



COMBATING ANTIMICROBIAL RESISTANCE - A ONE HEALTH APPROACH

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COMBATING ANTIMICROBIAL RESISTANCE - A ONE HEALTH APPROACH

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Editorial: Combating Antimicrobial Resistance - A One Health Approach

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Editorial on the Research Topic

Combating Antimicrobial Resistance - A One Health Approach

Antimicrobial resistance (AMR) is a life threatening and a very serious global health problem. There is an increasing alarming concern regarding the emergence of multi-drug resistant (MDR) superbugs. Infections emerging from such pathogens are in most cases unresponsive to treatment with few, if any, antimicrobial agents currently available and effective. This issue is causing the development of two eras, a pre-antimicrobial agent era and raises concerns regarding a post-antimicrobial agent era, where the last resort antimicrobial agents are not potent to treat infections caused by Gram-positive and Gram-negative MDR strains.

Given the complexity of the AMR challenge at the level of human and animal health, in addition to the impact on the environment, it seems very important to stress and emphasize the role of a “One Health” approach in tackling the problem of AMR. Therefore, unifying efforts to combat and overcome this alarming issue requires a many-sided approach. The key points of this mission are mainly highlighted in three major tasks, understanding the bacterial resistance mechanisms, in addition to the utilization of combinatorial therapeutic approaches for potential clinical options, and the discovery of novel antimicrobial agents and/or targets.

The Research Topic entitled: “Combating Antimicrobial Resistance—A One Health Approach” harbors 25 published manuscripts that include 197 authors, scientists and researchers from worldwide renowned institutions and research centers. These manuscripts mainly focus on understanding the mechanisms of resistance in Gram-positive and Gram-negative MDR strains, address combination antimicrobial therapy as a potential treatment against Gram-positive and Gram-negative bacilli, and the discovery of novel antimicrobial agents with potential novel targets or new mode of actions.

To understand the mechanisms of resistance in Gram-positive and Gram-negative MDR strains, the Research Topic consists of 13 outstanding manuscripts, reviews, and original research articles that aim to elucidate novel mechanisms of resistance in different MDR strains and widen our knowledge to further understand how Gram-positive and Gram-negative bacteria combat antimicrobial agents.

With respect to Gram-positive bacteria, molecular epidemiological studies in *Streptococcus pneumoniae* from children with pneumonia in “Shanghai, China” provided the microbiology community with precise prevalence of serotype modifications and diversity in the emergence of MDR international clones, where the most common serotypes documented are 19F, 6A, 19A, 23F, 14, and 6B (Zhao et al.). Interestingly, the epidemiological study in *S. pneumoniae*

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correlates with the emerging crisis of AMR in China. This crisis is worsening and has become a major public and global safety problem, causing serious danger to humans and animal health and to the environment. This is due to the fact that the emergence of bacterial resistance is much faster as compared to the discovery of new antimicrobial agents, which will lead humans to enter the “post-antimicrobial era.” This makes China one of the most nations at risk of augmented AMR (Qu et al.). Furthermore, poultry surveillance studies in the UK have indicated the presence of livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) which suggests an augmenting problem in the zoonotic transmission of LA-MRSA in Europe (Anjum et al.). In addition, AMR poultry studies in the US revealed the possible propagation of resistance genes to the soil through poultry waste. This highlights the high need to understand the migration and the spread of AMR genes in the environment and in particular the ones in direct relevance to the health of humans and animals using the One Health Approach (Yang et al.).

In relation to Gram-negative bacteria, in the past decade the Middle East has become a reservoir for extended-spectrum cephalosporin and carbapenem resistant Gram-negative bacilli (GNB) in hospitals and to a lower level in the environment. Carbapenemases producers are dominating in hospitals while, extended spectrum beta lactamases (ESBL) and colistin resistance are becoming an alarming concern in animals. This is mainly due to the abusive use of colistin in veterinary medicine, where minimal information is available regarding the level of antimicrobial agents' consumption in the whole community and in farms (Dandachi et al.).

Integrative and conjugative elements (ICEs) are self-transmissible mobile genetic elements that provide the bacteria with all necessary tools to acquire complicated and complex traits through horizontal gene transfer and encode resistance to antimicrobial agents and heavy metals in parallel through a process known as “co-selection.” Assessing the emergence of multidrug- and toxin-resistant bacteria via ICEs in *Vibrio parahaemolyticus*, isolated from aquaculture shrimp in China revealed that ICEs are not the major transmission facilitators of resistance to antimicrobial agents or heavy metals in Asia (He et al.). In addition to ICE, “Heteroresistance” is one of the mostly investigated aspects that are a characteristic of most MDR pathogens. One important issue is the absence of a universal guideline that clinicians could apply to treat heteroresistant *Helicobacter pylori* in clinical settings. Therefore, increased knowledge of gastroenterologists about the presence of the heteroresistance phenomenon is highly essential. This should be accompanied with accurate breakpoint measurements in order to have a firm statement of heteroresistance among various *H. pylori* isolates (Rizvanov et al.).

To further understand the resistance mechanisms to antimicrobial agents in Gram-negative MDR strains, several studies documented the molecular and physiological characterization of fluoroquinolone resistance and the mechanisms of adaptive resistance to aminoglycosides in *Salmonella enteritidis* and *Escherichia coli*, respectively. In *S. enteritidis*, two high-frequency mutations can take place in

the *gyrA* gene, leading to amino acid substitutions S83Y, S83F, and D87G. These mutations induce augmented resistance to fluoroquinolones as they upregulate the expression levels of the efflux pump associated genes *ramA*, *acrB*, and *tolC*, and downregulate the relative expression levels of the porin gene *ompF* and thus, lead to high levels of AMR (Vidovic et al.). In *E. coli*, *yhjX* encodes a putative transporter, known to be the only target of the YpdA/YpdB two-component system, which in turn is mainly induced by pyruvate availability. *yhjX* deletion improves the growth of *E. coli* in a medium containing sub-inhibitory concentrations of gentamicin. With time, *yhjX* mediates the adaptive resistance to aminoglycosides (Zhou et al.).

Overcoming AMR, inhibiting biofilm formation, and the inhibition of diarrhea and death caused by pathogenic *E. coli* in newborn piglets were documented in two research articles. In the former, the spore forming, rod-shaped, Gram-positive *Bacillus subtilis* was used as it is known to be a safe and a reliable probiotic in humans and animals. The isolation of *B. subtilis*, named WS-1, from healthy pigs inhibits the growth of pathogenic *E. coli* *in vitro* and protects the small intestine from injuries caused by an *E. coli* infection (Du et al.). In the latter study, seven gene-specific knockouts were produced by recombination in ocular *E. coli*. The mutations targeted three transmembrane genes *ytfR*, *mdtO*, and *tolA*, *ryfA* coding for non-coding RNA and three metabolic genes *mhpA*, *mhpB*, and *bdcR*. Interestingly, biofilm synthesis was inhibited in five mutants ($\Delta bdcR$, $\Delta mhpA$, $\Delta mhpB$, $\Delta ryfA$, and $\Delta tolA$) with no impact on their susceptibility profiles against a panel of antimicrobial agents such as ceftazidime, cefuroxime, ciprofloxacin, gentamicin, cefotaxime, sulfamethoxazole, and imipenem. Indeed, this was the first study to demonstrate the role of metabolic genes in biofilm formation (Ranjith et al.).

Although AMR genes can remain for decades in the environment even in an antimicrobial-deprived condition, reducing the use of antimicrobials is a key step to decrease AMR in livestock. Fecal complex microbial communities can out-compete bacteria harboring AMR genes. Interestingly, it was shown that changing the microbial surrounding can inhibit the transmission of AMR genes from one generation to another. For example, the proportion of resistant *Enterobacteria* declined from 93 to 9% accompanied with reduced prevalence of eight AMR genes upon testing different fecal complex microbial communities in rabbits (Achard et al.). In a similar study that focused on the effect of perinatal tulathromycin (TUL) metaphylaxis on the development of fecal microbiota and its correlation with AMR in pre-weaned piglets, it was shown that a total of 127 AMR genes linked to 19 different classes of antimicrobial agents are present. However, these results highlighted that perinatal TUL metaphylaxis has no essential impacts on fecal microbiota structure and the prevalence of AMR genes (Zeineldin et al.). These two studies suggest that different fecal microbial communities impact sensitivity to different classes of antimicrobial agents in addition to the abundance of AMR genes.

In the context of applying a combinatorial therapeutic approach for potential treatment options against MDR Gram-positive and Gram-negative bacteria and the discovery of novel antimicrobial agents and/or targets, the Research Topic consists

of 12 manuscripts from well-recognized research institutes and universities.

With respect to Gram-positive bacteria, *in vitro*, *ex vivo*, and *in vivo* studies to assess the efficacy of the antimicrobial peptide DPK-060 revealed promising results in treating *S. aureus* topical infections. DPK-60 significantly decreased bacterial counts and bacterial survival rates, highlighting DPK-60 as a potential safe drug pending further clinical trials (Håkansson et al.). In addition to DPK-60, the synthetic retinoid compound CD437 was shown to have strong bactericidal effects on *Enterococcus faecalis*, anti-biofilm effects on *Staphylococcus*, and reduced biofilm synthesis in *Pseudomonas aeruginosa*. This study was performed in China and interestingly, it highlighted synergistic effects when CD437 was combined with gentamicin (Tan et al.). This proves the effectiveness of combination therapies to further enhance the potency of specific drugs. In a similar study, Balsacone C was shown to inhibit MRSA isolates without inducing resistance. Balsacone C is a new dihydrochalcone isolated from *Populus balsamifera* that was shown to have strong inhibitory effects on *S. aureus*. Upon *in vitro* assessment, Balsacone C induced sensitivity on a panel of antimicrobial agents such as penicillin, amoxicillin/clavulanic acid, ciprofloxacin, moxifloxacin, levofloxacin, clindamycin, erythromycin, and cefoxitin. In addition, microscopic analysis revealed that Balsacone C can induce changes in the bacterial cell membrane and thus, demonstrating the mode of action of Balsacone C (Côté et al.).

In relation to Gram-negative bacteria, a study from China highlighted the usage of anti-outer membrane vesicle antibodies to increase antimicrobial sensitivity of Pan-Drug Resistant (PDR) *Acinetobacter baumannii*. Mice were immunized with *A. baumannii* outer membrane vesicles (AbOMVs) to produce high IgG levels. The produced antibodies were then injected in mice infected with *A. baumannii*. Interestingly, this process enhanced the susceptibility to quinolone antimicrobial agents, improved the survival rate of mice, and decreased bacterial load in organs. This sheds light onto a novel approach to treat PDR *A. baumannii* (Huang et al.). In a different study performed in Australia, the antimicrobial activity of Robenidine alone or in combination with ethylenediaminetetraacetic acid (EDTA) or polymyxin B nonapeptide (PMBN) against Gram-negative pathogens was assessed *in vitro* using the Broth Microdilution (BMD) assay and combinatorial tests such as the checkerboard method. Interestingly, the assessment of robenidine alone revealed bactericidal effects against *A. baumannii* and *Acinetobacter calcoaceticus*. In combination with either EDTA or PMBN, robenidine showed antimicrobial activity against *E. coli*, *Klebsiella pneumoniae*, and *P. aeruginosa* with no anti-biofilm effects. This study reveals for the first time the impact of the combination of robenidine with EDTA or PMBN for potential clinical application (Khazandi et al.).

Bacterial persister cells are unique cells that exhibit phenotypic modifications and are known to tolerate the effect of antimicrobial agents in a transient manner and play important roles in the emergence of AMR and chronic infections.

The formation of persister cells is not well-established. In a study from China, it was shown that hypoionic shock facilitates aminoglycoside killing of both nutrient shift- and starvation-induced bacterial persister cells in *E. coli*, but not *S. aureus*. Hypoionic shock facilitates aminoglycoside uptake and this study sheds light on the importance of further investigating persister cells eradication and aminoglycoside use (Chen et al.).

Candida albicans resistance to antifungal agents has been well-documented in the past years, which raised concerns and challenges in clinical therapeutic treatments. It was shown that N-butylphthalide extracted from *Apium graveolens* has potential antifungal activity and anti-biofilm effects against *C. albicans*. Interestingly, upon the combination of N-butylphthalide with fluconazole, further synergistic effects were observed. Additional experiments from this study reveal that the documented synergistic effects are due to increased cell uptake and decreased efflux pumping (Gong et al.).

Carbapenems are considered among the last-resort antimicrobial agents; however Carbapenem-resistant Gram-negative bacteria became major concerns in hospitals. β -Lactamases encoding genes such as KPC, NDM, and OXA were shown to encode for carbapenem resistance as well. A new therapeutic strategy to combat Carbapenem-resistant Gram-negative bacteria involves the usage of carbapenem/ β -lactamase inhibitor (β LI) combinations. Combining meropenem, imipenem, and ertapenem with the corresponding β LI restored *A. baumannii*'s sensitivity *in vitro* and increased survival rates *in vivo* when meropenem was combined with Avibactam or Relebactam. This study highlights carbapenem/ β LI combinations as a new option for antimicrobial combination therapies (El Hafi et al.). In a similar study, ceftazidime/avibactam enhanced the antibacterial potency of Polymyxin B against Polymyxin B heteroresistant KPC-2-Producing *K. pneumoniae* and inhibited the production of resistant bacteria *in vitro*. This study also suggested that the combination of polymyxin B and the ceftazidime/avibactam could be considered for treating heteroresistant bacteria (Ma et al.).

With respect to virulence in *K. pneumoniae*, the World Health Organization (WHO) has listed this bacterium as one of the major pathogens in immediate need for novel antimicrobial agents due to the wide spectrum of AMR. This was mainly highlighted in a review that focused on the challenges and concerns in establishing a universal *Klebsiella* vaccine and a novel drug due to the high frequency of mutations this pathogen acquires. This is represented via the various modifications *Klebsiella* strains can adopt at the level of the extracellular polysaccharides, such as lipopolysaccharides, capsular polysaccharides, and exopolysaccharides (Patro and Rathinavelan). In a different study focusing on MDR *K. pneumoniae* resistant to sulfonamides and β -lactams, the potency of commercially and clinically available sulfonamides animal feeds were examined to select for a model strain upon measuring the recovery rate in the presence of AMR markers *sul1* (sulfonamide) and *blaKPC-3* (meropenem). This research study highlights that in certain conditions the use of non-essential antimicrobial agents can result in "co-selection" mechanisms and the emergence of

resistance to essential antimicrobial agents, which might have direct consequences on the human health on the long-term (Brown et al.).

To predict clinical efficacy of novel antimicrobial agents, the VetCAST (the veterinary subcommittee of EUCAST) presents a semi-mechanistic time-kill curve (TKC) assay to assess drug potencies. For example, florfenicol, a long acting veterinary antimicrobial agent used against calf pneumonia organisms (*Pasteurella multocida* and *Mannheimia haemolytica*) was assessed in a collaborative study between France and US. It was shown that the semi-mechanistic approach provides precise estimation of bacterial growth, counts, and pharmacodynamic variables. Such studies are in high need nowadays in order to check whether novel antimicrobial agents are of significant impact to combat AMR (Pelligand et al.).

In conclusion, the published manuscripts included in this Research Topic help shed light on the growing problems of AMR, bacterial pathogenesis and virulence, in addition to understanding bacterial resistance, the utilization of combination

therapy for potential therapeutic options, and the discovery of novel antimicrobial agents and/or targets in Gram-positive and Gram-negative MDR strains.

AUTHOR CONTRIBUTIONS

GM drafted and edited the manuscript. AA edited the manuscript. WB assembled all needed information and contributed to drafting the manuscript.

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Selection of Multidrug-Resistant Bacteria in Medicated Animal Feeds

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Exposure to antimicrobial resistant (AMR) bacteria is a major public health issue which may, in part, have roots in food production practices that are conducive to the selection of AMR bacteria ultimately impacting the human microbiome through food consumption. Of particular concern is the prophylactic use of antibiotics in animal husbandry, such as the medication of feeds with sulfonamides and other antibiotics not considered clinically relevant, but which may nonetheless co-select for multi-drug resistant (MDR) bacteria harboring resistance to medically important antibiotics. Using a MDR *Klebsiella pneumoniae* strain exhibiting resistance to sulfonamides and beta-lactams (including carbapenem) as a model, we examined the ability of non-medicated and commercially medicated (sulfonamide) animal feeds to select for the model strain when inoculated at low levels by measuring its recovery along with key AMR markers, *sul1* (sulfonamide) and *blaKPC-3* (meropenem), under different incubation conditions. When non-medicated feeds were supplemented with defined amounts of sulfadiazine the model strain was significantly enriched after incubation in Mueller Hinton Broth at 37°C overnight, or in same at room temperature for a week, with consistent detection of both the *sul1* and *blaKPC-3* markers as determined by polymerase chain reaction (PCR) techniques to screen colony isolates recovered on plating media. Significant recoveries of the inoculated strain and the *sul1* and *blaKPC-3* markers were observed with one of three commercially medicated (sulfamethazine) feeds tested under various incubation conditions. These results demonstrate that under certain conditions the prophylactic use of so-called non-priority antibiotics in feeds can potentially lead to co-selection of environmental AMR bacteria with resistance to medically important antibiotics, which may have far-reaching implications for human health.

