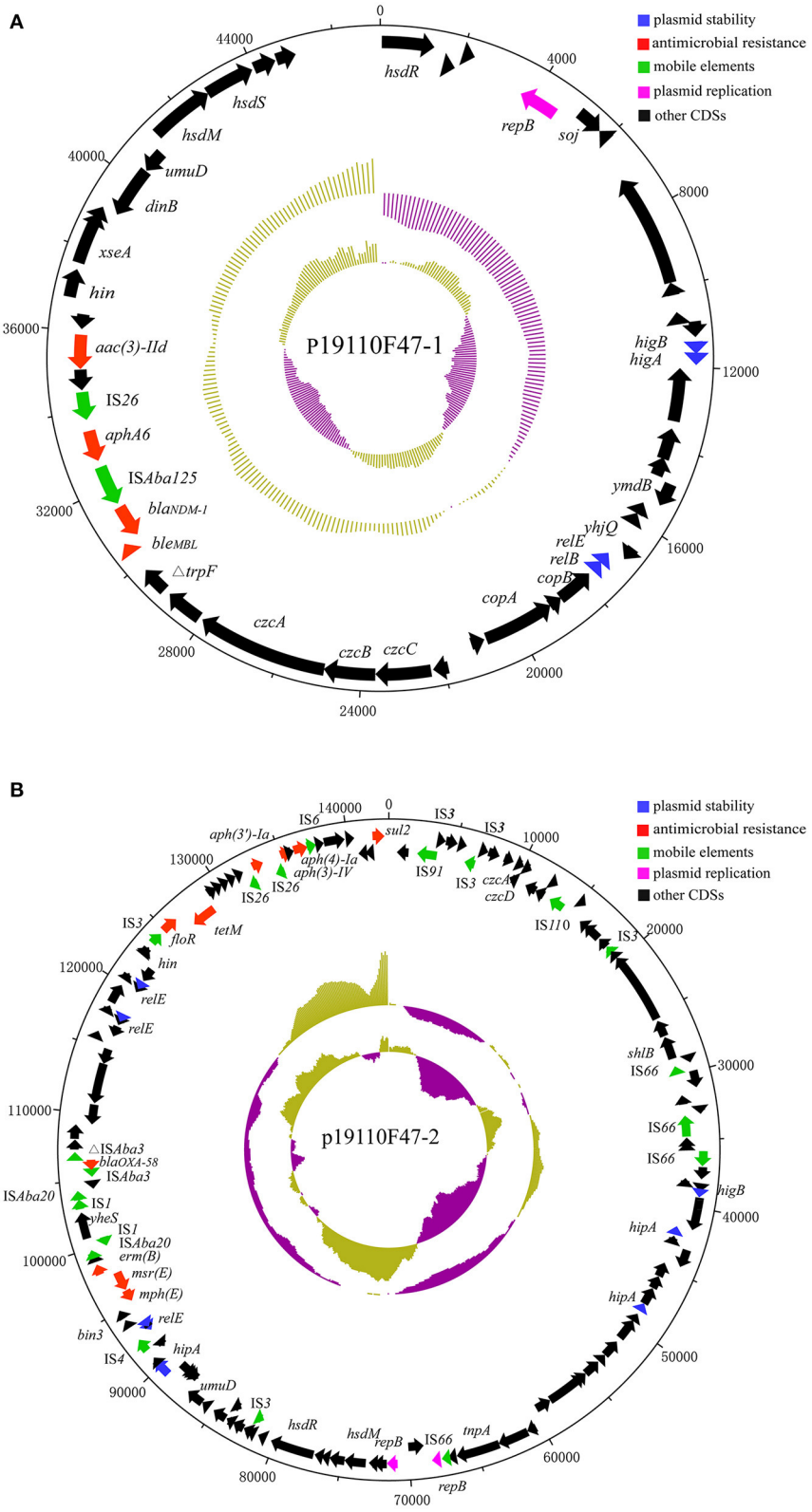

FIGURE 2
Structure of plasmids p19110F47-1 **(A)** and p19110F47-2 **(B)** from *A. towneri* 19110F47. The size scales are given in bp; genes are color-coded, depending on functional annotations: red, antimicrobial resistance; blue, plasmid stability; green, mobile elements; purple, plasmid replication; and black, other coding sequences (CDS).

within the chromosomal DNA and plasmids of *Acinetobacter* isolates (29, 30), suggesting that IS*Aba125* might be involved in the dissemination of bla_{NDM-1} among *Acinetobacter* spp. Numerous studies have documented that the bla_{NDM-1} was frequently present adjacent to truncated or intact IS*Aba125* as well as in other *Enterobacteriaceae*, including *E. coli*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Salmonella enterica* (31–33), indicating that IS*Aba125* plays a vital role in the spread of bla_{NDM-1} among different species of bacteria.

The bla_{OXA-58} was located on another plasmid p19110F47-2. An analysis for the genetic environments of bla_{OXA-58} showed that a truncated IS*Aba3* element was located upstream of bla_{OXA-58} and a complete IS*Aba3* element downstream of it. The genetic context found in the present study was consistent with that in plasmid pLHC22-2-tetX-162k (CP084298) in a previous study (19). In addition, the presence of two intact IS*Aba3* copies in opposite orientation up-stream and down-stream of bla_{OXA-58} was described in a series of studies and led to the assumption that IS*Aba3* might play a role in the transfer of bla_{OXA-58} (22, 34). It has been documented that transformation of a cloned bla_{OXA-58} gene on a low-copy-number vector into a susceptible *A. baumannii* strain increased the MICs of carbapenems, but not to levels considered as resistant (35, 36). For clinical carbapenem resistance in *Acinetobacter* spp., other carbapenem resistance mechanisms in addition to OXA-58-like β-lactamases are required (35). In the present study, the *A. towneri* isolate 19110F47 showed resistance to meropenem with a MIC of 16 μg/mL. It was speculated that bla_{NDM-1} played a dominant role in conferring the meropenem resistance phenotype.

The *tet*(X4), bla_{NDM-1} and bla_{OXA-58} genes have been documented in multiple studies solely or any two of them (14, 16, 20, 22, 37). Only one study by Zheng *et al.* described that a *tet*(X6) variant and the two carbapenemase genes bla_{NDM-1} and bla_{OXA-58} were located on the same plasmid in an *A. baumannii* isolate of chicken origin (15). In the current study, we identified the occurrence of *tet*(X4), bla_{NDM-1} and bla_{OXA-58} in the same *A. baumannii* isolate of swine origin, with *tet*(X4) being located on a novel chromosomal GI and bla_{NDM-1} and bla_{OXA-58} carried by two novel plasmids.

Conclusions

In conclusion, we described the co-existence of *tet*(X4), bla_{NDM-1} and bla_{OXA58} in a porcine *A. towneri* isolate that displays resistance to carbapenems and tigecycline among numeous other antimicrobial agents. The *tet*(X4) is part of active translocatable units in the chromosomal DNA, facilitating its transfer into other plasmidic or chromosomal sites. The emergence of chromosomal *tet*(X) genes combined with plasmid-mediated carbapenem resistance genes in the same isolate, as shown in this study, will further compromise the treatment options of severe infections caused by *Acinetobacter* spp.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Author contributions

X-DD and HY designed the research and supervised the study. AL, WZ, RY, and CL performed the experiments and analyzed the data. AL and HY wrote the manuscript. SS and X-DD reviewed and edited the manuscript. All authors revised the manuscript and approved the final version for submission.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.1002149/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Structure analysis of *bla*$_{NDM-1}$-bearing plasmid. Comparison analysis of the plasmid p19110F47-1 with other similar plasmids submitted in GenBank database.