Keywords: antibiotic, sulfonamide, carbapenem, resistance, selection, cross-resistance, medicated feeds

INTRODUCTION

The occurrence of food-borne bacteria with anti-microbial resistance characteristics (e.g., resistance to therapeutic antibiotics) is widely regarded as a serious public health threat as foods constitute a primary niche for human exposure to environmental microbiota. Outbreaks of foodborne illness caused by antimicrobial resistant (AMR) bacteria are becoming more common, for example, multi-drug resistant (MDR) strains of *Klebsiella pneumoniae* producing extended-spectrum β -lactamases were implicated in foodborne outbreaks in Spain (Calbo et al., 2011), and a recent outbreak of salmonellosis in the US was linked to chicken contaminated with an MDR strain of *Salmonella* Heidelberg (Gieraltowski et al., 2016). The question of how pathogenic bacteria acquire antibiotic resistance is the subject of intense research, and one possibility is that commensal bacteria

occurring in food production environments may serve as a reservoir for AMR genes residing on mobile genetic elements which can be passed on to human pathogens (Economou and Gousia, 2015).

In North America it has been common practice to supplement livestock feeds with antibiotics for growth-promotion and prophylaxis (disease prevention). There is some evidence indicating that this practice may select for the acquisition of AMR traits and their transfer to the microbiota of food production animals such as cattle, chickens and pigs (Economou and Gousia, 2015; Jahanbakhsh et al., 2015; Agga et al., 2016), and to human pathogenic bacteria (Marshall and Levy, 2011; Ter Kuile et al., 2016). To minimize the risk of fostering the development of AMR to medically important antibiotics the tendency has been to use those considered lower priority for clinical applications, for example, sulfonamides and tetracyclines. As a precautionary measure in 2006 the European Union banned the labeling of antibiotics as growth promotants in feeds (European Union, 2005; Levy, 2014), and Canada followed suit in 2017 (Mehrotra and Ireland, 2017). However, there remains considerable scope for the use of antibiotics in feeds as prophylactic agents to treat or prevent infections in food production animals (FDA, 2013). One potential pitfall of using non-priority antibiotics prophylactically is the fact that some environmental bacteria exhibit MDR traits due to carriage of multiple resistance genes on plasmids or within a combination of plasmids and chromosomal loci (Chakraborty, 2017). For MDR bacteria application of selective pressure for one trait may result in co-selection of other resistance genes harbored in a single cell. In the present context, co-selection occurs when an MDR bacterial population is expanded on exposure to a single antibiotic for which it carries a resistance gene, resulting in a concomitant increase of any other AMR genes harbored within the same cells.

We were interested in studying co-selection of a medically important resistance trait in an environmental MDR bacterial species capable of entering the food production chain (e.g., via animal feeds), under selection conditions involving the presence of a typical antibiotic used in commercially medicated feeds. For this purpose, we designated an MDR *K. pneumoniae* isolate recovered from raw sewage influent as a model for a naturally occurring environmental bacterium with the potential to serve as a reservoir for the transfer of AMR genes to the food animal microbiome. *K. pneumoniae* is considered an important source of AMR genes that can be readily transferred to other bacteria, including commensals and pathogens found in food production environments (Berendonk et al., 2015). The particular model strain chosen was determined to possess genes specifying resistance to sulfonamides, commonly used in feeds, as well as medically important beta-lactams, including meropenem, which is a member of the high priority carbapenem antibiotic class. A key objective of this study was to determine if this strain could be enriched after incubation under different conditions in animal feeds containing sulfadiazine, and also, whether the enriched bacteria carried both sulfonamide and meropenem resistance genes.

MATERIALS AND METHODS

Bacterial Strains

Bacterial strains were routinely grown on MacConkey (MAC) agar (BD Difco Inc., Belgium) at 37°C for 16–18 h. Suspensions for inoculation were prepared by growth in 10 mL Mueller Hinton Broth (MHB) (Oxoid Ltd., United States) at 37°C for 16–20 h, and counted by plating serial dilutions on nutrient agar (NA) (Oxoid Ltd., Basingstoke, United Kingdom) plates which were incubated for 16–20 h at 37°C. Bacterial isolates recovered from feed samples were identified using a VTEK 2 GN ID Card (bioMérieux, Canada) following the manufacturer's instructions.

Two *K. pneumoniae* strains were used for feed inoculation experiments. One strain (OLC-1237) was from the culture collection of the Ottawa Laboratory Carling (Canadian Food Inspection Agency) and determined to be susceptible to sulfonamides on the basis of phenotypic and genomic analyses (see below); whereas, another strain, determined to be MDR (including resistance to sulfonamides and carbapenem) on the basis of genomic and phenotypic analyses (Table 1), was isolated from an Ottawa, Ontario, waste water treatment plant using the method described by Zurfluh et al. (2013), and designated OLC-2685. Briefly, 250 ml of influent sample was concentrated by filtration through 0.80 and 0.22 µm S-Pak membrane filters (Millipore, France). Filters were aseptically transferred to a sterile stomacher bag containing 25 ml modified tryptone soya broth (mTSB) (Oxoid). Filters were rinsed by manually massaging stomacher bags for 5 min to release collected bacterial cells. An aliquot from each rinse was serially diluted in sterile phosphate buffered saline (PBS), and dilutions were plated in triplicate on MacConkey agar (Sigma-Aldrich, St. Louis, MO, United States) containing 4 µg/ml meropenem (MACMER) (USP, United States) to select for carbapenem resistant Gram-negative bacteria, and incubated at 37°C for 16–20 h. The *K. pneumoniae* isolate selected was identified by whole genome sequence analysis and subjected to AMR gene characterization (below). Antimicrobial susceptibility testing of the strains was determined using the Sensititre CMV4AGNF susceptibility plate (Thermo Fisher Scientific, United States) following the manufacturer's instructions.

TABLE 1 | ResFinder analyses of *K. pneumoniae* strains used in this study.

Antimicrobial	OLC-1237	OLC-2685
β-Lactams	<i>BlaSHV-11-like</i>	<i>blaCARB-2</i> , <i>blaKPC-3</i> , <i>blaOXA-9</i> , <i>blaSHV-11</i> , <i>blaTEM-1A-like</i>
Aminoglycosides		<i>aac(6')-Ib-like</i> , <i>aadA1</i> , <i>aadA2</i> , <i>aph(3')-Ia</i> , <i>strA-like</i> , <i>strB-like</i>
Sulfonamides		<i>sul1</i>
Quinolones	<i>OqxA-like</i> , <i>oqxB</i>	<i>oqxA</i> , <i>oqxB</i>
Fosfomycin	<i>fosA-like</i>	<i>fosA-like</i>
Macrolides		<i>mph(A)</i> , <i>mph(E)</i> , <i>msr(E)</i>
Trimethoprim		<i>dfrA18-like</i>
Phenicol		<i>cmlA1-like</i>
Fluoroquinolones		<i>aac(6')Ib-cr-like</i>

Antimicrobial susceptibility testing of bacterial strains was also performed using the Sensititre CMV4AGNF susceptibility plate (Thermo Fisher Scientific, United States), as well as VITEK 2 AST-GN98 and –N208 cards (bioMérieux), following the manufacturers' instructions.

Whole Genome Sequence Analysis

Whole genome sequence analysis of *K. pneumoniae* strains OLC-1237 and OLC-2685 was performed to determine the presence of AMR genes and confirm the bacterial species identity. Genomic DNA was extracted from bacteria grown in MHB (Oxoid) at 37°C for 16–20 h using the Promega Maxwell 16 Cell DNA purification kit (Promega, United States). Sequencing libraries were constructed using the Nextera XT DNA sample preparation kit (Illumina, Inc., United States) and paired-end sequencing was performed on the Illumina MiSeq platform using 600 cycle MiSeq reagent kits (v3) with 6% PhiX control. Quality of raw sequencing reads was assessed with FastQC version 0.11.4 (Andrews, 2010), quality trimmed with bbdduk version 37.66 (Bushnell, 2016), and error corrected using BayesHammer (Nikolenko et al., 2013) included with the SPAdes genome assembler version 3.7.1 (Nurk et al., 2013). Contigs were assembled from the trimmed and error-corrected reads using SPAdes version 3.7.1 (Nurk et al., 2013) with the “careful” setting enabled. Assembly quality was assessed with qualimap version 2.2 (Okonechnikov et al., 2015), and quast version 2.3 (Gurevich et al., 2013). MASH version 2.0 (Ondov et al., 2016), was used to determine the genera of the isolates. Core genomes, plasmid and prophage complement, vector contamination, in addition to 16S sequencing, MLST and rMLST profiles, as well as the presence of markers associated with virulence, and antimicrobial resistance were calculated using custom Python scripts¹. AMR gene detection was additionally conducted using the Center for Genomic Epidemiology ResFinder webtool (v2.1²) with the default settings for gene identity threshold (90 %) and minimum length (60%) (Zankari et al., 2012). Plasmid maps were generated using PlasMapper (Dong et al., 2004). The raw sequence data for strains OLC-1237 and OLC-2685 can be downloaded from the Sequence Read Archive using accessions SRR7788649 and SRR7796513, respectively.

Animal Feeds and Sulfonamide Residue Testing

A variety of feeds obtained through Canadian Food Inspection Agency testing programs and analyzed for sulfonamide content were used for this study. Feed OTT-FE-2017-0819 was a hog grower pellet with no detectable antibiotic content and determined to contain 10⁵ cfu/g total background microbiota. OTT-FE-2016-0651 was rolled corn containing 0.76 mg/kg sulfamethazine and determined to contain 9 × 10⁴ cfu/g total background microbiota. OTT-FE-2017-0006 and OTT-FE-2017-0007 were both pig starter meals containing 110 mg/kg sulfamethazine, and determined to contain 6 × 10⁴ and 9 × 10⁴ cfu/g total background microbiota, respectively.

¹<https://github.com/OLC-Bioinformatics/COWBAT/tree/v0.1.5>

²<https://cge.cbs.dtu.dk/services/ResFinder-2.1/>, last [accessed 16 August, 2018]

All feeds were provided in ground form. The presence of antibiotics and their concentrations were determined by liquid chromatography with post-column derivatization as previously described (Smallidge and Albert, 2000). Background microbiota counts were determined by plating serial dilutions of feed-in-broth suspension (below) on NA and incubating for 16–20 h at 37°C.

Enrichment of *Klebsiella pneumoniae* in Non-medicated Feed Mixed With Defined Levels Sulfadiazine

Ten grams sub-samples of a non-medicated feed (OTT-FE-2016-0819) were combined with 90 mL MHB to which defined levels (0, 0.5, or 2 mg/mL) of sulfadiazine (Sigma-Aldrich, St. Louis, Mo, United States) were added. The feed suspensions were then inoculated with 0 or 149 cfu of OLC-1237 (sensitive strain) or 150 cfu of OLC-2685 (resistant strain) and mixed. Two sets of samples were prepared in this manner, and one was incubated at 35°C for 16–20 h, while the other was left in the dark at room temperature (22°C) for a period of 7 days. After incubation decimal dilutions of the suspensions were plated on regular MAC agar, and incubated at 37°C for 16–20 h, followed by assay of the colonies (minimum 30 colonies sampled per plate) using the PCR methods described below.

Enrichment of MDR *K. pneumoniae* in Commercially Medicated Feeds

Feed samples commercially medicated with sulfadiazine (OTT-FE-2016-0651, OTT-FE-2017-0006 or OTT-FE-2017-0007) were combined at a ratio of 10 g with 90 mL of MHB as above. The feed suspension was then inoculated with 0 or 140 cfu of OLC2685. Two sets of samples were prepared in this manner, and one was incubated at 37°C for 16–20 h, while the other was left in the dark at room temperature (22°C) for a period of 7 days. After incubation decimal dilutions of the suspensions were plated on MAC agar, and incubated at 37°C for 16–20 h, followed by assay of the colonies (minimum 30 colonies sampled per plate) using the PCR methods described below.

Enrichment of MDR *Klebsiella pneumoniae* in “Moist” Commercially Medicated Feed

One gram portions of a feed sample (OTT-FE-2016-0651) were placed in individual sterile test tubes then inoculated with 100 µL of phosphate-buffered saline (PBS) containing 0 or 113 cfu of OLC-2685. To create a moist environment in the feed samples, 1 mL of sterile PBS was added to each tube, which were then placed in a 37°C incubator with 58–62% humidity for 6 days. Samples were then resuspended in 5 mL of PBS and serial dilutions of each suspension were plated on MAC agar, and incubated at 37°C for 16–20 h, followed by assay of the colonies (minimum 30 colonies sampled per plate) using the PCR methods described below.

Polymerase Chain Reaction (PCR) Colony Analyses

Sulfonamide and Carbapenem Resistance Gene Assays

Bacterial colonies recovered from feeds (below) were assayed by separate PCR techniques targeting the key genes conferring resistance to sulfonamides (*Sul1*) (Frank et al., 2007) and carbapenem (*blaKPC-3*) (Bogaerts et al., 2013; **Table 2**), which were determined by ResFinder analysis to occur in the MDR *K. pneumoniae* strain (OLC-2685) used in this study (**Table 1**).

Bacterial colony lysates were prepared by suspension of a colony in 200 μ L of 1% (v/v) Triton X-100 (Sigma-Aldrich), followed by heating at 100°C for 10 min, and 2 μ L of lysate was combined with 25 μ L of reaction mixture containing 2.5 units HotStar Taq, 1.6 X HotStar PCR buffer (giving a final MgCl₂ concentration of 2.4 mM) (Qiagen Inc., Canada), 200 μ M of each dNTP, and 0.2 μ M of each (forward and reverse) primer (**Table 2**). The PCR was carried out in a Mastercycler gradient thermal cycler (Eppendorf, Germany) using the following conditions: an initial denaturation cycle at 94°C for 15 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s, and elongation at 72°C for 1 min 30 s, with an additional 2 min at 72°C following the last cycle. Amplicons were analyzed using the QIAxcel (Qiagen) system (following the manufacturer's instructions).

Strain-Specific PCR Primers

The recovery of inoculum strains used in the above feed enrichment studies was confirmed by assaying colonies using separate strain-specific PCR methods targeting OLC1237 and OLC2685. Strain-specific DNA sequences were determined *in silico* using whole genome sequence data from target strains OLC1237 and OLC2685 analyzed by the SigSeekr tool previously described for the identification of strain-specific signature sequences for PCR primer development (Knowles et al., 2015).

Primers for the strain-specific PCR methods (**Table 2**) were produced from the signature sequences identified by SigSeekr using the NCBI primer-BLAST tool (Ye et al., 2012). The specificity of primers for the respective target strains was verified by ascertaining that the expected PCR products under

optimized PCR conditions (above) were only obtained with the intended target strains, and not with 21 different non-target *K. pneumoniae* and 7 different *K. oxytoca* strains, as well as 12 different *Escherichia coli* and 4 *Hafnia alvei* strains from the OLC culture collection.

Statistical Analyses

Data were analyzed using the two-proportions z-test with R version 3.3.1 (R Core Team, 2013). PCR positive results were considered successes out of *n* CFU tested for each experiment. One-tailed z-tests were used to test whether the proportion of OLC-2685 positives was greater in higher concentrations of sulfadiazine (non-medicated feed experiments, **Tables 3, 4**) and to compare inoculated and uninoculated commercially medicated samples (**Tables 5–7**). A two sample two-tailed z-test was used to compare growth of OLC-2685 and OLC-1237 in the absence of sulfadiazine (non-medicated feed).

RESULTS AND DISCUSSION

Model *K. pneumoniae* Strains

Both strains used in this study were determined to harbor a variety of genes conferring resistance to antibiotics (**Table 1**). Strain OLC-1237, which is a legacy strain from the CFIA culture collection, exhibited the presence of a small number of resistance genes specifying resistance to β -lactams, quinolones and fosfomycin, but did not contain any known sulfonamide resistance genes, nor did it exhibit phenotypic resistance to sulfadiazine, and for this reason was selected to serve as a (non-resistant) control in these studies. The MDR strain OLC-2685, recently isolated from a waste treatment plant, constitutes a “real world” strain which has not been laboratory-adapted, and therefore is highly representative of an environmental bacterium which can potentially occur in animal feeds. This strain was found to carry many different resistance genes, key among which (for present purposes) were genes specifying resistance to sulfonamides (*sul1*) and meropenem (*blaKPC-3*) (**Table 1**), the resistance phenotypes for which were confirmed (not shown). Genomic analysis of strain OLC-2685 determined the presence of multiple copies of *sul1* (though their location on the chromosome or plasmids could not be ascertained) and that the *blaKPC-3* gene was located on the *Klebsiella* plasmid pRYCKPC3.1, which did not contain a *sul1* gene, indicating that the two genes were not genetically linked. We were also able to determine the presence of genomic signature sequences for the development of strain-specific PCR methods (**Table 2**) as an aid in determining their selection and recovery in inoculated feed samples. PCR methods targeting the *blaKPC-3* and *Sul1* genes found in OLC-2685 (**Table 2**) were used as a means of verifying their selection or co-selection during the enrichment process.

Enrichment in Non-medicated Feed

We were interested in studying the enrichment of model MDR and sulfonamide-sensitive *K. pneumoniae* strains in feeds having unselected background microbiota, and to which defined amounts of sulfadiazine could be added at the start of the

TABLE 2 | Primers used for PCR colony assays.

Target gene	Primer Sequence (5' to 3') ^a	Amplicon Size (bp)	Source
<i>blaKPC-3</i>	TCGCCGTCTAGTTCGTGCTCTTG	353	Bogaerts et al., 2013
	ACAGCTCCGCCACCGTCAT		
<i>Sul1</i>	CGGCGTGGGCTACCTGAACG	433	Frank et al., 2007
	GCCGATCGCGTGAAGTTCGG		
OLC-1237	AGGACGCAGACCACCTATCT	314	This study
	TACTGACGCGCGCAGATATT		
OLC-2685	ACGTTTACGCTACCGGAGGA	299	This study
	ATCTCCCGCCCAAGTGAATG		

^aDegenerate bases: Y, pyrimidine; R, purine.

TABLE 3 | Recovery of inoculated *K. pneumoniae* strains grown at 37°C overnight in a non-medicated feed.

Inoculum	Sulfadiazine (mg/mL)	PE cell density (cfu/mL) ^a	No. of colonies assayed ^b	PCR-positive colonies (%) ^c			
				OLC-2685	OLC-1237	<i>blaKPC-3</i>	<i>Sul1</i>
Uninoculated	0	10 ⁸	30	0	0	0	0
	0.5	10 ⁷	30	0	0	0	100
	2	10 ⁴	33	0	0	0	0
OLC-2685	0	10 ⁸	30	0	0	0	0
	0.5	10 ⁷	30	10	0	10	70
	2	10 ⁵	34	100	0	100	100
OLC-1237	0	10 ⁸	30	0	13	0	0
	0.5	10 ⁷	30	0	0	0	0
	2	10 ⁴	33	0	0	0	0

^aPE, post-enrichment (total density of bacteria obtained after enrichment). ^bNumber of colonies isolated on plating media tested by PCR. ^cPercentage of colonies giving PCR-positive results.

TABLE 4 | Recovery of inoculated *K. pneumoniae* strains grown at room temperature for a week in a non-medicated feed.

Inoculum	Sulfadiazine (mg/mL)	PE cell density (cfu/mL) ^a	No. of colonies assayed ^b	PCR-positive colonies (%) ^c			
				OLC-2685	OLC-1237	<i>blaKPC-3</i>	<i>Sul1</i>
Uninoculated	0	10 ⁷	30	0	0	0	0
	0.5	10 ⁶	30	0	0	0	17
	2	10 ⁴	33	0	0	0	30
OLC-2685	0	10 ⁷	30	3	0	3	3
	0.5	10 ⁶	30	20	0	20	33
	2	10 ⁴	34	79	0	79	91
OLC-1237	0	10 ⁷	30	0	7	0	0
	0.5	10 ⁶	30	0	0	0	53
	2	10 ⁴	34	0	0	0	23

^aPE, post-enrichment (total density of bacteria obtained after enrichment). ^bNumber of colonies isolated on plating media tested by PCR. ^cPercentage of colonies giving PCR-positive results.

TABLE 5 | Recovery of inoculated MDR *K. pneumoniae* strain grown at 37°C in commercially medicated feeds.

Feed	Inoculum	PE cell density (cfu/mL) ^a	No. of colonies assayed ^b	PCR-positive colonies (%) ^c		
				OLC-2685	<i>blaKPC-3</i>	<i>Sul1</i>
OTT-FE-2016-0651	None	10 ⁵	30	0	0	0
	OLC-2685	10 ⁷	34	100	100	100
OTT-FE-2017-0006	None	10 ⁸	30	0	0	0
	OLC-2685	10 ⁸	30	7	7	7
OTT-FE-2017-0007	None	10 ⁸	30	0	0	0
	OLC-2685	10 ⁸	30	0	0	0

^aPE, post-enrichment (total density of bacteria obtained after enrichment). ^bNumber of colonies isolated on plating media tested by PCR. ^cPercentage of colonies giving PCR-positive results.

selection process. For these experiments we used sulfadiazine as a typical representative of the sulfonamide family of antibiotics. The amounts of sulfadiazine used were selected to bracket the known minimum inhibitory concentration range for sulfonamide-resistant *K. pneumoniae* (typically >2 mg/mL) (Lin et al., 2016).

Portions of a non-medicated hog pellet feed sample (OTT-FE-2017-0819) were inoculated with a relatively low number of *K. pneumoniae* cells (approximately 2–3 orders

of magnitude lower than the level of background bacteria), either OLC-1237 (sulfadiazine-sensitive) or OLC-2685 (MDR, including sulfadiazine- and carbapenem-resistant), or left un-inoculated, then mixed with nutrient-rich MHB containing various levels of sulfadiazine. Samples incubated at 37°C to foster rapid growth exhibited no discernible enrichment of OLC-2685 in the absence of sulfadiazine when colonies isolated on plating media from the enrichment broth cultures were assayed by PCR for the strain-specific, *Sul1* and *blaKPC-3*

TABLE 6 | Recovery of inoculated MDR *K. pneumoniae* strain grown at room temperature for a week in commercially medicated feeds.

Feed	Inoculum	PE cell density (cfu/mL) ^a	No. of colonies assayed ^b	PCR-positive colonies (%) ^c		
				OLC-2685	<i>blaKPC-3</i>	<i>Sul1</i>
OTT-FE-2016-0651	None	10 ⁹	30	0	0	0
	OLC-2685	10 ⁹	34	18	18	18
OTT-FE-2017-0006	None	10 ⁹	30	0	0	0
	OLC-2685	10 ⁹	30	0	0	0
OTT-FE-2017-0007	None	10 ⁹	30	0	0	0
	OLC-2685	10 ⁹	34	0	0	0

^aPE, post-enrichment (total density of bacteria obtained after enrichment). ^bNumber of colonies isolated on plating media tested by PCR. ^cPercentage of colonies giving PCR-positive results.

TABLE 7 | Recovery of inoculated MDR *K. pneumoniae* strain incubated at 37°C for a week in a moist commercially medicated feed.

Inoculum	PE cell density (cfu/mL) ^a	No. of colonies assayed ^b	PCR-positive colonies (%) ^c		
			OLC-2685	<i>blaKPC-3</i>	<i>Sul1</i>
None	10 ⁷	30	0	0	0
OLC-2685	10 ⁷	36	100	100	100

^aPE, post-enrichment (total density of bacteria obtained after enrichment). ^bNumber of colonies isolated on plating media tested by PCR. ^cPercentage of colonies giving PCR-positive results.

resistance markers (Table 3). This may be due to overgrowth by background bacteria, as evidenced by the fact that the final enrichment broth cultures reached high cell densities matching those observed for the un-inoculated portion. On the other hand, when sulfadiazine was added to the enrichment broth at a low concentration (0.5 mg/mL), the inoculated strain OLC-1237 was not recovered, whereas it was possible to recover the inoculated OLC-2685 strain, albeit at the low rate of 10 % of all colonies isolated on plating media ($p = 0.1181$). In this instance, all of the colonies bearing the OLC-2685 genetic marker also bore the *Sul1* and *blaKPC-3* genes, as determined by PCR. The majority of the remainder of the colonies recovered on the plates were background bacteria bearing the *Sul1* gene, which presumably was present among the background bacteria initially and enabled their selection in the presence of sulfadiazine. Analysis of twelve of these isolates revealed their identity as *K. pneumoniae* and *Enterobacter cloacae*.

When the sample inoculated with OLC-2685 was grown in the presence of a high concentration (2 mg/mL) of sulfadiazine all of the bacteria recovered from the enrichment broth on plating media proved to be the inoculated strain, as evidenced by detection of the strain-specific marker and the two antibiotic resistance genes in all of the colonies ($p < 0.0001$, compared to both uninoculated samples and those inoculated with OLC-1237) (Table 3). The ability of the inoculated strain to outcompete the background bacteria, even those bearing the *Sul1* gene, may be attributable to the fact the OLC-2685 was found by genomic analysis to harbor multiple copies of this gene, which may have provided a survival advantage in the presence of a higher concentration of sulfadiazine.

The total cell densities achieved in the enrichment broth cultures were consistently lower (by one to four orders of magnitude) in the presence of sulfadiazine, indicating that the antibiotic effectively suppressed growth of the background bacteria. Those cells which did grow at the lower sulfadiazine concentration were found to harbor the *Sul1* gene (Table 3), demonstrating the enrichment effect of sulfadiazine when conditions were suitable for the growth of bacteria in the feed sample. No significant difference was observed for recovery of OLC-1237 and OLC-2685 in the absence of sulfadiazine in non-medicated feed ($p = 0.1205$ and 1 for 37°C and room temperature, respectively) (Tables 3, 4). The inability to recover strain OLC-1237 – which is of the same species as the MDR strain – in the presence of sulfadiazine (Table 3) further supports the notion that selective enrichment of the MDR *K. pneumoniae* strain was due to the presence of its sulfonamide resistance trait, and not necessarily because *K. pneumoniae* are more competitive. It should be noted that in every instance (even in the absence of an inoculated strain) colonies were recovered at all concentrations of sulfadiazine, and the lack of a detectable *Sul1* gene in these colonies suggests that they were either species with intrinsic resistance to sulfonamides or bearing alternative resistance factors.

The implications of these experimental results are two-fold: (1) the presence of sulfadiazine in a feed sample imposes selective pressure which favors the outgrowth of *K. pneumoniae* (and possibly other bacteria) possessing resistance associated with *Sul1*, even in the presence of high levels of background bacteria; and (2) selection on the basis of a single resistance trait causes co-selection of other traits present in the same cell, such as the gene specifying carbapenem resistance, even though the gene resides on a separate genetic element (plasmid).

Selection of one strain in a complex population may be influenced by growth dynamics of the broader population. Therefore, we examined the impact of slow growth by carrying out the enrichments under sub-optimal conditions, where the samples were left at room temperature for 7 days. Under these conditions, a similar pattern of selective enrichment of OLC-2685 was observed as with enrichment under fast growth conditions, with all colonies recovered in the presence of sulfadiazine bearing the strain-specific marker as well as the *Sul1* and *blaKPC-3* markers (Table 4), demonstrating the same selective enrichment and resistance gene co-selection phenomenon as

before (Table 3). Altered population growth dynamics may account for quantitative differences in the results obtained with the two growth conditions, as well as the differential recoveries of background bacteria bearing *Sul1*.

Enrichment in Commercially Medicated Feeds

The observation that the endogenous feed community contains bacteria with sulfonamide resistance suggests that feeds with long-term exposure to the antibiotic may develop altered populations which have adapted to the selective pressure, in other words, a significant sulfonamide-enriched fraction. To examine whether this might have an impact on the recovery of an incidental strain, such as OLC-2685 cells added to a sample, we studied its enrichment in three different commercially medicated feeds for which sulfonamide levels were determined using a standard analytical chemistry technique. Enrichment of the MDR model strain under fast growth conditions in the feed sample with the low level of sulfamethazine resulted in a high level (100 %) of recovery of the strain on plating media, as evidenced by the presence of the strain-specific, *Sul1* and *blaKPC-3* markers in all of the colonies assayed ($p < 0.0001$) (Table 5). However, its recovery in the feeds containing high levels of the antibiotic was significantly lower, with a recovery level of 7 % in one sample ($p = 0.236$) and no recovery in the other. When the samples were subjected to slow growth conditions (room temperature for 7 days), only the feed with a low level of sulfamethazine produced significant recovery of the inoculated model strain ($p < 0.05$) (Table 6), albeit at a lower rate (18 %) than observed in the other experiments.

Enrichment in a Commercially Medicated Feed Under Nutritionally Limited, Moist Conditions

The previous experiments were conducted under conditions where bacteria were enriched in a nutrient-rich broth, which may not be representative of “real world” conditions where feeds and their endogenous bacteria exist in a low-nutrient environment which is stressful to bacterial cells and possibly not conducive to vigorous growth necessary for selection and enrichment to occur. Strain OLC-2685 was inoculated into a small sample of commercially medicated feed (OTT-FE-2016-0651, which had been shown to enable enrichment of the MDR model strain) moistened by the addition of sterile saline solution, and then incubated in a high moisture incubator at 37°C for 6 days. Significant background microbial growth was evidenced by the attainment of moderately high cell densities in both the uninoculated and inoculated samples, with all colonies recovered on plating media from the inoculated sample having the strain-specific, *Sul1* and *blaKPC-3* markers, and none of the colonies recovered from the uninoculated sample having these markers ($p < 0.0001$) (Table 7). These results demonstrate that selection of sulfonamide-resistant bacteria can occur under simulated feed storage conditions of high temperature and

humidity that are propitious for microbial growth even in the absence of nutrient-rich media.

This study focused on obtaining preliminary evidence of co-selection of medically relevant antimicrobial resistances occurring in an MDR bacterial strain in medicated feeds. The inadvertent selection of resistance to medically important antibiotics such as carbapenem, even in common bacteria such as *K. pneumoniae*, may pose a significant public health concern since their presence in the food production chain could ultimately lead to exposure of the human microbiome to AMR bacteria which can transfer their resistance traits to pathogenic bacteria. We have effectively demonstrated that selection and enrichment of AMR bacteria can occur in feeds, and most significantly, even when feeds are medicated with antibiotics not used in human medicine co-selection of resistance to clinically significant antibiotics can occur for bacterial strains harboring multiple resistance traits.

Animal feeds are immensely variable in terms of their ingredient composition, background bacteria profiles, storage and handling conditions, and any or all of these factors may impact the extent to which selection and enrichment of AMR bacteria might occur. However, we observed significant recovery of the MDR model strain under different conditions even with a small sampling of randomly acquired feeds. Furthermore, the MDR *K. pneumoniae* model strain used in these studies is a raw sewage isolate and thus a representative denizen of the environmental microbial communities found in urban and agricultural regions of North America, and therefore highly likely to come into eventual contact with food production systems. These factors point to a high likelihood that enrichment of AMR bacteria and co-selection of clinically significant AMR traits are significant events which may contribute to the emergence of AMR bacteria to which humans are exposed through the food chain.

AUTHOR CONTRIBUTIONS

AC isolated the strains used in the study as well as helped with experiment planning. EB performed the recovery and bacterial screening experiments. CC helped with project planning. BB did project planning and wrote the manuscript.

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Negligible Impact of Perinatal Tulathromycin Metaphylaxis on the Developmental Dynamics of Fecal Microbiota and Their Accompanying Antimicrobial Resistome in Piglets

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While the antimicrobial resistance profiles of cultured pathogens have been characterized in swine, the fluctuations in antimicrobial resistance genes (ARGs) associated with the developing gastrointestinal microbiota have not been elucidated. The objective of this study was to assess the impact of perinatal tulathromycin (TUL) metaphylaxis on the developmental dynamics of fecal microbiota and their accompanying antimicrobial resistome in pre-weaned piglets. Sixteen litters were given one of two treatments [control group (CONT; saline 1cc IM) and TUL group (TUL; 2.5 mg/kg IM)] directly after birth. Deep fecal swabs were collected at day 0 (prior to treatment), and again at days 5 and 20 post treatment. Shotgun metagenomic sequencing was performed on the extracted DNA, and the fecal microbiota structure and abundance of ARGs were assessed. Collectively, the swine fecal microbiota and their accompanying ARGs were diverse and established soon after birth. Across all samples, a total of 127 ARGs related to 19 different classes of antibiotics were identified. The majority of identified ARGs were observed in both experimental groups and at all-time points. The magnitude and extent of differences in microbial composition and abundance of ARGs between the TUL and CONT groups were statistically insignificant. However, both fecal microbiota composition and ARGs abundance were changed significantly between different sampling days. In combination, these results indicate that the perinatal TUL metaphylaxis has no measurable benefits or detriment impacts on fecal microbiota structure and abundance of ARGs in pre-weaned piglets.

Keywords: antimicrobial, tulathromycin, microbiota, resistome, sequencing, piglets

INTRODUCTION

In swine production industry, antimicrobials are the most common prescribed drug primarily for treatment and prevention of diseases (Cromwell, 2002). The over use of existing antimicrobial results in perturbations of gut microbiota, promote the selection of antimicrobial-resistant microorganisms, and increase the abundance of various ARGs (Czaplewski et al., 2016; Hoelzer et al., 2017). Recently, there is widespread concern regarding the contribution of antimicrobial

use in livestock to the development of antimicrobial resistance in people (Founou et al., 2016; Connelly et al., 2018). To overcome the resistance problem, the livestock production system must optimize the use of antimicrobial treatment (Maron et al., 2013). The key step in this optimization process is to understand the mechanism and extent by which antimicrobial intervention affects the resident microbiota, and their accompanying ARGs (Allen et al., 2014). Additionally, the ability to link the changes in the developmental dynamics of resident microbiota to their accompanying antimicrobial resistome is crucial in managing and preventing this global health threat. Most of the studies that evaluated the effect of antimicrobial interventions on the emergence of antibiotic resistant bacteria have frequently focused on phenotypic resistance in a single class of organism using culture methods (McEwen and Fedorka-Cray, 2002; Thanner et al., 2016). The advancements in high-throughput sequencing techniques, have improved our understanding about the gastrointestinal tract bacterial populations, and helped the researchers to quantitatively assess the dissemination of ARGs in different environments (Zhao et al., 2017).

Tulathromycin (TUL) is bacteriostatic macrolide act by inhibiting the biosynthesis of essential bacterial proteins and stimulates the disassociation of ribosomal peptidyl-tRNA during translocation process (Mazzei et al., 1993; Schokker et al., 2014). On the basis of its favorable antimicrobial characteristics, TUL is utilized therapeutically in neonatal piglets for control and prevention of infectious diseases at a single dosage of 2.5 mg/kg. BW (Pyörälä et al., 2014). Disruption of gut microbiota establishment and their accompanying antimicrobial resistome as a result of antimicrobial administration during this critical phase of production may produce important implications for swine health later in life (Kelly et al., 2017). Early-life TUL intervention in neonatal piglets exhibited limited effect on gastrointestinal microbial diversity and composition directly after administration but had a long-lasting impacts at day 176 after administration (Schokker et al., 2014). Similarly, exploring the change of fecal microbiota of growing piglets in response to TUL administration revealed that the fecal microbiota structure exhibited a pronounced shift after single dose of treatment and had returned rapidly (within two weeks) to a distribution that closely resembled that observed on day 0 prior to treatment (Zeineldin et al., 2018). To fully understand the swine gastrointestinal microbial ecosystem during early life, it is important to understand the dynamics of gastrointestinal microbiota development and prevalence of ARGs in response to perinatal antimicrobial metaphylaxis. Consequently, the aim of this study was to investigate the short-term impact of perinatal TUL metaphylaxis on the developmental dynamics of fecal microbiota and their accompanying ARGs in neonatal piglets.