SUPPLEMENTARY FIGURE 2
Structure analysis of *bla*$_{OXA-58}$-bearing plasmid. Comparison analysis of the plasmid p19110F47-2 with other similar plasmids submitted in GenBank database.

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Xin Zhao,
McGill University, Canada

REVIEWED BY
Getahun E. Agga,
Agricultural Research Service (USDA), United States
Ursula Höfle,
University of Castilla-La Mancha, Spain

*CORRESPONDENCE
Taras Günther
taras.guenther@bfr.bund.de

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Environmental factors associated with the prevalence of ESBL/AmpC-producing *Escherichia coli* in wild boar *(Sus scrofa)*

Taras Günther[1,2]*, Stephanie Kramer-Schadt[3,4], Marcel Fuhrmann[1] and Vitaly Belik[2]

[1]Department of Biological Safety, German Federal Institute for Risk Assessment, Berlin, Germany, [2]System Modeling Group, Institute for Veterinary Epidemiology and Biostatistics, Freie Universität Berlin, Berlin, Germany, [3]Department of Ecological Dynamics, Leibniz-Institute for Zoo and Wildlife Research, Berlin, Germany, [4]Institute of Ecology, Technical University of Berlin, Berlin, Germany

Antimicrobial resistances (AMR) in bacteria, such as ESBL/AmpC-producing *E. coli,* are a burden to human and animal health. This burden is mainly driven by the consumption and release of antimicrobial substances into the environment. The pollution and contamination of habitats by AMR in bacteria and antimicrobial substances can lead to the transmission of bacterial AMR to wildlife. Therefore, it is necessary to understand the transmission cycle of antibiotics and resistant bacteria between humans, and animals as well as their occurrences in the environment. Environmental factors associated with the occurrence of bacterial AMR in wildlife can lead to a better understanding of the distribution of bacterial AMR in humans and animals using One Health approaches. Here, we analyzed data gathered in the framework of the German zoonoses monitoring program in 2016 and 2020 using spatiotemporal statistics to identify relevant environmental factors (e.g., livestock density, climatic variables, and human density) in association with the spatial distribution of ESBL/AmpC-producing *E. coli*. For this purpose, we developed a generic data integration and analysis pipeline to link spatially explicit environmental factors to the monitoring data. Finally, we built a binomial generalized linear mixed model (GLMM) to determine the factors associated with the spatial distribution of ESBL/AmpC-producing *E. coli*. In 2016 and 2020, 807 fecal samples from hunted wild boar (*Sus scrofa L.*) were randomly taken in 13 federal states and selectively analyzed for ESBL/AmpC-producing *E. coli*. Forty-eight isolates were identified in 12 German federal states, with an overall prevalence of 6%. We observed an almost three times higher probability of ESBL/AmpC-producing *E. coli* isolates in wild boar in counties with high cattle densities (OR = 2.57, $p \leq 0.01$). Furthermore, we identified a seasonal effect in areas with high precipitation during the off-hunting seasons (OR = 2.78, $p = 0.025$) and low precipitation throughout the years (OR = 0.42, $p = 0.025$). However, due to the low amount of identified isolates, confidence intervals were wide, indicating a high level of uncertainty. This suggests that further studies on smaller scales need to be conducted with multiannual data and improved metadata, e.g., on

the location, the hunting procedure, and species characteristics to be collected during field sampling.

KEYWORDS

antimicrobial resistance, One Health, wildlife, spatial analysis, *E. coli*

Introduction

Antibiotics are used for the treatment of most bacterial infectious diseases and are an important instrument in veterinary and human medicine. However, the consumption and release of antimicrobial substances into the environment can foster the development of antimicrobial resistance (AMR) in bacteria (1–4). While the development of resistances is an evolutionary process in bacteria known as the "arms race" to survive, the overconsumption and use of antimicrobial substances can further increase the selection pressure in bacteria to develop resistances against several antibiotics (2, 5, 6). This leads to a high exposure of bacterial AMR in humans, animals, and the environment (2, 3, 7, 8). In 2019, approximately 4.95 million people died as a direct consequence of infections with antimicrobial-resistant bacteria worldwide (8). Therefore, one key element to prevent the uncontrolled development of AMR in bacteria is to understand the transmission cycles of antibiotics and resistant bacteria between humans and animals, as well as their occurrences in the environment.

The role of antimicrobial-resistant bacteria in wildlife and the environment is of particular interest for One Health approaches considering the human-wildlife-livestock interface. Various studies show that multiple antimicrobial-resistant bacteria can be found globally in wildlife and the environment, i.e., soil, water bodies, feed, and food (1, 3, 9–13). In recent years, major efforts have been made to identify the reservoirs and sources of antimicrobial-resistant bacteria in wildlife.

In 2019, Torres et al. (12) highlighted that antimicrobial-resistant bacteria are not ubiquitously distributed among wildlife species. In particular, the wild boar (*Sus scrofa*) was suggested as an appropriate sentinel species to investigate the distribution and transmission cycles of antimicrobial-resistant bacteria in wildlife and the environment due to their omnivorous feeding habits and their high abundances in various habitats (12, 14, 15). The transmission of antimicrobial-resistant bacteria and antimicrobial residues to wild boar is most likely caused through the consumption of contaminated feed and water. Various studies have identified different sources as the origin of the occurrence of AMR in bacteria and antibiotics in the environment, such as manure-based fertilizers in agriculture applied to fields or pastures, sewage wastewater treatment plants, landfills, waste and aquacultural facilities (6, 16–18). Therefore, environments characterized by livestock farming and densely populated areas are often more predisposed to be contaminated by antimicrobial-resistant bacteria and antimicrobial substances, increasing the exposure of wildlife species living in proximity to these areas, such as wild boar (7, 11, 15, 19, 20).

However, the amount, composition, and durability of antimicrobial-resistant bacteria and antimicrobial substances in these environments often depend on additional environmental influences. In sewage treatment facilities, for example, the amount and composition of microorganisms, including antimicrobial-resistant bacteria, depends on different climatic factors, such as temperature (21). Furthermore, facilities such as hospitals, slaughterhouses, and residential areas in the catchment area of sewage plants can release high amounts of antimicrobial-resistant bacteria and antibiotics into sewage. This contaminated sewage can then enter nearby water bodies, such as rivers, due to insufficient filtration (4).

In livestock farming environments the accumulation of antimicrobial substances and antimicrobial-resistant bacteria is even more complex and influenced by climate, weather, soil properties and farm management, as well as the types of farm animals (22). For example, the storage conditions of manure and the timing of fertilization can have a significant effect on the amount of antimicrobial-resistant bacteria that reach fields and pastures (23). In the agroecosystem, environmental factors such as soil composition, local weather conditions, adjacent drainage ditches and habitats are also important factors leading to the accumulation of antimicrobial substances and antimicrobial-resistant bacteria in the environment (22). In addition, wild boar ecology needs to be considered; as social group living species they might serve as mobile links for AMR in bacteria (24) which is another possible driver of spread. This leads to a complex and dynamic transmission cycle, causing the presence of antimicrobial-resistant bacteria in wildlife species such as wild boar.

The German Federal Institute for Risk Assessment (BfR), the German Federal Office for Consumer Protection and Food Safety (BVL) and the German Federal States conduct routine monitoring programs for zoonoses in Germany. Due to the role of wild boar for game meat production, in 2016 and 2020, the German monitoring program included wild boar as a target species, testing for antimicrobial-resistant bacteria and other zoonotic agents (25–27). During the monitoring programs, 807 fecal samples of hunted wild boar (2016 $n = 547$ samples, 2020 $n = 260$ samples) were randomly taken across Germany and specifically tested for the presence of "extended spectrum (ESBL)

and ampicillin class C (AmpC) beta-lactamase-producing *Escherichia coli*" (in the following: ESBL/AmpC *E. coli*) (25–27). The distribution pattern of ESBL/AmpC *E. coli* isolates seemed to be regionally concentrated and raised the question of whether there were spatiotemporal differences between isolates and negative samples.

In this study, we hypothesized that anthropogenic and environmental factors within the sampling regions could serve as indicators for the complex transmission cycle of ESBL/AmpC *E. coli* in wild boar. Regional density of livestock or human population might be an indicator for the exposure of the environment to contaminated sewage or fertilizers, and further, a possible transmission to wild boar. Here, we used the data of the German zoonoses monitoring program from 2016 and 2020 to develop an analysis pipeline that can be used in upcoming years as a tool for a standardized analysis (25, 27). The pipeline extracts and links environmental data to the sampling location and tested samples on ESBL/AmpC *E. coli*. For this purpose, a review of scientific literature was conducted to identify suitable environmental factors. Based on these results, the relevant environmental data were gathered from public online data portals.

Thus, the primary objectives of this study were i) to link data of the monitoring program with spatially related environmental factor data that were collected from different public online data sources, ii) to analyze the associations between the occurrence of ESBL/AmpC *E. coli* in wild boar and the environmental variables *via* geospatial analysis, and iii) to propose recommendations on data collection for monitoring of antimcirobial-resistant bacteria in wildlife using wild boar as a study model. This study will support a better understanding of the role of antimicrobial-resistant bacteria in wildlife and the environment following the framework of One Health.

Materials and methods

Sampling data and study area

In the German zoonoses monitoring programs in 2016 and 2020, 899 fecal and nasal swab samples from hunted wild boars were randomly collected and tested for different zoonoses and antimicrobial-resistant bacteria (25–27). In 2016, 547 out of 551 fecal samples of wild boar were tested specifically for ESBL/AmpC *E. coli*. In 2020 260 fecal samples out of 384 were tested specifically for ESBL/AmpC *E. coli*. The samples were collected in 14 out of the 16 German federal states, excluding Bremen, and Hamburg. We decided to exclude five samples taken in 2016 in Berlin since the provided information on the hunting area was not sufficient for conducting analyzes and because Berlin was the only sampling area comprising a metropolitan area. The minimum sample size defined for the monitoring programs was based on the hunting bags of the federal states 2013/2014 for the monitoring program 2016 and the hunting bags of 2017/2018 for the monitoring program 2020 (25, 27). However, the sample collection within the different federal states was not mandatory and depended on the availability of samples (Figure 1). The sample size differed from 1 to 23 per sampled county. The samples were taken monthly in 2016 and 2020, with a focus during the hunting seasons in the winter months of January, February, October, November, and December, where 78% of all samples were taken. Therefore, we defined two main seasons: the first season is defined as main hunting season (October–February) and the other one as the off-hunting season (March–September). The sampling locations were notified to the authorities at municipality or county level. Unfortunately, the samples were collected with the intention of aggregating the monitoring data at federal and national level. Therefore, detailed species information such as sex and age of the shot animals, as well as the exact locations, were often not concise or sufficiently reported. Furthermore, no information was provided on the hunting method within the different sampling regions. The results of the zoonoses monitoring programs have previously been published by Plaza-Rodriguez et al. (26) and in the report on the respective zoonoses monitoring in 2016 and 2020 (25, 27). These studies describe their findings on a national level for different animal species and zoonotic bacteria. Our study is based on the raw data of the monitoring program focusing on the wild boar samples tested for ESBL/AmpC *E. coli*. We extended the reported data with additional information to allow detailed spatial analysis.

Laboratory analysis

The primary isolation of ESBL/AmpC *E. coli* in collected fecal samples was carried out in accredited state laboratories according to the EU reference laboratory protocol for the isolation of ESBL-, AmpC- and carbapenemase-producing *E. coli* in caecal samples (28). These results were reported to the German Federal Office for Consumer Protection and Food Safety (BVL) for aggregation and reporting at national level. The confirmation, characterization, and phenotypic resistance testing of ESBL/AmpC *E. coli* isolates was performed at the National Reference Laboratories for Antimicrobial Resistance (NRL-AR) at the BfR. The antimicrobial susceptibility testing (AST) was conducted with broth microdilution method according to CLSI M07-A10 and CLSI M45-A, using the standardized EUVSEC and EUVSEC2 plates (TREK Diagnostic Systems) for 14 antibiotics (Commission Implementing Decision (CID) 2013/652/EU) (26). A further characterization in regard to harbored ESBL/AmpC genes within *E. coli* isolates resistant to third generation cephalosporins was conducted in three steps (26). First, a prescreening by real-time PCR was performed for the detection of the typical beta-lactamases