MATERIALS AND METHODS

Ethics Statement, Animals and Samples Collection

The present study was conducted in a commercial swine farm in the Midwestern United States with consent from the

facility owner. All procedures were carried out in agreement with principles and guidelines of the Institutional Animal Care and Use Committee of University of Illinois at Urbana-Champaign. The protocol was evaluated and approved by the Ethical Committee for Institutional Animal Use and Care of the University of Illinois at Urbana-Champaign. A total of 16 sows with their newborn piglets (220 piglets in total) were used in this study. Approximately five days before farrowing, the pregnant sows were transferred to the farrowing pens and kept there until the end of the experiment. Sows were given *ad libitum* water and fed a standard lactation diet via an automatic dry feeding system. No antimicrobials were administered to the sows before or after farrowing. Sows followed the normal farrowing procedures established by the farm and any piglet escaped this protocol was not enrolled in the study. Additionally, if more piglets were present than the dam milk glands, piglets were removed. All litters contained 12 to 14 piglets after this procedure. Directly after birth (< 6 h), litters were randomly assigned to one of two groups; CONT ($n = 8$ litters) and TUL group ($n = 8$ litters). In TUL group, a total of 108 piglets were treated with 2.5 mg TUL/kg IM (Draxxin[®], Zoetis US, Chicago Heights, IL, United States). In CONT group, a total of 112 piglets were treated with saline 1cc IM. The piglet's tails were docked, and 200 mg of Iron dextran was administered at three days of age. Males were surgically castrated at the same time according to the farm protocols. Daily physical examination was performed individually to evaluate the attitude and appetite of all piglets and their dams by farm staff. The piglets were individually identified with in litter. The weights of all piglets and mortality percent were recorded throughout the study. Individual deep fecal swabs (Pur-Wraps[®], Puritan Medical Products, Guilford, Maine) were collected immediately prior treatment (day 0), and again on days 5 and 20 post treatment (Supplementary Figure S1). The fecal swabs were kept in dry ice-chilled boxes, transported to the laboratory on the same day and stored at -80°C until further processing.

Fecal DNA Extraction and Shotgun Metagenomic Sequencing

Genomic DNA was extracted from subgroups of fecal swabs (4 piglets per sampling day in each group) and from negative control samples (sterile cotton swab and extraction kit reagent) using Power Fecal DNA Isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, United States) according to manufacturer's standard protocol (Zeineldin et al., 2017a,b). The fecal swabs were randomly selected from the piglets that remained healthy throughout the sucking period. For each sample, total DNA concentration and integrity were evaluated using a Nanodrop[™] spectrophotometer (NanoDrop Technologies, Rockland, DE, United States) at wavelengths of 260 and 280 nm, and agarose gel electrophoresis (Bio-Rad Laboratories, Inc., Hercules, CA, United States). Extracted DNA was immediately stored at -20°C and then shipped on dry ice for sequencing at the W. M. Keck Center for Comparative and Functional Genomics (University of Illinois at Urbana-Champaign, Urbana, IL, United States).

DNA libraries were constructed using the Nextera DNA Flex Library Preparation Kit (Illumina, Inc., San Diego, CA,

United States). Briefly, 100 ng of DNA were tagged, cleaned up with magnetic beads and amplified for 5 cycles of PCR using Illumina Enhanced PCR Mix and Nextera FS dual indexed primers. Amplified DNAs were cleaned, and size selected for fragments 250 to 750 bp in length, using a double-sided bead purification procedure. The final libraries were quantitated using Qubit High-Sensitivity DNA (Life Technologies, Grand Island, NY, United States) and the average size was determined on the AATI Fragment Analyzer (Advanced Analytics, Ames, IA, United States). Libraries were pooled evenly, and the pool was cleaned using a 1:1 ratio with AxyPrep Mag PCR Cleanup beads (Axygen, Inc., Union City, CA, United States), then evaluated again on AATI Fragment Analyzer (Advanced Analytics, Ames, IA, United States). The final pool was diluted to 5 nM concentration and further quantitated by qPCR (Bio-Rad Laboratories, Inc., CA, United States). The pool was then denatured and spiked with 4% non-indexed PhiX control library and loaded onto the MiSeq V3 flowcell at a concentration of 10 pM for cluster formation and sequencing. Finally, DNA libraries were sequenced from both ends of the molecules to a total read length of 250 nt from each end following manufacturer's guidelines (Illumina, Inc., San Diego, CA, United States).

Sequence Data Processing and Microbial Community Analysis

Raw sequence data files were de-multiplexed and converted to fastq files using Casava v.1.8.2 (Illumina, Inc. San Diego, CA, United States). Sequence reads quality were assessed using FastQC software (Andrews, 2010). Adaptor sequence and low-quality reads with Phred score <30 were trimmed from the raw sequence data using Trimmomatic software (Bolger et al., 2014). The trimmed sequence files were then uploaded to the Metagenome Rapid Annotation Using Subsystems Technology (MG-RAST) webserver to determine the taxonomic composition of fecal microbiota at the phylum, genus, and species levels, and to predict the metabolic functional gene profiles (Glass et al., 2010). The MG-RAST webserver utilizes a high-performance data-mining algorithm along with curated genome databases that rapidly disambiguates millions of short reads of a metagenomics sequence into discrete microorganisms engendering the identified sequences. In MG-RAST, sequence reads were subjected to additional quality control filtering, including dereplication (removal of sequences produced by sequencing artifacts), removal of host-specific species sequences, length filtering (removal of sequences with a length > 2 standard deviations from the mean), and ambiguous base filtering (removal of sequences with > 5 ambiguous base pairs). Normalization was performed using a log₂-based transformation [$\log_2(x + 1)$], followed by standardization within each sample and linear scaling across all samples (Gaeta et al., 2017). We used a nonredundant multisource protein annotation database (M5NR) as annotation source for microbial classification. Microbiota abundance was analyzed using a best-hit classification approach with a maximum *e* value of 1×10^{-5} , a minimum identity cutoff of 60%, and a minimum

alignment length cutoff of 15. We used SEED subsystem as the annotation source for predicted metabolic functional gene profiles. To be publicly available, the sequence reads were deposited in MG-RAST webserver under the following accession numbers: from mgm4779141.3 to mgm4779164.3.

Fecal microbiota alpha diversity indices were calculated within PAST version 3.13 using Chao 1, Shannon, Simpson and Evenness indices. Beta diversity was computed using principal component analysis (PCA) based on non-phylogenetic Bray–Curtis distance metrics implemented in MicrobiomeAnalyst (Dhariwal et al., 2017). The difference in overall microbial composition between the CONT and TUL groups was determined using non-parametric multivariate analysis of variance (PERMANOVA) with 9999 permutations and Bonferroni corrected *P* values in PAST version 3.13. The difference in fecal microbiota relative abundance and alpha diversity metrics between the two groups (CONT and TUL) at each time point (Day 0, 5, and 20) were analyzed using Mann–Whitney pairwise comparison test with sequential Bonferroni significance in PAST version 3.13. Significance difference was stated at *P* < 0.05. To further quantify the overall microbial composition similarities between the two groups at each time point, the relative abundance of fecal microbiota at genus level were assessed using the linear discriminant analysis effect size (LEfSe) pipeline using Galaxy¹ (Segata et al., 2011). The difference in overall predictive function gene profiles between the CONT and TUL groups were compared using STAMP (Statistical Analysis of Metagenomic Profiles) software (Parks et al., 2014). For two-groups analysis, two-sided Welch's *t*-test and Benjamini–Hochberg FDR correction were used, while for multiple-groups analysis, ANOVA with the Tukey–Kramer test and Benjamini–Hochberg correction were chosen. Differences were stated significant at *P* < 0.05. PCA and heatmap diagram were also performed using STAMP software.

Antimicrobial Resistance Genes Identification

To assess and quantify the relative abundance of the ARGs in our data, we used SRST2 pipeline (Inouye et al., 2014). The SRST2 pipeline was used to map the raw sequence reads and cluster the similar sequences against a database of preference, using CD-hit with an identity threshold of 80% (Clausen et al., 2016). For ARGs identification, we used antibiotic resistance gene database (ARG-ANNOT) that incorporated all sequences of known antibiotics resistance genes (Lopez-Rojas et al., 2013). Antimicrobial resistance genes alpha diversity metrics were computed using the Shannon index, Simpson's index, Chao1 richness estimate and Pielou's evenness index. The difference in the relative abundance and diversity of ARGs between the CONT and TUL groups at the different sampling days were analyzed using Mann–Whitney pairwise comparison test with sequential Bonferroni significance in PAST version 3.13. Additionally, two-sided Welch's *t*-test and Benjamini–Hochberg FDR correction were used to compare the overall difference in ARGs abundance between the CONT and TUL groups using

¹<https://huttenhower.sph.harvard.edu/galaxy/>

STAMP software (Parks et al., 2014). Differences were considered significant at $P < 0.05$. PCA and heatmap diagram were also performed using STAMP software. The overall difference in ARGs abundance between the CONT and TUL groups was determined using PERMANOVA with 9999 permutations and Bonferroni corrected P values in PAST version 3.13.

RESULTS

Impact of TUL Metaphylaxis on the Body Weight Gain and Overall Mortality Percent

There was no significant change in the average daily weight gain between day 0 and day 20 in the TUL group compared to CONT group (means $\pm$ SE; 4.61 ± 0.18 vs. 4.54 ± 0.26 , **Supplementary Figure S2A**). The TUL-treated piglets showed also non-significant changes in the overall mortality rate (day 0 to 20) compared to the CONT (means $\pm$ SE; 0.028 ± 0.005 vs. 0.021 ± 0.009 , **Supplementary Figure S2B**). Our results showed that the early-life TUL administration has no advantage in increasing the average daily weight gain in the neonatal piglets or reducing the piglet's mortality during the neonatal periods.

Shotgun Metagenomic Sequencing Summary

Across all fecal samples, shotgun metagenomic sequencing generated a total of 19,236,952 raw sequence reads (mean number of sequences per sample: 400,742.88; median: 394,675; range: 358,524–464,985). The average Phred quality score of raw sequence reads across all samples was 33.7 and only 1.01% of all reads were removed due to low quality. Using the criterion of MG-RAST taxonomic classification, 3,833,882 taxonomic hits were identified among all samples, all of which were taxonomically assigned according to RefSeq classification. Collectively, a total of 2,010,187 and 1,829,585 hits were identified in the CONT and TUL piglets, respectively.

Taxonomical Classification of the Fecal Microbiota

Across all samples, 29 different bacterial phyla, 586 genera, and 1468 species were detected using MG-RAST webserver. Collectively, the fecal microbiota composition at both phylum and genus level in the CONT and TUL piglets varied greatly according to the age. At the phylum level, *Proteobacteria* was the most predominant phylum at day 0, representing 62 and 70% of all bacterial populations in CONT and TUL-treated piglets, respectively. While at day 20, *Firmicutes* was the most abundant phylum, representing 52% and 60% of all bacterial populations in the CONT and TUL-treated piglets, respectively. Distribution of the most abundant bacterial phyla in both CONT and TUL groups at different sampling days are depicted in (**Figure 1**). When selectively comparing changes between the CONT and TUL-treated piglets, there was no significant change in bacterial phyla that averaged more than 1% of the relative abundance.

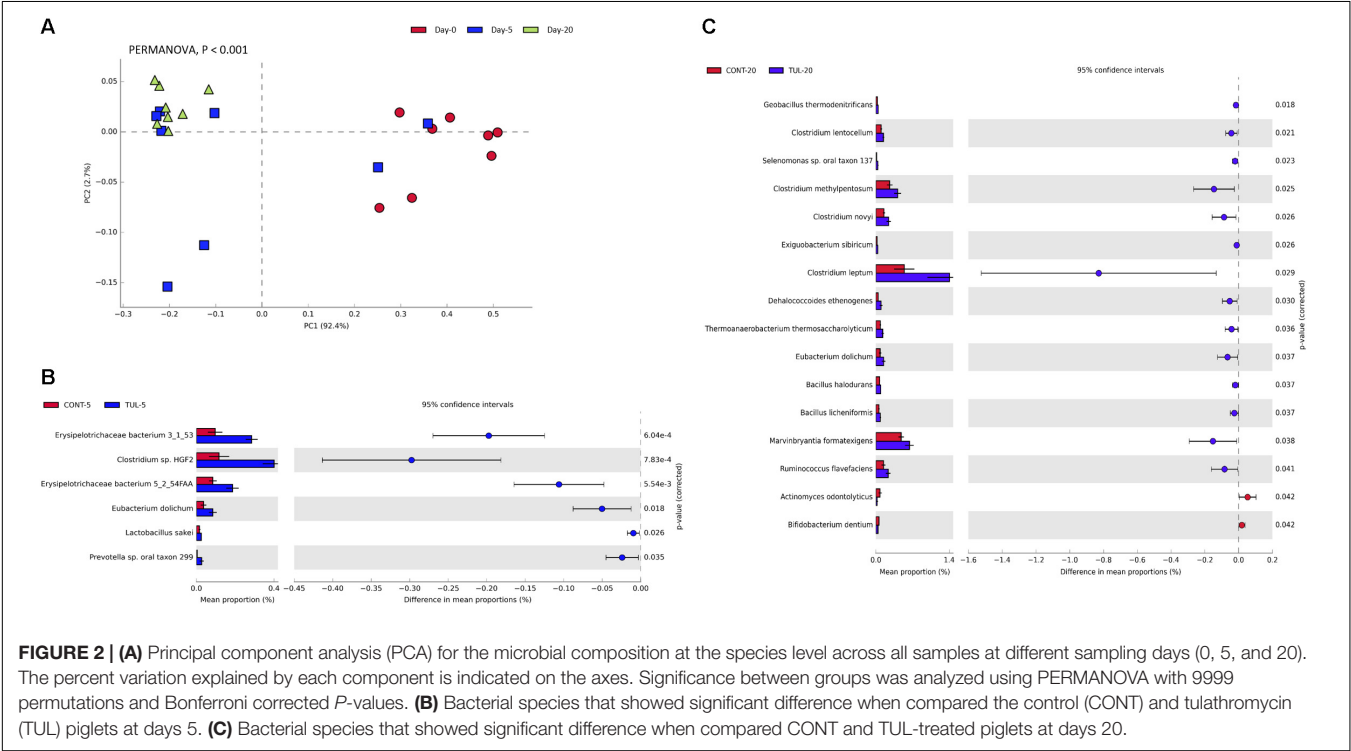
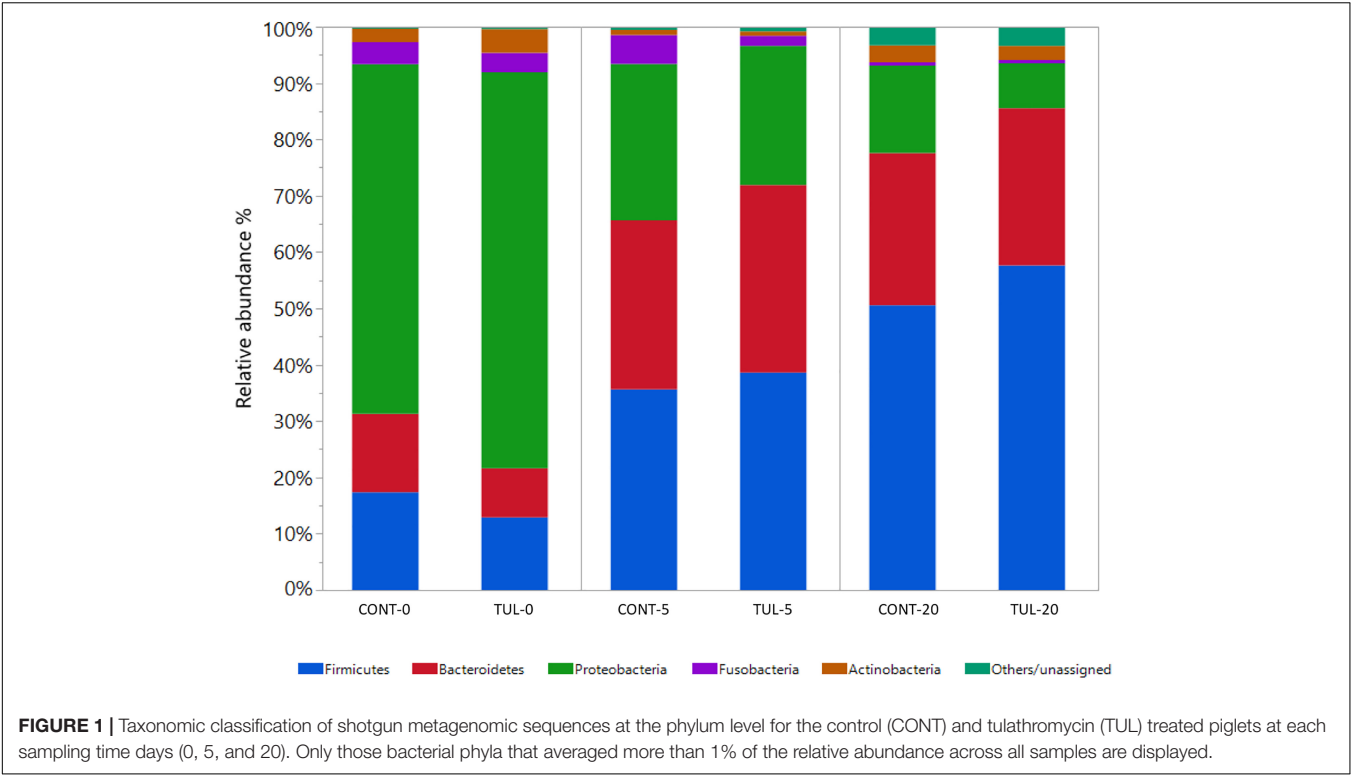
At the genus level, the predominant bacterial genera that averaged more than 1% across all samples at the baseline (day 0) was comprised of common fecal microbial genera including *Escherichia* (50.72%), *Bacteroides* (7.73%), *Clostridium* (7.03%), *Shigella* (5.61%), *Streptococcus* (2.18%), *Fusobacterium* (1.74%), *Salmonella* (1.31%), and *Lactobacillus* (1.01%). Distribution of the most abundant bacterial genera in both CONT and TUL groups at different sampling days are depicted in (**Supplementary Figure S3**). Even though there were no significant changes detected in bacterial genera that averaged more than 1% between the two groups, in-depth analysis at genera-level suggested that TUL treatment was associated with minor changes in the fecal microbiota of these young piglets. At the species level, the most predominant 100 microbial species across all samples are depicted in (**Supplementary Table S1**). Collectively, the microbial composition at species level in the CONT and TUL piglets varied greatly according to the age (**Figure 2A**). Additionally, the relative abundance of some bacterial species showed significant difference when compared to the CONT and TUL-treated piglets at days 5 and 20 (**Figures 2B,C**).