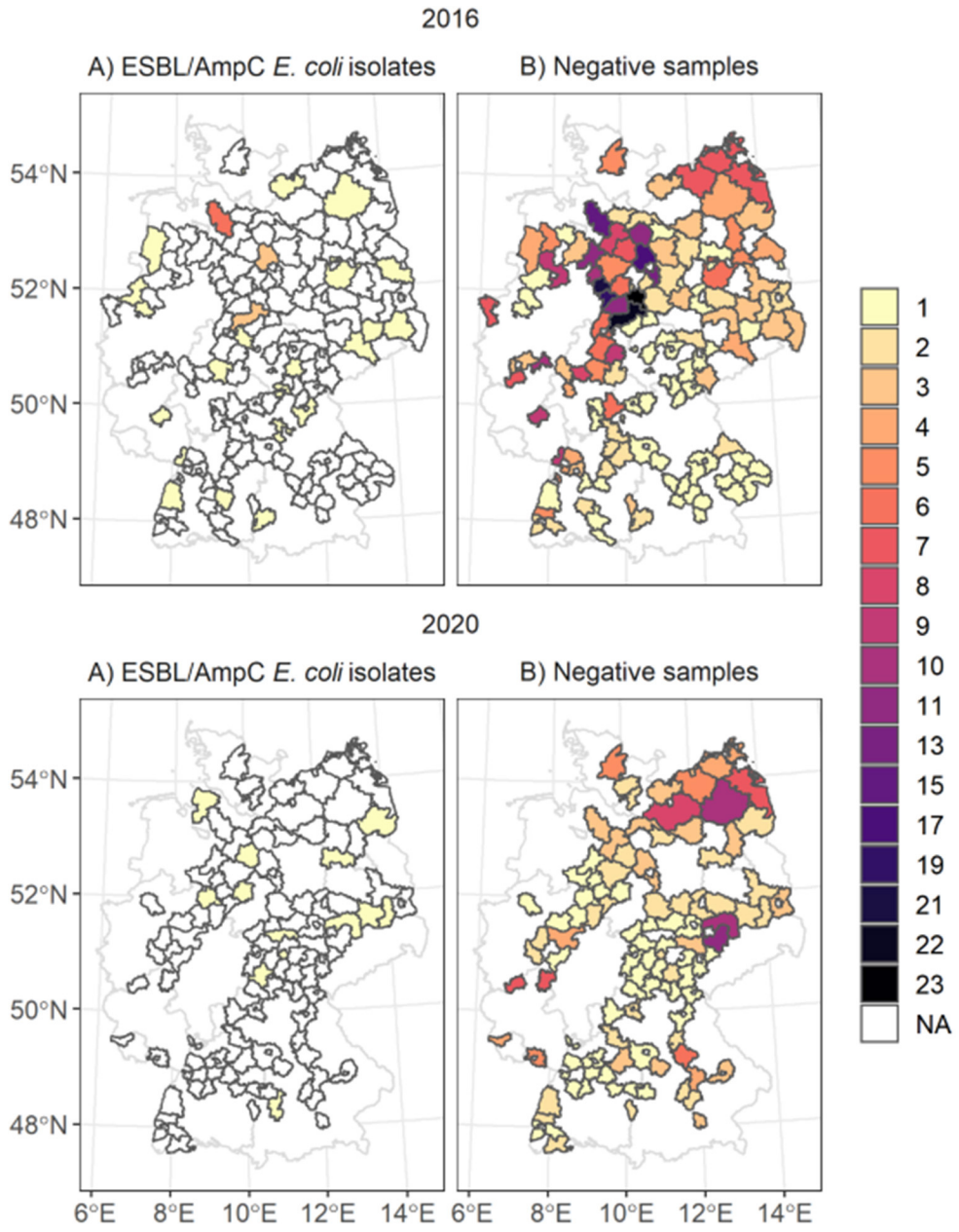

FIGURE 1
Spatial distribution of ESBL/AmpC *E. coli* isolates **(map A)** and negative samples taken **(map B)** in the counties. The color of the polygons represents the number of **(A)** number of isolates taken and in **(B)** the number of negative samples taken. The white areas marked as NA represent areas with no isolates identified.

TEM, CTX, SHV, and CRY (26). Thereafter, the PCR products were analyzed by Sanger sequencing for the determination of the ESBL variant (26). In a third step, isolates that were negative with real time PCR were screened by PCR for the presence of bla_{FOX}, bla_{MOX}, bla_{CIT}, bla_{DHA}, and bla_{EBC} genes (26). Since some discovered beta-lactamase types differed within the primer regions, it was not possible to distinguish between CTX-M-14 and *-17* (CTX-M-14 like), between CTX-M-65 and 90 (CTX-M-65-like), and between CMY-2/-22 and−66 (CMY-2-like) (26). The detailed laboratory analysis is already published in Plaza-Rodriguez et al. (26). We used the discovered resistance genes in our study to look for specific spatial patterns for further validation of the origin of resistances.

Data analysis pipeline

The data analysis pipeline (Figure 2) consists of three main steps: i) data cleaning, ii) data linking and extraction of environmental predictor variables and iii) statistical data analysis (29). During the data cleaning step, we deleted duplicated data from the same wild boar sample. Subsequently, the information on the sample origin was merged according to the county code with the spatial vector data of the Federal Agency for Cartography and Geodesy (BKG, scale 1: 250,000) (30). This vector data contains the polygon information of the borders of all counties of Germany. Data on explicit environmental factors were collected from different public data sources and formats (raster, csv and Excel). Tabular data were linked based on the reported county keys. We harmonized raster datasets using Lambert azimuthal equal-area projection (LAEA: EPSG-Code 3035). The template raster resolution was 100 square meters (m^2), which represents the specific information about the transformed data set in each grid cell. The processed environmental raster files were stacked and extracted according to the reported counties, months, and years using the "*exactextractr*" R-package (31). The data analysis pipeline was developed in R 4.1.1 (32), combining all steps in one code base. We used, among others, the packages *raster*, *sf*, *exactextractr* and *tidyverse* (31, 33–35).

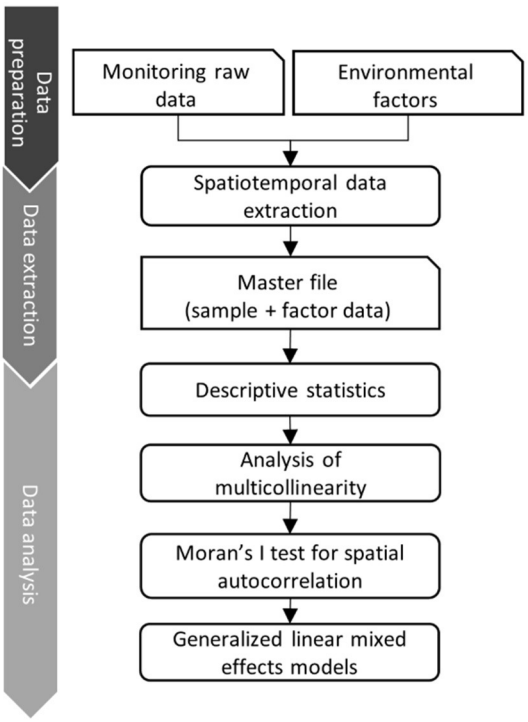

FIGURE 2
Data analysis pipeline that combines monitoring raw data with data of explicit environmental factors (right vertical axis). On the left the 3 main steps of the data pipeline are described.

Environmental predictor variables

We based the selection of environmental predictors on a review of current scientific literature focusing on the development and contamination of bacterial AMR in the environment. In several publications, the role of agriculture and densely populated areas are described, but also climatic factors such as high temperatures and precipitation (11, 12, 15, 20–22, 26). Therefore, we collected meteorological, agricultural and geographical data from publicly available websites listed in Table 1. The meteorological data originate from the "Deutscher Wetterdienst" (DWD), a federal authority that is responsible for weather and climate information in Germany (36). We derived monthly information on air temperature (measured in 2016 and 2020 in 2 m height) and precipitation (from 2016 and 2020). We also included the climatic precipitation and temperature (both measured and averaged from 1991 to 2020) to identify weather anomalies during the years 2016 and 2020. This information was provided as raster files for each month and year. We projected the raster files into the target LAEA projection (see Section 2.3), and the values of the weather data were extracted as median values for each notified county of origin and the month of sampling. Information on the human population density and livestock production systems in 2016 and 2020 were collected from the data portal "www.regionalstatistik.de" provided by the German federal statistical offices. We downloaded information on the human population density, cattle density, and pig density per km^2 in each county from the "Regional Atlas of Germany" as csv files. The downloaded data were merged according to the county code (NUTS) with the monitoring data. To account for the transmission of ESBL/AmpC *E. coli* within the wild boar population, we used raster data of the MaxEnt model prediction based on presence-background data describing suitability for wild boar occurrence (2014–2017) published by the *ENETWILD*-consortium 2019 (37). The wild boar presence probability raster data was also reprojected into the target LAEA projection and extracted as median for each county (see Section 2.3). Thus, all data sources were summarized in one master file that contained the information on ESBL/AmpC *E. coli* isolates obtained from fecal samples, the environmental variables and the spatial information given as the geometry of the county.

Statistical analysis

The statistical analysis was performed in four main steps. We first conducted a descriptive analysis of the spatial

TABLE 1 Collected data and their origin.