Based on LEfSe algorithm, the changes in the fecal microbiota structure caused by perinatal TUL intervention are limited to a particular group of microbial taxa (**Figure 3**). Compared to the CONT group, 3, 3 and 8 OTUs were identified as indicator taxa in the TUL-treated piglets at days 0, 5, and 20, respectively (**Figure 3**). At day 5, the TUL-treated piglets exhibited a high contribution of *Erysipelotrichaceae*, *Bacteroidetes*, and *Mucilaginibacter* taxa. While at day 20, *Ruminococcus*, *Ethanoligenens*, *Butyrivibrio*, *Lachnospiraceae*, *Dehalococcoides*, *Thermoanaerobacterium*, *Abiotrophia*, and *Cellulosilyticum* taxa were enriched in the TUL piglets.

We next investigated the effects of early life TUL metaphylaxis on the fecal microbiota diversity. Alpha diversity metrics showed non-significant changes between the CONT and TUL groups (**Figure 4**). However, the metagenomic analysis in both experimental groups revealed that the microbial diversity and richness indices were increased with the age (**Figure 4**). Beta diversity analysis also showed that the TUL-induced changes in the microbial community composition were not sufficient to cluster the microbial populations at days 0, 5, and 20 as shown by PCA of Bray–Curtis distance (**Figure 5**).

Effect of TUL Metaphylaxis on Microbial Functional Profiles

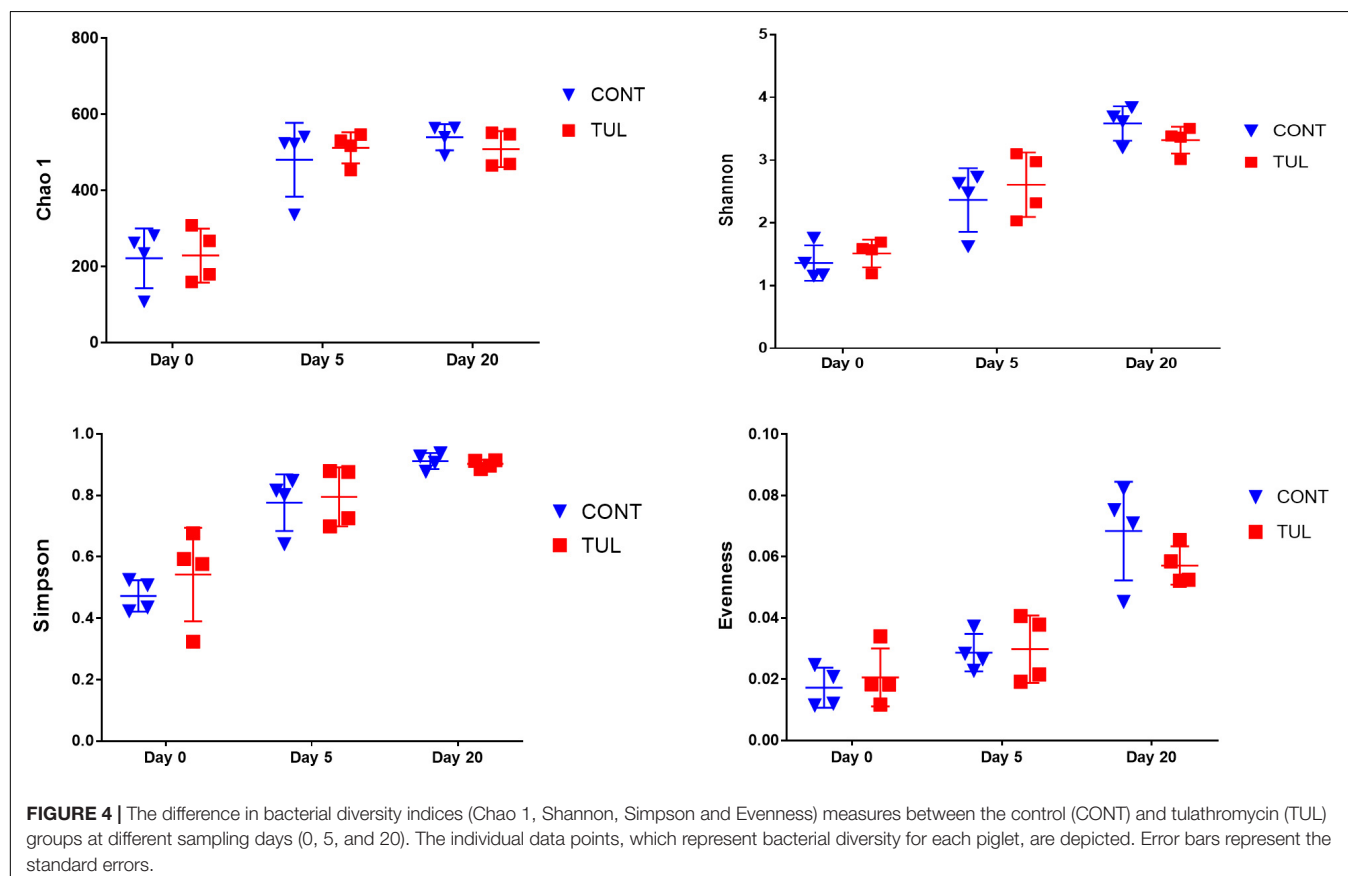
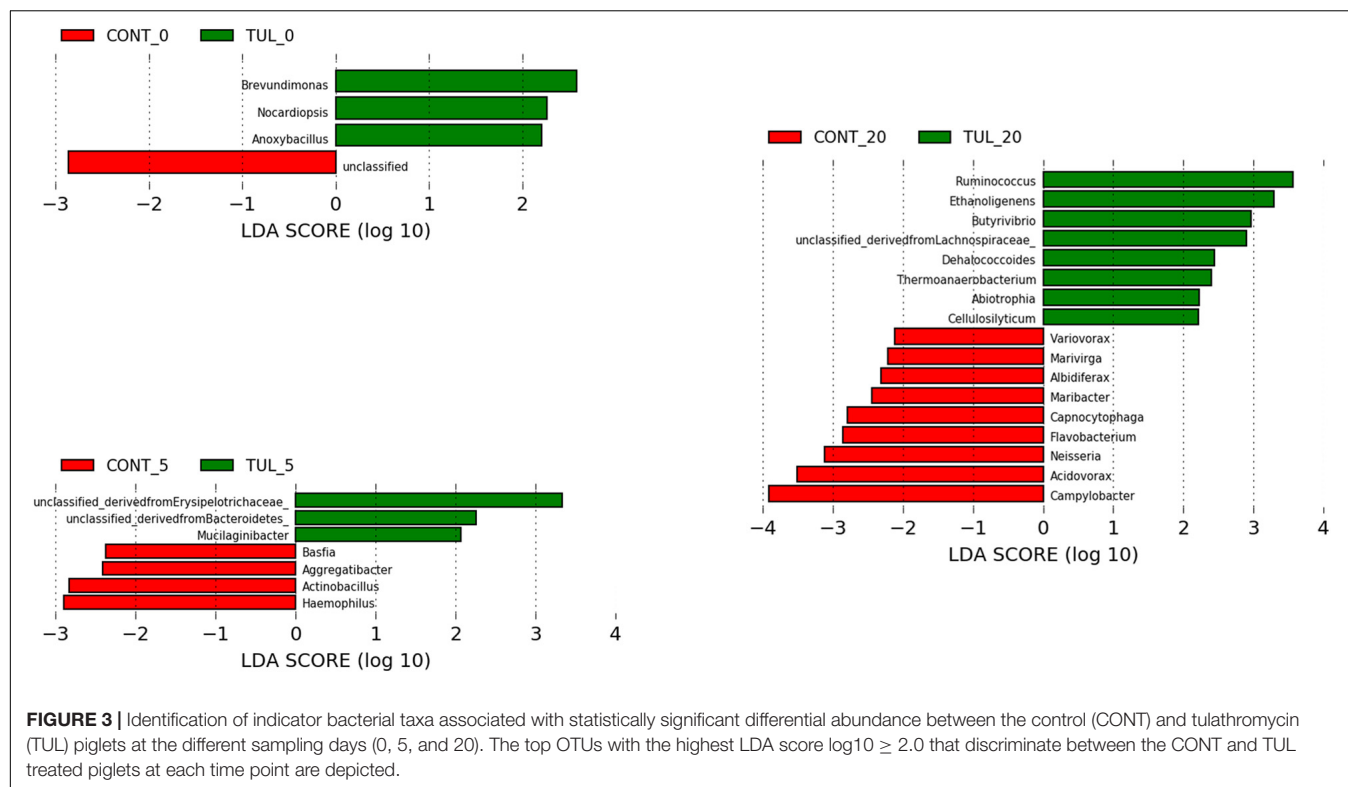
The relative abundance of the microbial functional profiles at level 2 KEGG pathway is depicted in (**Supplementary Figure S4A**). There was no significant difference in the overall metabolic functional capability at level 2 pathway between the CONT and TUL groups (**Supplementary Figures S4B,C**). However, the overall predicted functional profiles in both CONT and TUL were varied greatly according to the age (**Figure 6A**). Furthermore, the extended bar plot of the functional potential at level 3 KEGG pathways revealed significant difference in the relative abundance of some metabolic and antibiotic resistance functional genes between the CONT and TUL-treated piglets (**Figures 6B,C**).

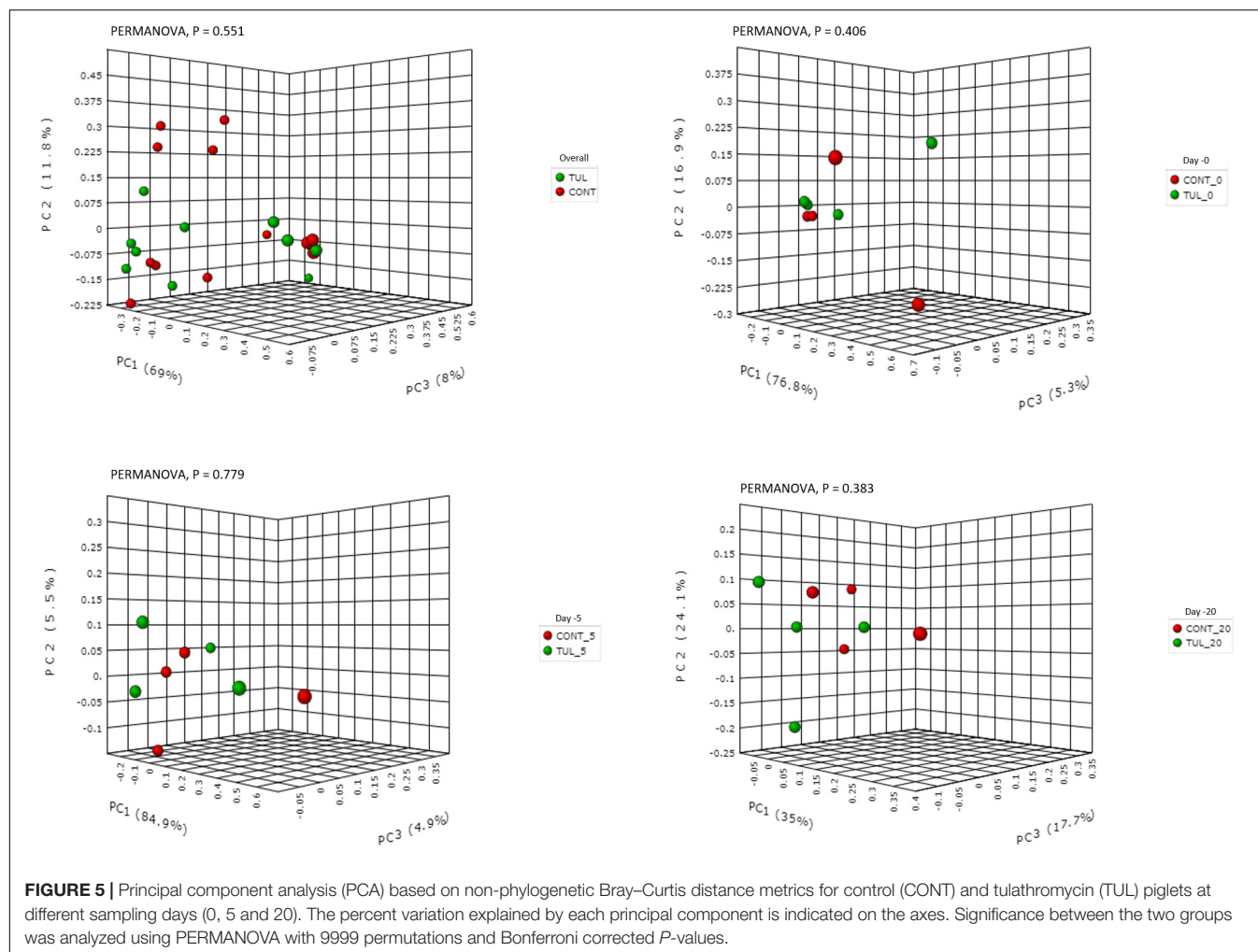


Effect of TUL Metaphylaxis on Antimicrobial Resistance Genes

Across all samples, a total of 127 ARGs related to 19 different classes of antibiotics were identified. The detected

ARGs confer resistance to lipopeptide, aminocoumarin, tetracycline, fluoroquinolone, beta-lactam, aminoglycoside, streptogramin, macrolide, lincosamide, lipopeptide, rifamycin, phenicol, peptide, glycopeptide, nucleoside, sulfonamide,





fluoroquinolones, coumarin, rifampin, and diaminopyrimidine antibiotics. A heatmap of identified ARGs relative abundance in the fecal microbiota at class level in both CONT and TUL groups was depicted in (Supplementary Figure S5). The identified ARGs were observed in both CONT and TUL groups and at all-time points. The highest level of ARGs across all samples were associated with *tetQ* (10.22%), *tetO* (7.21%), and *tetW* (6.24%), *PmrC* (4.65%), and *APH(3')-IIIa* (3.77%). The magnitude and extent of differences in the 50-predominant ARGs, between the CONT and TUL groups were statistically insignificant (Figure 7).

To gain further insight, we calculated several alpha-diversity indices for ARGs in both CONT and TUL groups. Alpha diversity analysis showed non-significant changes in the Chao1, Shannon, Simpson and Pielou's evenness indices between the CONT and TUL groups (Supplementary Figure S6). However, the metagenomic analysis revealed that the ARGs diversity and richness indices were increased with age. PCA also showed that the overall fecal ARGs did not differ significantly between the TUL and CONT groups (PERMANOVA, $P = 0.353$; Figure 8A). However, the ARGs abundance across all samples varied greatly according to the age (PERMANOVA, $P < 0.001$; Figure 8B).