Category	Factors	Unit	Year	Source	Reference
Weather/Climate	Average monthly temperature	Degree Celsius	2016, 2020	*DWD*	cdc.dwd.de
	Average monthly precipitation	mm	2016, 2020	*DWD*	cdc.dwd.de
	Average monthly temperature (climate) (1991–2020)	Degree Celsius	1991–2020	*DWD*	cdc.dwd.de
	Average monthly precipitation (climate) (1991–2020)	mm	1991–2020	*DWD*	cdc.dwd.de
Sources/Proxys	Human population density	Human population density/ Km^2	2016, 2022	*Regionalstatitik.de*	www.regionalstatistik.de
	Cattle density	Cattle density /Km^2	2016, 2020	*Regionalstatitik.de*	www.regionalstatistik.de
	Pig density	Pig density /Km^2		*Regionalstatitik.de*	www.regionalstatistik.de
Wild boar	Wild boar presence probability	Suitability (Low:0/High:1)	2014–2017	*ENETWILD*-consortium	https://enetwild.com/maps/
Geographic	County Germany GE250 data base		2016	*BKG*	www.bkg.bund.de/
Time	Month		2016	Raw Data	
	Main hunting season and off-hunting season			generated	

distribution of samples. Before model fitting, we tested all extracted environmental variables for multicollinearity with the Spearman's rank correlation test ("*ggally*" R-package) and kept all variables with $|rho| < 0.7$ (Supplementary Figure 1). We tested the samples taken on spatial autocorrelation using Moran's I, which relies on a comparison of the distance between the center points of each county. All counties sampled were spatially independent ($p = 0.01$). Next, we used generalized linear mixed-effects models (GLMM) with the presence or absence of ESBL/AmpC *E. coli* represented as binary response variable (1/0), with logic link function, binomial error distribution. As explanatory predictor variables, we included the environmental factors (covariates) such as cattle density, pig density, human population density, the average monthly precipitation, the climatic mean precipitation, the wild boar presence probability, and the average monthly temperature. All explanatory predictor variables were normalized using the scale function to account for different units. To assess the temporal dynamics of the occurrences of ESBL/AmpC *E. coli* in wild boar, we included season as an interaction term combined with each environmental covariate. To account for regional and yearly differences, we used the county ID as well as the year as random effects. We ran the GLMM using the R-package "*glmmTMB*" (38). Estimates were transformed to odds ratios (OR) with the *sjPlot* package (39). The standard significance threshold was set to $p < 0.05$ (95% confidence interval, CI). Finally, we examined the model for linearity of predictors, independence of errors and dispersion with the *DHARMa* package (40) (Supplementary Figures 2, 3).

Results

Spatial distribution of the samples

We linked and analyzed the data of 802 fecal samples of dead wild boars tested for ESBL/AmpC *E. coli* as part of the zoonoses monitoring program in 2016 and 2020. The sample size differed between the years: for 2016, 542 samples, and for 2020, 260 samples. The samples originated from 181 German counties in 13 federal states (Figure 1). Most samples, were collected in the federal state of Lower Saxony in the northwestern part of Germany ($n = 271$, 33.6%). At the county level, most fecal samples were collected in 2016 in Goslar, where all 23 samples were negative. In 2020, most samples originated from the federal state Mecklenburg-Western Pomerania ($n = 40$) and the counties Leipzig ($n = 11$) and North Saxony ($n = 11$) (Saxony). Most samples tested for ESBL/AmpC *E. coli* (78.4%) were taken during the main hunting season, especially in November (56%). During the off-hunting season (March-September), 15.8% of the samples were taken.

A total of 48 ESBL/AmpC *E. coli* isolates out of 802 fecal samples were detected in 39 counties and 11 federal states (Figure 1). In 2016, 37 isolates out of 544 samples and in 2020, 13 isolates out of 260 samples were identified. The highest number of ESBL/AmpC *E. coli* isolates in 2016 were found in the federal state Lower Saxony with 13 isolates out of 241 fecal samples and on the county level in Rotenburg (Wümme) (5 isolates out of 15 fecal samples) (Supplementary Figure 5). The distribution of the sample size and the sample regions differed between 2020 and 2016, and most isolates of 2020 were identified in the federal

state Brandenburg, with four isolates. No county had more than one identified isolate in 2020.

The overall temporal distribution of the taken samples was characterized by the hunting season (Figure 3). In November 2016, 22 isolates were found in the 306 samples taken, which is the highest number of isolates found. In January ($n = 83$), February ($n = 15$) and May ($n = 8$) no isolates were identified. The overall proportion of identified isolates during the off-hunting season was 30% higher than in the main hunting season. However, the samples taken during the off-hunting season represented only 16% of all collected samples (Figure 3). The proportion of isolates ranged from 33% ($n = 6$, isolates $= 2$) in June, 50% in July ($n = 6$, isolates $= 3$), 22% in August ($n = 9$, isolates $= 2$) and 25% in September ($n = 4$, isolates $= 1$) (Figure 3). In 2020, isolates were identified only in 3 months: March, September, and November. In September ($n = 6$) and November ($n = 6$) 12 out of 13 identified isolates were found. Hence, 53% of all isolates were identified within the off-hunting season.

In 2016, 25 isolates of the 35 identified ESBL/AmpC *E. coli* isolates were additionally tested for their resistance genes. Fifty-seven percent of the isolates were contained $bla_{\mathrm{CTX-M-1}}$ ($n = 11$) genes, 17% $bla_{\mathrm{CTX-M-15}}$ ($n = 5$) genes and 13% as $bla_{\mathrm{CTX-M-14}}$ -like genes ($n = 3$). Furthermore, one isolate contained $bla_{\mathrm{CTX-M65}}$, and another isolate was classified as AmpC phenotype bla_{CMY2}-like gene. Sixty-four percent of the 25 analyzed isolates were identified during the hunting season and 28% during the off-hunting season. Most isolates identified in 2016 contained $bla_{\mathrm{CTX-M-1}}$ genes, with nine isolates in the main hunting season and four in the off-hunting season. At the county level, most ESBL/AmpC *E. coli* were identified during the main hunting season in Rotenburg (Wümme) in Lower Saxony, with the ESBL genes $bla_{\mathrm{CTX-M-1}}$ ($n = 3$) and $bla_{\mathrm{CTX-M-15}}$ ($n = 2$). Most of the analyzed resistance genes (40%) originated from the federal state of Lower Saxony. The resistance genes $bla_{\mathrm{CTX-M-1}}$ ($n = 5$), $bla_{\mathrm{CTX-M15}}$ ($n = 3$), $bla_{\mathrm{CTX-M-14}}$ -like ($n = 1$) and one $bla_{\mathrm{CTXM-M-65}}$ were identified in the isolates from Lower Saxony. These isolates were collected during the main hunting season. Most samples during the off-hunting season originated from the federal state of North Rhine-Westphalia, with one isolate detected with $bla_{\mathrm{CTX-M-1}}$ and another with $bla_{\mathrm{CTX-M-15}}$ collected during