DISCUSSION

In this study, we used shotgun metagenomic sequencing to assess the developmental dynamics of fecal microbiota and their accompanying antimicrobial resistome in the newborn piglets in response to TUL metaphylaxis soon after birth. This study was performed in a commercial swine farm to improve the practical relevance of our results. The findings of this study revealed that single dose of TUL prophylaxis immediately after birth had no advantage in reducing the mortality and/or increasing the average daily weight gain in the neonatal piglets. The early-life microbial composition soon after birth was predominantly comprised of *Escherichia*, *Bacteroides*, *Clostridium*, *Shigella*, *Fusobacterium*, and *Streptococcus*. These taxa create an anaerobic environment that play an important role in establishing the other health beneficial strict anaerobes (Pantoja-Feliciano et al., 2013). The piglets fecal microbiota composition observed in this study soon after birth was similar to that published by (Rodríguez et al., 2015; Slifierz et al., 2015; Kubasova et al., 2017; Maradiaga et al., 2018). In 20-day-old piglets, *Clostridium*, *Bacteroides*, *Escherichia*, *Lactobacillus*, and *Prevotella* were the most abundant

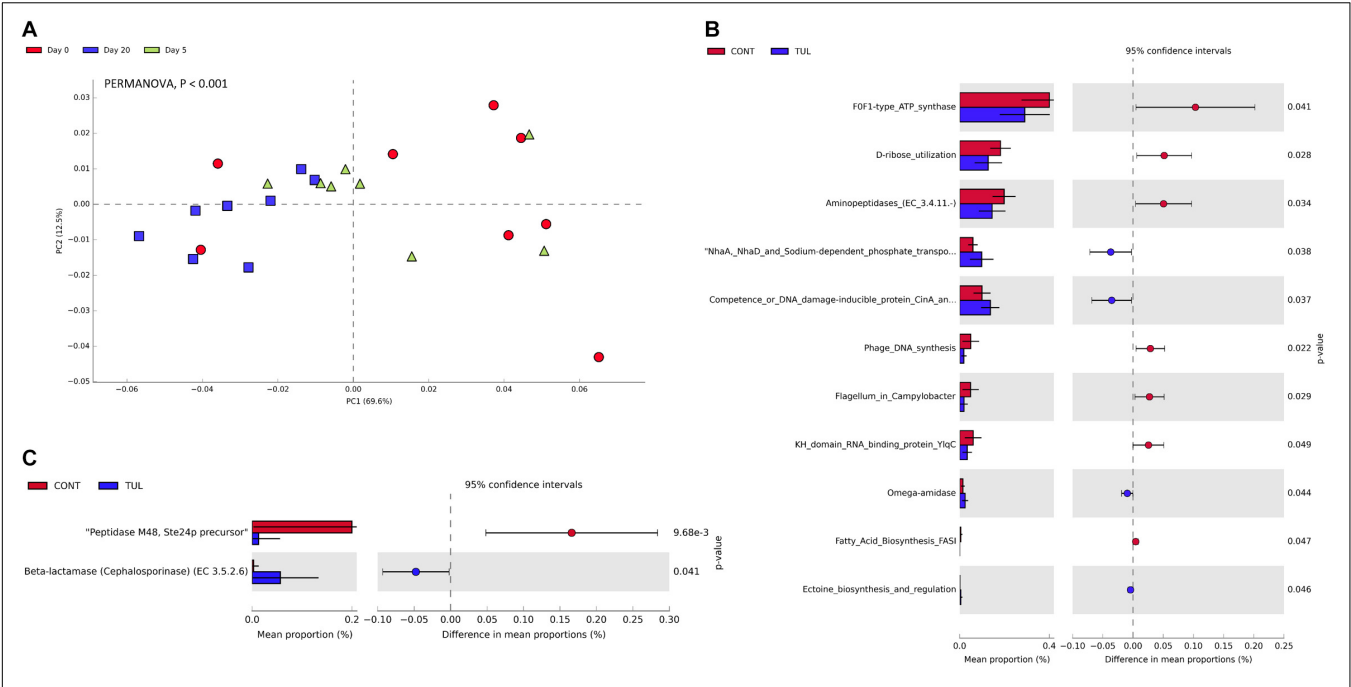


FIGURE 6 | (A) Principal component analysis (PCA) for the predicted functional profiles at level 3 across all samples at different sampling days (0, 5, and 20). The percent variation explained by each component is indicated on the axes. Significance between groups was analyzed using PERMANOVA with 9999 permutations and Bonferroni corrected P -values. **(B)** Extended bar plot showed the statistically significant difference in functional gene features in the tulathromycin (TUL) piglets compared to the control (CONT) group. P -value < 0.05 was considered significant. **(C)** Extended bar plot showed the statistically significant difference in the resistance to antibiotics functional genes in the tulathromycin (TUL) piglets compared to the control (CONT) group. P -value < 0.05 was considered significant.

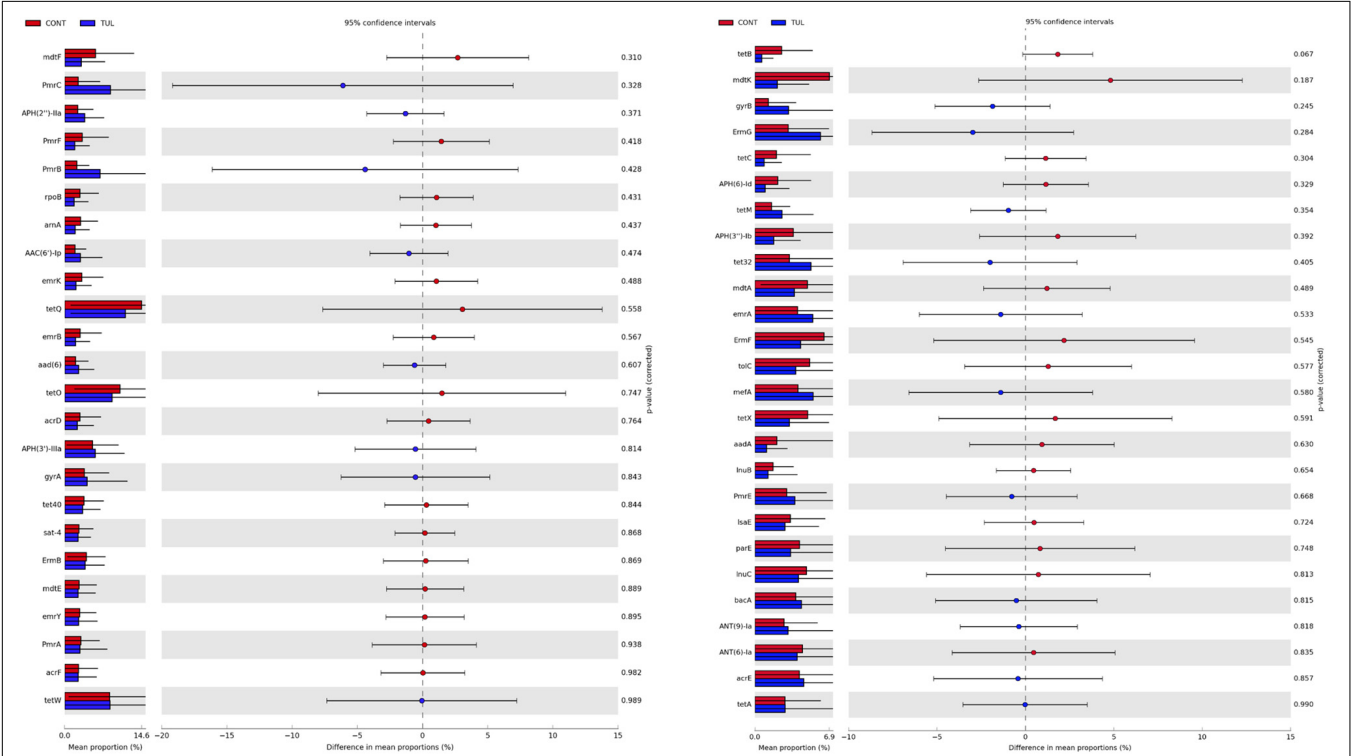
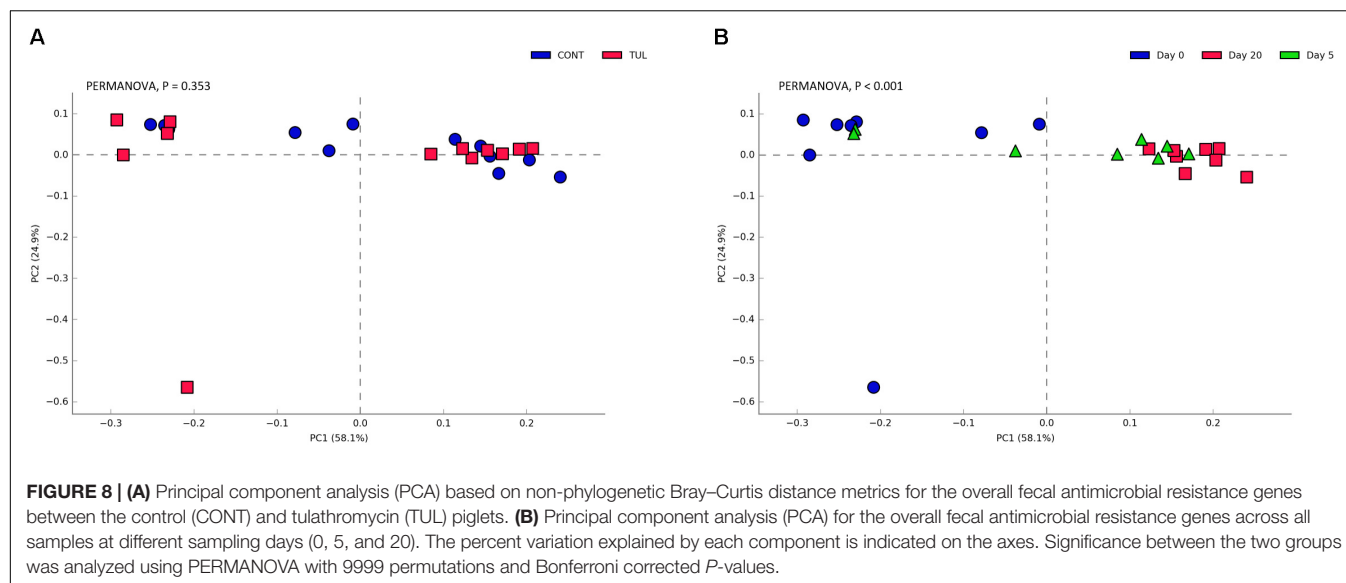


FIGURE 7 | Extended bar plot showed the 50-predominant antimicrobial resistance genes, between the control (CONT) and tulathromycin (TUL) groups. P -value < 0.05 was considered significant.



microbiota member, and were similar to the previous reports (Slifierz et al., 2015; Kubasova et al., 2017). While our study revealed that the age is the most significant contributor in the fecal microbiota development, understanding the early colonization pattern of gut microbiota will open the door to new perspectives related to the impacts of early life antimicrobials administration on the health of neonates in the swine management systems.

While, there have been contradictory reports regarding the impact of antimicrobial interventions on fecal microbiota, our results are broadly consistent with a previous study that assessed the effects of TUL intervention on fecal microbiota and their accompanying ARGs in commercial feedlot calves (Doster et al., 2018). Doster and his colleagues reported that the fecal microbiota structure and ARGs relative abundance were not significantly different between the control and TUL-treated cattle using shotgun metagenomic sequencing. Similarly, our results revealed that the single dose of TUL metaphylaxis in the neonatal piglets has no measurable benefits or detriment impacts on either the overall fecal microbial community or emergence of ARGs.

In this study, the use of TUL metaphylaxis was associated with non-significant changes in microbial diversity compared to the CONT piglets. Similar to our findings, antibiotic administration in cattle showed non-significant changes the fecal microbiota composition and diversity in the lower gastrointestinal tract (Thomas et al., 2017). This might be due to the rapid absorption and distribution of TUL from the injection site to the target tissues particularly the respiratory tract, with exceptionally long elimination half-life in the lung tissue (6 days in pigs) (Benchaoui et al., 2004). Moreover, TUL excretion is somewhat slow (about 70% within 23 days), with the excreted route being divided between urine (40%) and feces (32%). The modest changes in the fecal microbiota composition following early life TUL metaphylaxis are likely to reflect a combination between the resident microbiota resistance mechanism and the relatively

weak gastrointestinal selective pressure of single-dose of TUL (Choo et al., 2018).

Similar to the highly diverse and developed fecal microbiota composition, the overall predicted functional profiles in both CONT and TUL-treated piglets varied greatly according to the age. The age variability in functional profiles has also been previously detected in RNA and DNA -based metagenomic analysis (Phillips et al., 2004; Qin et al., 2010). While these are only statistical presumptions in functional features of the taxonomically assigned microbial population in our study, similar changes have been declared after antimicrobial treatment in human (Pérez-Cobas et al., 2013). Further investigations into the functional profiles either by direct metabolites measurement or by transcriptome analysis will be an essential next step to better understand the effect of the early life antimicrobial interventions on gut microbiota function in swine.

One important consequence of overuse of antimicrobials in livestock production is the spread of ARGs, which is a serious public health issue (MacKie et al., 2006). Recently, the use of functional metagenomic provides a potential resource for detecting the existence of ARGs in gastrointestinal microbial community (Thomas et al., 2017). In this study, the identified ARGs were observed in both CONT and TUL-treated piglets across all-time points. Similarly, previous studies have reported that the newborn infants harbor ARGs that potentially acquired from their mothers (Yassour et al., 2016). Some ARGs were also detected in the absence of antimicrobial exposure in both human (Tsukayama et al., 2018) and cattle (Chambers et al., 2015). Interestingly, the magnitude and extent of differences in the proportion of macrolide resistance genes sequence between the TUL and CONT groups were statistically insignificant ($P > 0.10$; **Figure 8**). Similarly, macrolide treatment did not result in a significant increase in the macrolide resistance genes (*erm(A)*, *erm(B)*, *erm(C)*, *erm(F)*, *mef(A/E)*, and *msrA* in people (Choo et al., 2018). In combination, there was no measurable effect

of TUL treatment on ARGs in this group of piglets. Since we used only single dose of TUL and the total study duration in this study was only 20 days, the ARGs profile we determined here may not be representation of longer-term effect of such antimicrobial metaphylaxis.

While the results of this study could open a new avenue in understanding the impact of antimicrobial administration on the early-life developmental dynamics of fecal microbiota and resistome in piglets, our study had number of experimental limitations that should be considered. Our analysis focused only on the fecal microbiota and their accompanying ARGs. Whereas the impact of antimicrobial treatment on microbiota within other gastrointestinal regions is likely to be consistent with the results reported here, changes in composition and ARGs characteristics in other commensal populations in different biogeographic locations should be considered. Our analysis also focused on the short-term impact of TUL administration on fecal microbiota (first 20 days of life). It would have been interesting to continue to sample the piglets for a longer period after weaning to define how these minor changes impact the future health and productivity of growing piglets. Finally, the major limitation in this study was the low sequence reads and sequencing depth per sample compared to other metagenomic study (Ferguson et al., 2013). Despite these experimental limitations, our study results provide preliminary insight into an area of investigation that could be of great relevance to swine gut health.

CONCLUSION

This study demonstrated that TUL metaphylaxis at birth had relatively minor effects on the developmental dynamics of gut microbiota and their accompanying antimicrobial resistome in suckling piglets. This study suggests that a single dose of metaphylactic TUL treatment may be employed at birth without incurring drastic changes to the fecal microbiota and their accompanying ARGs in swine. However, further long-term studies across larger populations should be conducted to determine the beneficial and/or the detrimental effects of early life antimicrobials prophylaxis on gut microbial community structure and ARGs in pigs. Understanding when and how the gut microbiota responds to the antimicrobial administration will open the door to new perspectives on the utility of early life antimicrobial to healthy neonates in our livestock management systems.

ETHICS STATEMENT

The present study was conducted in a commercial swine farm in the Midwestern United States with consent from the facility owner. All procedures were carried out in agreement with principles and guidelines of the Institutional Animal Care and Use Committee of University of Illinois at Urbana-Champaign. The protocol was evaluated and approved by the Ethical Committee for Institutional

Animal Use and Care of the University of Illinois at Urbana-Champaign.

AUTHOR CONTRIBUTIONS

JL and BA designed the experiments. BBl, BBu, AM, and MZ conducted the experiments. MZ, BBl, and BBu performed the laboratory analyses. MZ and AM conducted the data analysis and wrote the manuscript. JL and BA edited the manuscript. All authors approved the manuscript 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/fmicb.2019.00726/full#supplementary-material>

FIGURE S1 | The detailed experimental design.

FIGURE S2 | (A) Bar graph illustrating the body weight (kg) at day 0 and day 20, and the average weight gain from day 0 to day 20 of age. (B) Bar graph illustrating the Mortality percent of piglets from day 0 to day 5 (day 0–5), from day 5 to day 10 (day 5–10), from day 10 to day 15 (day 10–15), and from day 15 to day 20 (day 15–20). The piglets were treated with a single dose of TUL at day 0 soon after birth (TUL, $N = 108$), or treated with saline (CONT, $N = 113$). There was no significant change in the average daily weight gain and overall mortality ratio ($P > 0.05$).

FIGURE S3 | Taxonomic classification of shotgun metagenomic sequences at the genus level for the control (CONT) and tulathromycin (TUL) treated piglets at each sampling time days (0, 5, and 20). Only those bacterial genera that averaged more than 1% of the relative abundance across all samples are displayed.

FIGURE S4 | Inferred predictive functional features of piglet's fecal microbiota. (A) Heatmap cluster analysis of metagenomic functional capability at level 2 KEGG pathway based on differentially abundant functional features between the control (CONT) and tulathromycin (TUL) groups, and at different sampling days (0, 5, and 20). The yellow/blue color of the X axis of the heat map represent the degree of similarities and cluster between the related class of assessed parameters. (B) The relative abundance of the functional profiles at level 2 in the TUL treated piglets compared to the CONT group (P -value > 0.05). (C) Principal component analysis (PCA) based on non-phylogenetic Bray–Curtis distance metrics for the overall functional gene profiles between THE CONT and TUL-treated piglets. The percent variation explained by each principal component is indicated on the axes.

FIGURE S5 | Heatmap of identified antimicrobial resistance genes in the fecal microbiota at class level of each piglet in both control (CONT) and tulathromycin

(TUL) groups. The yellow/blue color of the X axis of the heat map represent the degree of similarities and cluster between the related class of assessed parameters.

FIGURE S6 | The difference in bacterial diversity indices (Chao 1, Shannon, Simpson and Pielou's evenness indices) measures between the control (CONT)

and tulathromycin (TUL) piglets at different sampling days (0, 5, and 20). The individual data points, which represent bacterial diversity for each piglet, are depicted. Error bars represent the standard errors.

TABLE S1 | The most predominant 100 microbial species across all the samples in both control (CONT) and tulathromycin (TUL) treated piglets.