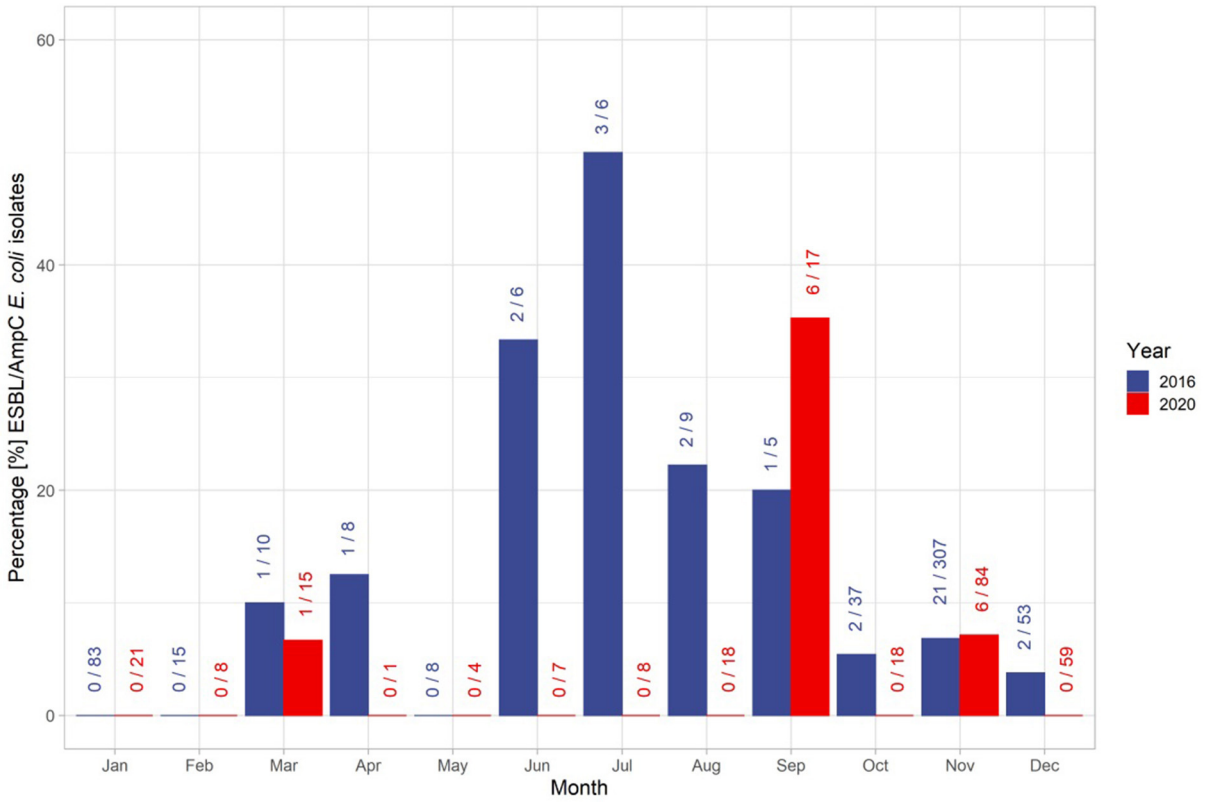

FIGURE 3
Distribution of isolates and the total number of samples throughout the years 2016 and 2020. The first number at each bar represent the number of identified ESBL/AmpC *E. coli* isolates within the month, and the second number after the slash sign represents the total number of samples taken within the month. The colors of the bars and numbers represent the associated year.

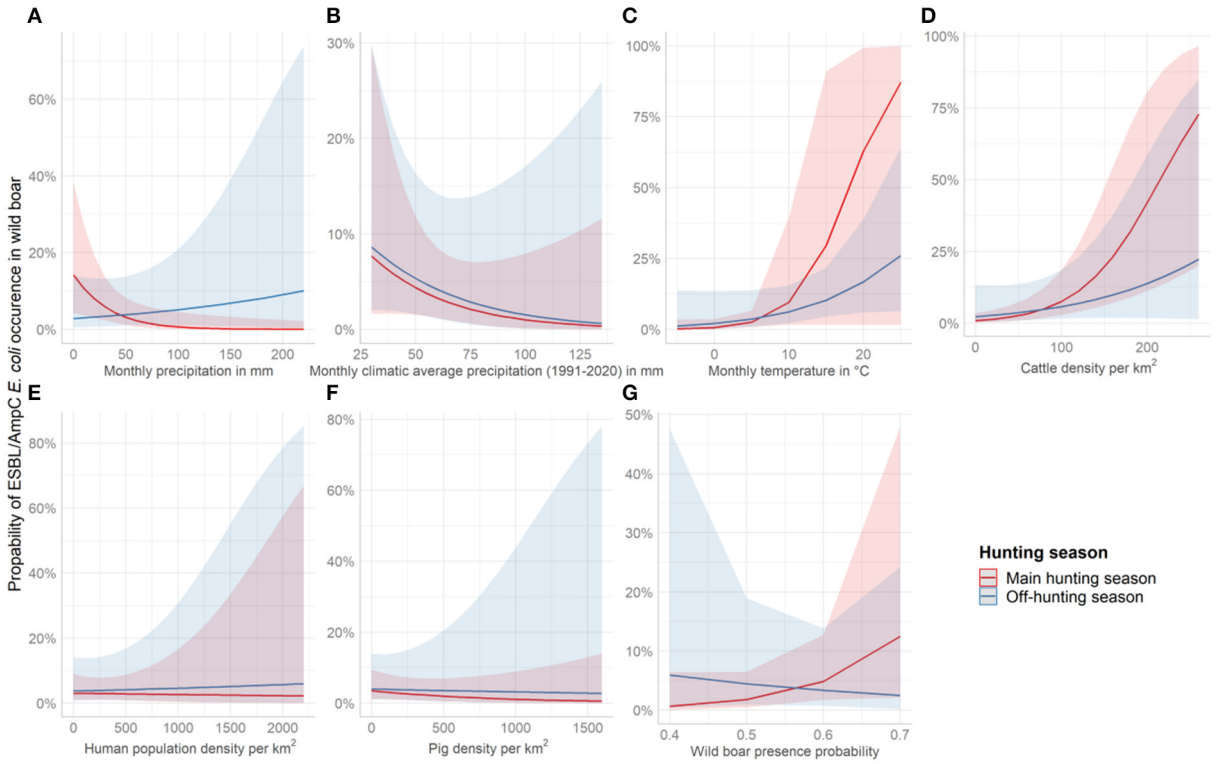

FIGURE 4
Effect plots showing the increase or decrease in the probability to identify ESBL/AmpC *E. coli* in wild boar per covariate and season. The red line represents the course of the main hunting season and the blue line the course of the off-hunting season. The covariates shown are **(A)** average monthly precipitation 2016 and 2020 in mm, **(B)** average monthly climatic precipitation (1991-2020) in mm, **(C)** average monthly temperature of 2016 and 2020 in °C, **(D)** cattle density per km^2, **(E)** Human population density per km^2, **(F)** pig density per km^2, and **(G)** wild boar abundance.

June 2016. Most samples in 2020 contained genes classified as $bla_{\text{CTX-M-1}}$ ($n = 6$) and $bla_{\text{CTX-M14}}$-like. In the other isolates, the AMR genes were classified as $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-27}}$, $bla_{\text{SHV-12}}$, and $bla_{\text{TEM-52-B}}$.

Environmental factors associated with the distribution of ESBL/AmpC *E. coli*

ESBL/AmpC *E. coli* in wild boar was almost three times more likely (OR = 2.78, $p = 0.03$) in counties with high average precipitation during the off-hunting season. In contrast, the overall probability to identify ESBL/AmpC *E. coli* in areas with low monthly average precipitation was significant as a single effect (OR = 0.42, $p = 0.02$) (Figure 4A and Table 2). The climatic average precipitation showed no significant effect in our model (Figure 4B and Table 2). Furthermore, areas with a higher average monthly temperature showed an almost four times higher occurrence of ESBL/AmpC *E. coli* in wild boar (OR = 3.61, $p = 0.06$) (Figure 4C and Table 2). Nevertheless, this effect was not significant.

In counties with a high cattle density, an almost three times higher (OR = 2.57, $p = <0.01$) (Figure 4D and Table 2) probability of ESBL/AmpC *E. coli* occurrence in wild boar was estimated. However, no effect in interaction with the hunting season was observed (Figure 4D and Table 2). The human population density (OR = 0.97, $p = 0.89$) (Figure 4E and Table 2) and pig density (OR = 0.79, $p = 0.29$) (Figure 4F and Table 2) showed no significant association with the likelihood of ESBL/AmpC *E. coli* occurrence as single effects, as well as in interaction with the hunting seasons (Table 2). The wild boar presence probability estimated by the MaxEnt prediction of the *ENETWILD*-consortium showed a slight positive effect (OR = 1.55, $p = 0.10$) even though it was non-significant (Figure 4G and Table 2). The overall model explained 35% of the variance in the data, whereas another 7% was explained by the random effects, year and county.

Discussion

The occurrence of ESBL/AmpC *E. coli* in wild boar feces had seasonal and spatial differences in 2016 and 2020 in Germany. We identified anthropogenic and seasonal effects such as cattle density, and precipitation associated with the occurrence of ESBL/AmpC *E. coli* isolates in wild boar.

TABLE 2 Estimates of fixed effects influencing the presence of ESBL/AmpC *E. coli* in wild boar (*Sus scrofa L.*) with the county as random effect (significance threshold $p < 0.05$).

Predictors	Odds ratios	CI	*p*
(Intercept)	0.03	0.01–0.09	**<0.001**
Hunting season [Off-hunting season]	1.30	0.33–5.19	0.709
Average monthly precipitation in mm	0.42	0.21–0.87	**0.020**
Average monthly precipitation in mm (climate)	0.61	0.29–1.28	0.193
Human population density per km^2	0.97	0.65–1.47	0.893
Average monthly temperature	3.61	0.92–14.18	0.066
Wild boar presence probability	1.55	0.91–2.62	0.104
Cattle density per km^2	2.57	1.53–4.32	**<0.001**
Pig density per km^2	0.79	0.51–1.23	0.293
Hunting season [Off-hunting season] * Average monthly precipitation in mm	2.78	1.13–6.80	**0.025**
Hunting season [Off-hunting season] * Average monthly precipitation in mm (climate)	1.07	0.38–3.04	0.899
Hunting season [Off-hunting season] * Human population density per km^2	1.07	0.61–1.89	0.810
Hunting season [Off-hunting season] * Average monthly temperature	0.46	0.11–1.97	0.299
Hunting season [Off-hunting season] * Wild boar presence probability	0.57	0.25–1.28	0.171
Hunting season [Off-hunting season] * Cattle density	0.59	0.25–1.39	0.226
Hunting season [Off-hunting season] * Pig density	1.21	0.58–2.54	0.618
Random effects			
σ^2	3.29		
$\tau_{00\ County}$	0.20		
$\tau_{00\ Year}$	0.21		
ICC	0.11		
N_{County}	180		
N_{Year}	2		
Observations	802		
Marginal R^2/Conditional R^2	0.357/0.429		

The fixed effects explained ~ 35% of the variance (marginal R2), while adding the random structure explained another 7%.

In June 2016, the precipitation in the counties Borken and Recklinghausen was 120 mm higher than the climatic monthly average (1991–2020) (DWD) (Supplementary Figure 4). During that time, heavy rainfalls and floods were reported in the regions where the isolates were identified (41). These events and hence the significant model outcome indicated a positive association between high precipitation in summer 2016 and the occurrence of ESBL/AmpC *E. coli* in wild boar. The transmission might be caused by the overflow of sewage water in sewage water plants. This is one of the main sources leading to the uncontrolled spread of bacterial AMR and other zoonotic pathogens into water bodies (4). We assume that wild boars came into contact with contaminated sewage water or with water from rivers next to sewage plants where an overflow occurred. The genes $bla_{CTX-M-1}$ and $bla_{CTX-M-15}$ were detected in isolates taken during the flooding event in June 2016 in Borken and Recklinghausen in the federal state of North Rhine-Westphalia. $Bla_{CTX-M-1}$ genes were previously found in non-clinical isolates of humans as well as in livestock (42), while $bla_{CTX-M-15}$ genes are predominantly found in clinical isolates from humans (43). The region of Borken and Recklinghausen has a high number of human settlements, which supports the assumption of transmission from sewage to wild boar. This positive association is in line with other studies that demonstrated a connection between high human population density and bacterial AMR emergence (11, 12, 15, 26). However, in our model, the human population density did not show a positive association with the isolates identified, and we were unable to distinguish the effects of the human population density and the pig density on the ESBL/AmpC *E. coli* occurrences in wild boars within our model. In contrast, lower average monthly precipitations had a significant effect on the probability of ESBL/AmpC *E. coli* occurrence in wild boar for the whole year. This may indicate that single weather events can significantly change the outcome of the ESBL/AmpC *E. coli* occurrence in wild boar. For example, for 2020 we were unable to identify a difference in the average monthly precipitation and the average climatic monthly precipitation within the counties. Moreover, we saw an almost four times higher probability of ESBL/AmpC *E. coli* occurrence in wild boars hunted in counties with a

higher average temperature. Even though this effect was not significant with a *p*-value of 0.07. Higher temperatures can enhance biological activity in soils and water and therefore might also influence the occurrence of AMR in bacteria in the environment (4, 18, 22). This can also lead to seasonal differences in the occurrence of ESBL/AmpC *E. coli* in sewage water of sewage cleaning plants (6). Even during winter, the occurrence in counties with milder temperatures was higher than in colder ones. However, in 2020 in May, June, and July no isolates were identified in the sampled areas with overall similar sample sizes compared with 2016.

Interestingly, the cattle density showed a highly significant association with the occurrence of ESBL/AmpC *E. coli* in wild boar. This is in line with the report of the zoonoses monitoring in 2015 and 2017, where cattle populations had a prevalence of 60 and 68% ESBL/AmpC *E. coli* isolates, respectively (44, 45). The high prevalence of ESBL/AmpC *E. coli* isolates in cattle populations also appears to impact the incidence of ESBL/AmpC *E. coli* isolates in wild boar, suggesting exposure from manure, farms, and cattle. In addition, 66% of the identified isolates were found during November. During this month, the spread of slurry is not allowed. However, prior to November, farmers attempt to apply manure to empty storage capacities before winter. This may lead to a temporary increased exposure of wildlife to manure contaminated with antimicrobial-resistant bacteria. Pastures were the only areas where fertilization was allowed until November 1st. Therefore, this would also mean that the prevalence of antimicrobial-resistant bacteria in wildlife would increase at the beginning of the vegetation period when farmers fertilize crops like wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) with nitrogen and slurry. In March, we saw in both years one ESBL/AmpC *E. coli* isolate in wild boars, but not during April and May. Another possible transmission route might be the release of contaminated sewage water from sewage plants, for example, from the slaughtering production industry releasing antimicrobial-resistant bacteria to the environment. Even though, this is not specific to the month of November itself (46, 47). To determine valid statements on the origin of the isolates, further studies are needed on smaller sampling areas to investigate such relations and to link, for example, genetic information of isolates identified in samples of sewage and farms with isolates of wild boar. Farm management might be another factor influencing AMR in bacteria release into the environment. Unfortunately, the monitoring data for 2016 and 2020 is too limited to provide information on the direct transmission of ESBL/AmpC *E. coli* between wild boar and cross-species transmission with other wildlife and livestock species. However, we observed a non-significant positive trend of ESBL/AmpC *E. coli* occurrence in areas with a higher wild boar presence probability by using the MaxEnt model prediction of the *ENETWILD*-consortium. This result may indicate that transmission of ESBL/AmpC *E. coli* may also be driven in part by the wild boar population itself, even though the association was non-significant. Accordingly, wild boar ecology needs to be considered in future studies to gain a better understanding of the transmission cycles of ESBL/AmpC *E. coli* within the wild boar population.