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Conflict of Interest Statement: 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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Molecular and Physiological Characterization of Fluoroquinolone-Highly Resistant *Salmonella* Enteritidis Strains

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Four clinical isolates of *Salmonella* Enteritidis, susceptible to ciprofloxacin, and their spontaneous ciprofloxacin resistant (MICs from 8 to 16 $\mu\text{g/mL}$) and highly resistant (MIC 2048 $\mu\text{g/mL}$) mutants were used to gain an insight into the dynamics of development of fluoroquinolone (FQs) resistance in *S. Enteritidis* serovar. The first two high-frequency (i.e., mutations that occurred in each tested strain) mutations occurred in the *gyrA*, resulting in amino acid substitutions S83Y and S83F as well as D87G. Amino acid substitution D87G was significantly associated with the highly resistant mutants. Another high-frequency mutation, deletion in the *ramRA* intergenic region, was determined among the same group of highly resistant mutants. More importantly, each of these deletion mutations affected the RamR binding site. The effect of one 41 bp deletion mutation was empirically tested. The results showed that the deletion was responsible for resistance to ceftiofur and amoxicillin/clavulanic acid and decreased susceptibility to azithromycin and tetracycline. Performing gene expression assays across all ciprofloxacin susceptible groups, we found a consistent and significant upregulation of the *ramA*, *acrB*, and *tolC* (efflux pump associated genes) and downregulation of *ompF* (porin), clearly illustrating the importance of not only efflux but also porin-mediated permeability in the development of FQs resistance. Our data also showed that *S. Enteritidis* could acquire multiple mutations in QRDR region, further resulting in no up regulation of the *ramA*, *acrB* and *tolC* genes. These QRDR mutations and no activation of the AcrAB efflux pump seem to preserve the fitness of this organism compared to the *S. Enteritidis* strains that did not acquire multiple QRDR mutations. This report describes the dynamics of FQ-associated mutations in the highly resistant in FQ mutants in *S. Enteritidis*. In addition, we characterized a deletion in the *ramRA* intergenic region, demonstrating that this frequent mutation in the highly resistant FQ mutants provide resistance or reduce susceptibility to multiple families of antibiotics.

Keywords: *Salmonella*, quinolone resistance, multidrug resistance, AcrAB efflux pump and small-colony variant phenotype, *OmpF* porin

INTRODUCTION

Non-typhoidal *Salmonella* (NTS) is a major zoonotic pathogen worldwide (Bangtrakulnonth et al., 2004; Scallan et al., 2011). Infections caused by this pathogen have been mainly associated with gastroenteritis, an acute self-limiting intestinal infection. However, it has been shown that in different regions of the world, especially in places with high percentages of immunocompromised populations, NTS is a frequent cause of bacteremia (Graham, 2010; Reddy et al., 2010; Okoro et al., 2012), an invasive life-threatening extra intestinal infection. Once acquired, this invasive salmonellosis may result in a fatality rate of 20% (Gordon, 2008). Multidrug resistance and in particular resistance to fluoroquinolones (FQs), a potent broad-spectrum family of antibiotics used for the primary treatment of invasive salmonellosis, play a key role in the treatment failure (Pers et al., 1996; Rupali et al., 2004).

Fluoroquinolones resistance in NTS can be acquired through transmissible quinolone-resistance mechanisms (Martinez-Martinez et al., 1998; Redgrave et al., 2014; Hooper and Jacoby, 2015). Transmissible quinolone-resistance occurs via the horizontal transfer of plasmids, which carry a family of *qnr* genes (i.e., *qnrA*, *qnrB*, *qnrS*, *qnrC*, and *qnrD*)—also known as plasmid-mediated quinolone resistance (PMQR) genes. It has been shown that the PMQR genes confer modest resistance against FQs (Martinez-Martinez et al., 1998). *Salmonella* spp., most commonly vertically acquired resistance to FQs through *de novo* mutations, which occurs in the quinolone resistance-determining region (QRDR) of the *gyrA* and *parC* genes (Piddock et al., 1998; O'Regan et al., 2009; Ricci and Piddock, 2009) and in genes encoding the *acrAB-tolC* efflux system (Baucheron et al., 2004) as well as in genes that encode regulators of the efflux system (Abouzeed et al., 2008; O'Regan et al., 2009; Blair et al., 2015). Vertically acquired mutations play a critical role in antimicrobial treatments, as these mutations occur quickly under the selective pressure of the drug, resulting in a high level of resistance against FQs, which may subsequently lead to antimicrobial treatment failure. The vertical evolution of resistance-conferring mutations is very complex. The outcome, antimicrobial susceptibility, depends on a sum of the interactions between antagonistic and synergistic resistance-conferring mutations rather than on an independent effect of a single mutation (Hartl, 2014). Understanding the development of *de novo* mutations, their interactions, and physiological adaptation of the bacterial organism to the selective pressure imposed by the drug is critically important in antimicrobial stewardship programs.

In this study, we examined the development of the resistance and the high-level resistance to FQs using four ciprofloxacin susceptible *Salmonella enterica* serovar Enteritidis (*S. Enteritidis*) isolates and their spontaneous ciprofloxacin resistant (MIC ranging from 8 to 16 $\mu\text{g/mL}$) and highly resistant (MIC 2048 $\mu\text{g/mL}$) mutants. The aim of this study was to determine the type and dynamics of the mutations and the overall physiological adaptations associated with the development of extremely high resistance to ciprofloxacin.

MATERIALS AND METHODS

Strains of *S. Enteritidis*

Out of 88 *S. Enteritidis* strains, implicated in human and avian infections, we selected four strains that exhibited the most profound susceptibility to ciprofloxacin. The following four *S. Enteritidis* strains: A-5 from Texas (MIC 0.015 $\mu\text{g/mL}$), A-7 from Pennsylvania (MIC 0.06 $\mu\text{g/mL}$), A-21 from Iowa (MIC 0.03 $\mu\text{g/mL}$), and A-33 from Wisconsin (MIC < 0.0009375 $\mu\text{g/mL}$) were selected for the study. All four strains were implicated in the primary infections of avian hosts. The strains were received from the National Veterinary Services Laboratories (NVSL), Ames, IA. The *S. Enteritidis* strains were diagnosed at the NVSL using standard microbiological and serological methods for *Salmonella* identification and classification. The strains were checked for their purity upon their arrival by plating them on Luria-Bertani (LB) agar (Difco) plates, followed by an overnight incubation at 37°C. After confirmation of the strains' purity, they were stored at -80°C in LB broth (Difco) with 10% glycerol.

Antimicrobial Susceptibility Tests

Four *S. Enteritidis* strains, A-5, A-7, A-21, and A-33, were examined for their antimicrobial susceptibilities using Sensititre CMV3AGNF plates (TREK Diagnostic Systems, Cleveland, OH, United States). The Sensititre plates each contained 14 antimicrobial agents dosed in 96 wells at appropriate dilutions, as specified by NARMS (National Antimicrobial Resistance Monitoring System) of the CDC. Each well of the Sensititre microtiter plate was inoculated according to the instructions of the manufacturer, followed by incubation at 37°C for 18–22 h. The minimal inhibitory concentration (MIC) breakpoints were determined according to the National Committee for Clinical Laboratory Standards (NCCLS) M07-A10 (Clinical and Laboratory Standards Institute [CLSI], 2015a) and M100-S25 (Clinical and Laboratory Standards Institute [CLSI], 2015b).

Induction of FQ Resistance and Selection for Resistant and Highly Resistant Mutants

To obtain this collection, we exposed each parental strain at its mid-exponential growth phase to 0.02 $\mu\text{g/mL}$ of ciprofloxacin, followed by an overnight incubation period. After the overnight incubation, ciprofloxacin-challenged cultures were inoculated (1:100) into LB broth, containing a twofold higher concentration of ciprofloxacin compared to the previous ciprofloxacin challenge. Further, a series of twofold increase ciprofloxacin concentrations (from 0.04 to 2.4 $\mu\text{g/mL}$) challenges were undertaken, and a selection of the ciprofloxacin resistant mutants took place on LB agar plates with 4 $\mu\text{g/mL}$ of drug concentration. Similarly, after a series of twofold increase ciprofloxacin concentrations (from 8 to 32 $\mu\text{g/mL}$) the highly resistant strains were selected on LB agar plates, which contained 40 $\mu\text{g/mL}$ of ciprofloxacin.

Minimal Inhibitory Concentration (MIC) Determinations

The MICs of ciprofloxacin for the parental strains and their resistant and highly resistant spontaneous mutants were determined using broth macrodilution method, as described by the NCCLS M07-A10 (Clinical and Laboratory Standards Institute [CLSI], 2015a) and M100-S25 (Clinical and Laboratory Standards Institute [CLSI], 2015b).

Cross-Resistance Assay

After selecting the resistant, A-5 (R), A-7 (R), A-21 (R), and A-33 (R) and the highly resistant mutants, A-5 (HR), A-7 (HR), A-21 (HR), and A-33 (HR), they were examined for any additional acquired antimicrobial resistance phenotype, which is not relevant to FQ class of antibiotics. The susceptibility of the resistant and the highly resistant strains was determined using Sensititre CMV3AGNF plates, as described above.

gyrAB, *parCE*, *acrAB-tolC*, *ramAR*, *rpoE*, *cpxR*, *ompFC*, and *lpxA* Full-Length Genes Sequencing and Single Nucleotide Polymorphisms (SNPs) Analysis

The list of the primers used for the full-length gene sequencing of *gyrA*, *gyrB*, *parC*, *parE*, *acrA*, *acrB*, *tolC*, *ramA*, *ramR*, *rpoE*, *cpxR*, *ompF*, *ompC*, and *lpxA* is shown in **Table 1**. The primers for *acrA*, *acrB*, *tolC*, *ramA*, *ramR*, *rpoE*, and *cpxR* are designed to target the promoter regions of these genes as well. The amplicons were generated by Platinum *Taq* DNA polymerase (Thermo Fisher Scientific) and were prepared for DNA sequencing by the Prism BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, United States). The nucleotide sequences on both strands were determined using an ABI 3730 x 1 DNA analyzer (Genomics Center, University of Minnesota, Minneapolis, MN, United States). Each strand was checked and then aligned with its complementary strand. A consensus DNA sequence was obtained using Clustal Omega (Larkin et al., 2007). The annotated DNA sequences were exported into Molecular Evolutionary Genetics Analysis (MEGA) version 7 (Tamura et al., 2007) for the identification of non-synonymous single nucleotide polymorphisms (nsSNPs) within the coding regions and SNPs within the promoter regions of *acrA*, *acrB*, *tolC*, *ramA*, *ramR*, *rpoE*, and *cpxR*. Nucleotide sequence translation was carried out using EMBOSS Transeq (Kearse et al., 2012) (the European Molecular Biology Laboratory–European Bioinformatics Institute; Hinxton, Cambridge, United Kingdom).

Gene Expression Assay

Overnight cultures of the susceptible, resistant, and highly resistant *S. Enteritidis* A-5, A-7, A-21, and A-33 strains were diluted 1/100 in 100 mL of LB and grown at 37°C with constant shaking at 190 rpm to optical density at 600 nm of 0.5. These mid-exponential growth phase cultures were exposed to ciprofloxacin at a final concentration of 0.010 µg/mL. The cultures were additionally incubated for 30 min and harvested by centrifugation. The total RNAs were extracted using the RNeasy Mini kit (Qiagen), following the manufacturer's

instructions. The synthesis of complementary DNA (cDNA) was carried out using iScript™ Reverse Transcription (Bio-Rad Laboratories, Inc. Hercules, CA, United States). Quantitative PCR was performed using Power SYBR green master mix kit (Applied Biosystems). The primers used to detect transcripts of *ramA*, *ramR*, *acrA*, *tolC*, *rpoE*, *cpxR*, *ompA*, *ompW*, *ompF*, *ompC*, and *slyB* are listed in **Supplementary Table S1**. The *gapA* gene was selected as an internal reference control and the data were reported as the fold change relative to the levels in the susceptible strains using the comparative C_T method (Schmittgen and Livak, 2008).

Complementation Assay

The *ramRA* sequence, including its intergenic region, was amplified by PCR using genomic DNA of *S. Enteritidis* 5-A highly resistant strain, Q5 High-Fidelity DNA polymerase (New England BioLabs, Ipswich, MA, United States), and the primers *ramRA* F (5'-GCG GGA TCC GAC AGT GAT GTT CAG TGA AC-3') and *ramRA* R (5'-TCA GTC GAC CTC TTG CTC GGC GCG CTG GA-3'); the *Bam*HI and *Sal*I sites are underlined. The *ramRA* fragment was double digested and cloned between the *Bam*HI and *Sal*I restriction sites of the low copy expression vector pTrc99A (Life Science Market). The recombinant pTrc99A vector was sequenced and an insertion of the *ramRA* haplotype of *S. Enteritidis* 5-A highly resistant strain was confirmed. The *S. Enteritidis* 5-A susceptible strain was transformed with the recombinant pTrc99A vector and also with an empty pTrc99A vector, respectively. Both *S. Enteritidis* A-5 susceptible strains, complemented with the recombinant and non-recombinant pTrc99A vectors, were tested for susceptibility to the panel of 10 antimicrobial agents, as described above.

Growth Curve Assay

The difference in the growth kinetics between the parental ciprofloxacin susceptible strain and its spontaneous highly resistant mutant with a small colony variants (SCVs) phenotype was determined by measuring biomass at 600 nm. The overnight cultures of the parental and highly resistant mutant strains were diluted and normalized to an optical density equivalent to 0.5 McFarland standard (1.5×10^8 CFU/mL). These normalized cultures were used to inoculate 50 mL of freshly prepared LB, followed by incubation at 37°C with shaking at 180 rpm. The OD₆₀₀ values were measured every 60 min for 6 h. In addition, the differences in the growth kinetics between the parental strains A-5, A-7, A-21, and A-33 and their resistant mutants were measured. This growth assay was carried out with addition of 0.005 µg/mL of ciprofloxacin as described above.

Statistical Analysis

To test for differences in gene expression between resistant and highly resistant strains, compared with their parental susceptible strains, *t*-tests were performed for each strain/gene/resistance combination. To better estimate variability, variance was pooled for data from the same strain. Additionally, *p*-values were adjusted for multiple comparisons using the Bonferroni–Holm correction, again, within each strain separately. All tests were performed on the ddCt scale, and results transformed to fold

TABLE 1 | Primers used for the amplification and full gene sequencing.