There are further limitations in this study to be acknowledged. We observed wide confidence intervals for all factors that indicate statistical uncertainties. Furthermore, there are possible biases in the sampling related to the non-reported hunting procedures. This, together with the low numbers of ESBL/AmpC *E. coli* isolates, might lead to confounding effects. Firstly, the hunting strategy itself might contribute to the unequal distribution of the samples throughout the years. While traditionally large drive hunts dominate in winter and whole sounders are shot, hide hunts in summer target single individuals (48). Repeated samples from the same sounder might lead to clustering, e.g., if the entire sounder had not encountered sources of bacterial AMR, the likelihood of finding many isolates might be on average lower and vice versa. Moreover, we need to consider that the samples in summer were only taken in few municipalities. For this reason, we can only speculate if the higher prevalence observed during the off-hunting season in 2016 (Figure 3) is truly representative or just subject to sampling bias, especially when considering the data of 2020, where only during May, September, and November isolates were identified, and the model outcome was not significant for the hunting season. Additionally, we cannot provide detailed information on how the sampling process was affected by the corona pandemic throughout 2020, which caused an overall smaller sample size during the year.

Not only was the sampling different throughout the years, but also in space. For example, the authorities in Lower Saxony took the required minimum sample of wild boar samples in 2016 in periods of time instead of during the whole main hunting season. Lower Saxony is one of the most important regions in Germany for livestock production. However, during the off-hunting season, the federal state of Lower Saxony was underrepresented in the samples taken, while during the winter of 2016 it was overrepresented (Supplementary Figure 5). Likewise, samples taken in areas with a high cattle density are temporarily underrepresented. This may bias our model results even though we integrated the sample region as a random effect. Therefore, further studies and upcoming monitoring programs of wildlife need a standardized annual data collection and should report information on the type of hunting as well as the exact locations.

Moreover, the livestock industry in our model is not completely represented. The poultry industry, one of the largest users of antibiotics in livestock farming, was not included in the model (49, 50). In the study of Urra et al. (23), the authors show that in fresh manure from chickens, high proportions of AMR in bacteria can be identified. It is then likely that poultry can contribute to the distribution of AMR in bacteria to wildlife as

well. We were unable to identify suitable data sources on the density of poultry for the years 2016 and 2020 and therefore did not include this as a covariate. Thus, the poultry industry should also be considered in future studies to determine the impact of different livestock species on the distribution of antimicrobial-resistant bacteria in wildlife. A comparison of the spatiotemporal prevalence data of livestock with the data of wildlife species could be beneficial as well, to better understand the transmission pathways from agriculture to wildlife.

Overall, our data pipeline allowed an extension and spatiotemporal analysis of the monitoring data. The analysis reveals the value of linking different environmental open data sources with data of monitoring programs in order to determine effects that might be associated with the distribution of bacterial AMR in wildlife. Our study demonstrates that monitoring programs targeting the distribution of antimicrobial-resistant bacteria in wildlife require further adjustments regarding the metadata collected during sampling to account for biases. Food safety monitoring programs are often designed based on livestock and food products for aggregation on a national level, considering privacy protection. Therefore, metadata on the individual level such as information on the correct sampling location is limited. In the case of free-ranging wildlife, sufficient harmonized information on age, sex, hunting methods and the correct sampling location is important. Furthermore, regarding wildlife the report of national units such as municipality or county level is not as beneficial as the report of specific coordinates (longitudes and latitudes), where the animals were sampled. Accurate coordinates are much more flexible to use regarding data extraction, linking, and processing on different spatial scales. It would also improve the comparability with other studies in the field and retrospective analyses, even though analytical methods might differ (15).

Conclusion

The distribution of ESBL/AmpC *E. coli* in wild boar is associated with environmental and anthropogenic factors. We showed that seasonal effects such as temperature and precipitation can have a significant association with the occurrence of ESBL/AmpC *E. coli* isolates in fecal samples from wild boar. Our results hint that weather events such as heavy rainfalls, floods, and high temperatures may increase the abundance of bacterial AMR in the environment, which is relevant to the potential effects of climate change. Interestingly, we also identified a positive effect on the distribution of ESBL/AmpC *E. coli* in wild boar by cattle density. This suggests that wild boars are exposed to antimicrobial-resistant bacteria in areas with large cattle populations. However, our analysis showed a high level of uncertainty, suggesting that multiannual data and small-scale studies in wildlife are needed to verify our findings.

Data availability statement

The datasets presented in this article are not readily available because the data analyzed in this study is subject to the following licenses/restrictions: The dataset used in this article belongs to the German National Zoonoses Monitoring Program. Currently these data are not publicly available, however work is currently underway to create a public database that contains this data along with many other data from other programs and years. The developed data integration and analysis pipeline in R is not publicly available and still in development. Requests to access the datasets should be directed to taras.guenther@bfr.bund.de.

Author contributions

TG, SK-S, MF, and VB: conceptualization and review and editing. TG and SK-S: methodology. TG: formal analysis and original draft preparation. All authors contributed to the article writing and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.980554/full#supplementary-material

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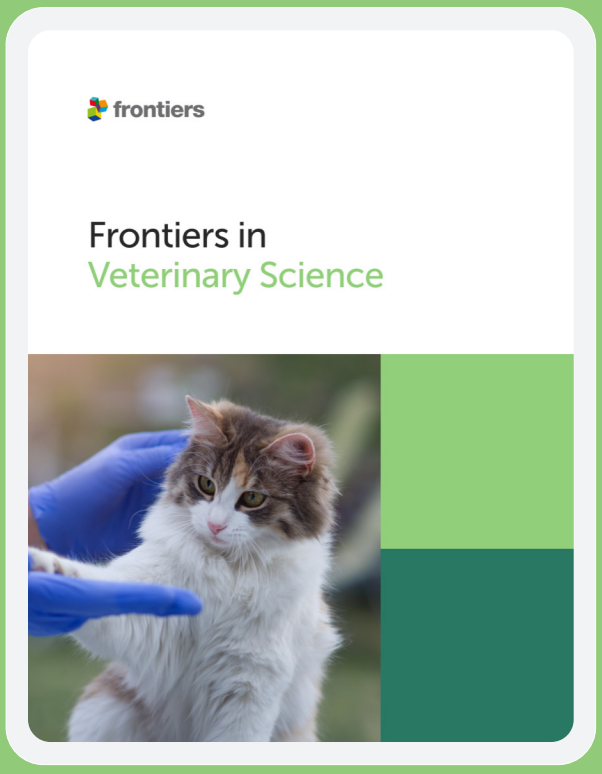

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