Gene	Purpose	Name of primer	Forward primer sequence (5'–3')	Name of primer	Reverse primer sequence (5'–3')	Amplicon size (bp)
<i>gyrA</i>	PCR	<i>gyrA</i> F	CGCCAATAAACGCCAAGACC	<i>gyrA</i> R	AGCGGTAAATGACGTAGCCC	2769
	Sequencing	F-1	GGTGAACCTCAACGACGGC	R-1	ACTGCGATACCGGAAGAACC	
	Sequencing	F-2	CTATGACGGTACGGAATAAT	R-2	CGGTGGCGCACGAACGCTGAAATG	
	Sequencing	F-3	GACCCAGCTACAGGTTTCCTTC	R-3	CTGGCTAATCAGATCTTCGATATT	
	Sequencing	F-4	ATCCGCGAAGAGATGGAGTTA	R-4	GACGGCGTCTTCTTTGAAGC	
<i>gyrB</i>	PCR	<i>gyrB</i> F	TAGCGCTGAACACGTTATAGAC	<i>gyrB</i> R	GCCACTCAGCGCTTTAGAGAT	2584
	Sequencing	F-1	CAGCGAGATGGCAAAATTCAC	R-1	CGCAATCAGACCTTCACGGG	
	Sequencing	F-2	TGGACAAAGAAGGCTACAGCA	R-2	CTTGTCGGGTTGTACTCGT	
	Sequencing	F-3	TCGACAAGATGCTTCTCTCCC	R-3	CATCGAACTTCCACTGACTGC	
	Sequencing	F-4	GCTGGGTGAATGCGCTG	R-4	GCCAGAAACGTACCATCGTG	
<i>parC</i>	PCR	<i>parC</i> F	CCACCTGTCTCACGTTTGT	<i>parC</i> R	GGCCGGATAAGACGCTACTG	2419
	Sequencing	F-1	AATACGCCGAGCTGCTGTTA	R-1	GTGGTGGCGAACAGATGGTT	
	Sequencing	F-2	ACCCGACGCGTTTGTAGTATT	R-2	GCTTTAGCTTCTTCGCGCTC	
	Sequencing	F-3	GTCCGAACGCAAAATGAATACC	R-3	TCTTCAATCACCAGCGGCG	
	Sequencing	F-4	GCTACGGCTTCGTTGTACG	R-4	CGCCGGTAACATTTTCGGTT	
<i>parE</i>	PCR	<i>parE</i> F	GCATTACGGGCTTCGAAGATT	<i>parE</i> R	ATGCGCAAGTGTGCGCATCA	2060
	Sequencing	F-1	CGCGACGGTCAGGTCTATAA	R-1	CGACAGACGCTCTTTGGTCT	
	Sequencing	F-2	ATACCGCAATATTCTGCCGC	R-2	GACTCAGATCGTCGCTGTCC	
	Sequencing	F-3	CTTCGATGAAGTGCTGGC	R-3	AATGGACGCTGGTTCAGTA	
<i>acrA</i>	PCR	<i>acrA</i> F	AAACGCAGGGCCACATCCAG	<i>acrA</i> R	TGATGGCGATCACCCACGCA	1230
	Sequencing	F-1	GATTGATCCTGCGACCTACCA	R-1	GGCGCGCAAAGTAATAGACC	
	Sequencing	F-2	AAACAGGAAAACGGCAAGCG	R-2	TTCACCGTCAGTTCAGCGAT	
<i>acrB</i>	PCR	<i>acrB</i> F	GCGCACAGGTAAAGTACA	<i>acrB</i> R	AATAATCAATCTAACAATAAGCGT	3295
	Sequencing	F-1	CGTGAGCGTTGAGAAGTCC	R-1	GGCGTGGTGTACATACGGG	
	Sequencing	F-2	TTGGCATCAAACCTGGCTACC	R-2	CGTCAGGATCAGCGCGAC	
	Sequencing	F-3	CAACCGGGGCAATTTATCGT	R-3	ATTTGTGAAAACGCTGCGG	
	Sequencing	F-4	CCGGTATTGCATTCGTGTG	R-4	TTATAGCGTTCCAGACGCGG	
	Sequencing	F-5	AACGACTGGTACGTTCTGTGG	R-5	ACCCATGGTTGTGAGCAGG	
	Sequencing	F-6	CTATCCCGTTCTCCGTAATG	R-6	AGAGGTACGGCTGATCGGA	
<i>tolC</i>	PCR	<i>tolC</i> F	CTGCTGTTTTATACCCCTGTGG	<i>tolC</i> R	TCAGGCGCGGCTTTATGAC	1692
	Sequencing	F-1	CTTTCCTATACCCAGGCGCA	R-1	CTTTCAGCTGTTGCGTTGC	
	Sequencing	F-2	TGGGATGGTTAACTCGCAGG	R-2	TTGCGGTATTGAGGATCAGC	
<i>ompC</i>	PCR	<i>ompC</i> F	CCGTTGATTTTAAAGTTTCGT	<i>ompC</i> R	GGACGCAAGCGTATATCAA	1301
	Sequencing	F-1	GATGGCGACCGACCTACAT	R-1	TACTGCGCTGCCAGATAGAT	
	Sequencing	F-2	GGCGCTATCACACGCTCTAAA	R-2	TTCAGTCTGGTTGCCCTGAA	
<i>ompF</i>	PCR	<i>ompF</i> F	ACCAAATCTTTATCTTTGTAGCA	<i>ompF</i> R	CCTGTTTTTGAAGACGCAC	1245
	Sequencing	F-1	CTAAAGCAGACCGCGCTGA	R-1	AACGGCCCACGATTCTGC	
	Sequencing	F-2	AGCGTACAGCAACAGCAAGC	R-2	CGACCATAATCGATTGAACCC	
<i>ramRA</i>	PCR	<i>ramRA</i> F	CATCGTACTGTGGGCGGAA	<i>ramRA</i> R	TGGTTTCTGTTGCTCGGCG	
	Sequencing	F-1	ACGAGTCATCATTTTGGCATC	R-1	AAGCATAGTAATAACCACACAAA	
	Sequencing	F-2	TTGACGGCGTATCTTTGCTTT	R-2	GCGACCAAAGATGAGCTGATTA	
<i>rpoE</i>	PCR	<i>rpoE</i> F	AACATGGTTGCGGCAGATTAG	<i>rpoE</i> R	ACGTTTCGCCATCCATCAAAG	1014
	Sequencing	F-1	GAGCGAGCAGTTAACGGACCA	R-1	GGAAACCAGACTCGCCACT	
<i>cpxR</i>	PCR	<i>cpxR</i> F	TAACTTTGCGCATCGCTTGC	<i>cpxR</i> R	ATCGAGCTTGGGCAACATCA	917
	Sequencing	F-1	CGCGGACGACTATTTACCCA	R-1	GGCGCAAAATAGCCCTGATG	
<i>lpxA</i>	PCR	<i>lpxA</i> F	CGTGGCCTGACCCGCTTTA	<i>lpxA</i> R	CACGAATTAAGCCTGCGCCA	1128
	Sequencing	F-1	GTCGCACATGATTGTACGGT	R-1	TCATCGACTGATACGTGCCCC	

change for presentation. Growth curves kinetics were analyzed by CoStat version 6.4 software (Co-Hort Software, Monterey, CA, United States) using homogeneity of linear regression slopes method to test for significant ($p < 0.05$) differences.

Nucleotide Sequence Accession Numbers

Nucleotide sequences of each gene haplotype were deposited in GenBank under accession numbers MH933946 to MH933963.

Also, accession numbers for six *ramAR* haplotypes, including their intergenic regions, range from MK024405 to MK024410.

RESULTS

Selection and Characterization of Spontaneous Ciprofloxacin-Resistant and Highly Resistant *S. Enteritidis* Mutants

Four clinical strains of *S. Enteritidis* with a high level of ciprofloxacin susceptibility, A-5 (MIC 0.015 µg/mL), A-7 (MIC 0.06 µg/mL), A-21 (MIC 0.03 µg/mL), and A-33 (MIC < 0.0009 µg/mL), were used to create a collection of spontaneous ciprofloxacin resistant and highly resistant mutants. Following the selection of the ciprofloxacin resistant and highly resistant mutants, we tested these two groups of mutants for their MIC values to ciprofloxacin. The resistant mutant strains exhibited MIC values ranging from 8 to 16 µg/mL, while their highly resistant counterparts achieved an MIC of 2048 µg/mL, indicating much higher MIC values compared to those of the resistant mutant strains (Table 2).

Exposure of *S. Enteritidis* to Ciprofloxacin Leads to the Development of Cross-Resistance

To determine the potential side effect of the ciprofloxacin treatment on antimicrobial susceptibility of the spontaneous mutants to antibiotics that are not related to the family of FQs, we carried out the antimicrobial susceptibility tests for the parental strains and their spontaneous resistant and highly

resistant mutants using ten, non-FQs-related antibiotics. The susceptibility tests illustrated the existence of four distinct phenotypes associated with the antimicrobial susceptibility profiles of the parental and their spontaneous mutant strains (Table 3). The first phenotype included no change of MICs for amoxicillin/clavulanic acid and ampicillin between the parental strains and their mutants (Table 3). The second phenotype showed a slight increase of MICs for ceftriaxone and gentamycin between the parental and their mutant strains (Table 3). The third phenotype was characterized by a variable change of MICs for tetracycline and streptomycin among the four *S. Enteritidis* parental strains and their spontaneous mutants (Table 3). For instance, the parental A-5 strain and its resistant and highly resistant mutant strains had the same MICs values of < 4 µg/mL for tetracycline. In contrast to this group of A-5 strains, parental A-7 strain exhibited an MIC of < 4 µg/mL; however, its resistant and highly resistant mutants acquired MICs of 8 µg/mL and 32 µg/mL, respectively, for the same antibiotic. The fourth phenotype was characterized by an increase in the MICs between the parental strains and their resistant and highly resistant mutants (Table 3). The MICs values for amikacin, chloramphenicol, cefoxitin, azithromycin, and ceftiofur of all of the *S. Enteritidis* strains have been accurately correlated to their ciprofloxacin MICs. The highest MICs values for these five antibiotics demonstrated ciprofloxacin highly resistant strains, whereas the lowest MICs values for the same five antibiotics exhibited the ciprofloxacin susceptible (i.e., parental) strains.

Mutations Associated With the Ciprofloxacin Resistant and Highly Resistant Strains

To identify mutations that contribute to the development of FQs-resistance (MIC from 8 to 16 µg/mL) and high resistance (MIC 2048 µg/mL), we sequenced the genes that encode topoisomerase II (*gyrA*, *gyrB*, *parC*, *parE*), efflux pump (*acrA*, *acrB*, *tolC*), efflux pump regulators (*ramR*, *ramA*), porins (*ompF*, *ompC*), extracytoplasmic stress response regulators (*rpoE*, *cpxR*), and liposaccharide component (*lpxA*) of the four parental strains along with their resistant and highly resistant mutants. No mutation was detected in the coding sequences of *gyrB*, *parE*, *acrA*, *acrB*, *tolC*, *ramA*, *ramR*, *ompF*, *ompC*, *rpoE*, *cpxR*, and *lpxA*. In addition, the entire promoter regions of the *acrA*, *acrB*, *tolC*, *rpoE*, and *cpxR* genes were intact, with no introduced mutations in either ciprofloxacin-resistant or ciprofloxacin-highly resistant mutants. A single non-synonymous substitution, G78D, was present in the *parC* of the highly resistant A-7 mutant strain. Another two amino acid substitutions, S80R and S80I, were present in the same gene of the resistant A-5 and highly resistant A-5 strains, respectively. In addition to these three amino acid substitutions, no other mutation was detected in the *parC* of the resistant A-7, resistant A-21, highly resistant A-21, resistant A-33, and highly resistant A-33 mutant strains. In contrast to the sporadic occurrence of mutations in *parC*, the *gyrA* was found to be a common target for the development of FQs resistance in the tested *S. Enteritidis* strains (Figure 1A and Table 2). The two FQs-resistant mutants, A-7 and A-21, acquired the same

TABLE 2 | Summary of MICs, amino acid substitutions, deletion mutations in the *ramRA* intergenic region and gene expression fold changes for the *ramA*, *acrB* and *ompF*.

	MIC(ug/mL)	Amino acid substitutions		Deletion mutations	Gene expression fold change		
	Ciprofloxacin	GyrA	ParC	RamRA	<i>ramA</i>	<i>acrB</i>	<i>ompF</i>
A5-S	0.015				1	1	1
A5-R	16	S83Y; D87G	S80R		1.3	2.7	−2.9
A-5-HR	2048	S83Y; D87G	S80I	41 nt + 1 mutation	80	6.6	−7.7
A-7-S	0.06				1	1	1
A-7-R	16	S83Y			11	3.6	−25
A-7-HR	2048	S83Y; D87G	G78D	9 nt	83.3	10.2	−3.8
A-21-S	0.03				1	1	1
A-21-R	8	S83Y		11 nt	72.2	8.3	−6.7
A-21-HR	2048	S83F; D87G		12 nt +1 mutation	205.8	14.5	−16.7
A-33-S	<0.0009				1	1	1
A-33-R	16	S83F			35.8	5.6	−100
A-33-HR	2048	S83F; D87G		4 nt	22.6	7.4	−43.3

TABLE 3 | Antimicrobial susceptibilities profiles of four parental *S. Enteritidis* strains and their resistant and highly resistant ciprofloxacin mutants.

Strain IDs	FQ Susceptibility groups											
	Susceptible				Resistant				Highly resistant			
	A-5	A-7	A-21	A-33	A-5	A-7	A-21	A-33	A-5	A-7	A-21	A-33
No change of MIC ($\mu\text{g/mL}$)												
Amoxicillin/ Clavulanic acid	<1/0.5	<1/0.5	<1/0.5	<1/0.5	<1/0.5	<1/0.5	<1/0.5	<1/0.5	<1/0.5	<1/0.5	<1/0.5	<1/0.5
Ampicillin	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Slight change of MIC												
Ceftriaxone	<0.25	<0.25	<0.25	<0.25	0.5	1	0.5	1	0.5	1	1	1
Gentamicin	2	2	1	4	4	2	1	2	4	1	4	2
Variable change of MIC												
Tetracycline	<4	<4	<4	<4	<4	8	<4	8	<4	32	8	<4
Streptomycin	64	64	64	>64	>64	>64	64	>64	>64	>64	64	>64
Increase of MIC (Cross-resistance)												
Amikacin	8	32	8	16	32	>64	>64	>64	>64	>64	>64	>64
Azithromycin	0.12	0.25	0.12	0.25	4	8	8	8	8	>16	>16	>16
Chloramphenicol	4	8	4	4	8	32	16	32	32	>32	>32	32
Cefoxitin	2	4	1	2	8	>32	>32	>32	32	>32	>32	>32
Ceftiofur	1	1	1	1	2	8	4	8	4	8	8	8

amino acid substitution, S83Y, the resistant A-33 mutant received another amino acid substitution, S83F, while the resistant A-5 mutant obtained two amino acid substitutions, S83Y and D87G (Table 2). The highly resistant mutants acquired a high-frequency mutation, the second amino acid substitutions, D87G (Table 2). The second high-frequency mutation associated with an extremely high level of ciprofloxacin resistance was identified in an intergenic region of *ramRA* (Figure 1B). This mutation was first identified among the resistant mutants, a single A-21 strain showed an 11-nt deletion (from 150 to 161 bp upstream of the *ramA*), which corresponds to the RamR binding site (Figure 1C and Table 2). This mutation progressed as the drug concentration increased and reached its high frequency among the highly resistant mutant strains. All the highly resistant mutant strains acquired a deletion that disrupted the RamR binding site (Figure 1C). To further investigate the effect of the *ramRA* intergenic deletion, the *ramRA* haplotype of the highly resistant A-5 strain was cloned into pTrc99-A and transformed into the parental A-5 strain. The complemented strain exhibited resistance to ceftiofur, amoxicillin/clavulanic acid and decreased susceptibility to azithromycin, tetracycline and ciprofloxacin, compared to the same strain transformed with the empty vector (Table 4). It is interesting that the A-5 parental strain complemented with the pTrc99A::*ramRA* vector could not develop a full ciprofloxacin resistance but only a decreased susceptibility to this antibiotic.

Gene Expression Analysis of the *acrAB-tolC* Efflux Pump, Its Regulators *ramRA*, Outer Membrane Components and Extracytoplasmic Regulators

To compare the effect of the ciprofloxacin treatment on the gene expressions of the parental, resistant, and highly resistant

strains, a subinhibitory concentration of this antibiotic for the A-5 parental strain was used. The selected ciprofloxacin concentration did not have any effect on the growth rate of this strain (data not shown). The expressions of the efflux pump genes, *acrA*, *acrB*, *tolC*, efflux pump regulators, *ramR*, *ramA*, extracytoplasmic regulators, *rpoE*, *cpxR*, and genes encoding porins, *ompA*, *ompW*, *ompF*, *ompC*, *slyB* for the parental strains and their resistant and highly resistant mutants are shown in Figure 2. Most notably, the expression of the *ramA* gene significantly increased in the all-highly resistant and resistant mutants except for the A-5 resistant mutant (Figure 2). The upregulation of the *ramA* gene ranged from 205 folds in the highly resistant A-21 strain to 10 folds in the resistant A-7 strain. Both genes associated with the *acrAB-tolC* efflux pump, *acrB* and *tolC*, showed significant ($p < 0.05$) levels of expression across the highly resistant and resistant strains. In general, the *acrB* exhibited a higher level of upregulation compared to that of the *tolC* gene. The highly resistant strains exhibited a higher expression of genes, *acrB* and *tolC*, compared to their resistant counterparts (Figure 2). In contrast to the efflux pump, gene encoding outer membrane porin F, *ompF*, showed significant ($p < 0.05$) downregulation across the all-highly resistant and resistant mutant strains (Figure 2). Another gene-encoding outer membrane protein C, *ompC*, was downregulated in all the resistant and highly resistant mutants but at a less significant rate compared to that of the *ompF* (Figure 2). The other five genes, *ompA*, *ompW*, *slyB*, *cpxR*, and *rpoE*, displayed a strain dependent pattern of expression (Figure 2).

Bacterial Growth Assay

Exposure to a high concentration of ciprofloxacin (e.g., 40 $\mu\text{g/mL}$) resulted in the acquisition of the SCV phenotype among the highly resistant A-7, A-21, and A-33 mutants (Figures 3A,B). To characterize the growth kinetics of the SCV



FIGURE 1 | The common mutations associated with the ciprofloxacin resistant and highly resistant mutants. **(A)** Comparative amino acid analysis of the GyrA identified amino acid substitutions S83Y and S83F among all four resistant mutants as well as double amino acid substitutions S83Y, S83F and D87G among all four highly resistant strains and one resistant strain. **(B)** Genetic organization of the *ram* locus, showing orientations of the *ramR* and *ramA* gens, their intergenic region with the RamR binding site, the *ramA* promoter sequences, inverted repeat sequences and deletion region associated with the highly resistant mutants. **(C)** Nucleotide sequence alignments of the *ramRA* intergenic regions of all three ciprofloxacin susceptible groups. Dash lines indicate deletion mutations, while the red color of nucleotide indicates nucleotide base substitution. The RamR binding site for *S. enterica* was determined by Baucheron et al. (2011).

and non-SCV mutants, we compared the growth curves of the A-21 and A-5 parental strains with their highly resistant mutants. Our data showed that the growth curve of the highly resistant A-21 mutant strain was significantly impaired ($p < 0.05$) compared to the growth curve of its parental strain (Figure 4), whereas the A-5 highly resistant mutant did not show significant growth alteration compared to its parental strain (Figure 4). To determine the effect of multiple QRDR mutations on *S. Enteritidis* fitness, we compared the growth curves of the A-5 resistant strain (i.e., GyrA S83Y, D87G; ParC S80R) and its A-5 parental strain (i.e., no QRDR mutations). There was no significant difference in the growth rate between these two strains (Figure 4). Next we compared the growth curves of the A-7 (GyrA S83Y), A-21 (GyrA S83Y) and A-33 (GyrA S83F) resistant strains with their parental strain (i.e., no QRDR mutations). The A-7, A-21 and A-33 resistant strains showed an impaired growth compared to those of other three strains (Figure 4).

DISCUSSION

Resistance to FQs class of antibiotics can be acquired vertically through *de novo* mutations and horizontally via the introduction of FQs resistance genes. The vertical evolution of FQs resistance can be very fast to allow the bacterial organism to develop resistance during a single antimicrobial treatment, which can lead to the treatment failure and death of a patient. Recently, Blair et al. (2015), examining the genomes of pre- and post-therapy isolates of *S. Typhimurium* from a patient who failed antimicrobial therapy, revealed a single mutation in the efflux pump gene, *acrB*, which was acquired during the antimicrobial treatment. This novel G288D substitution in the drug-binding pocket of AcrAB-TolC multidrug efflux pump caused resistance

to the FQs group of antibiotics but susceptibility to the other drugs (e.g., doxorubicin and minocycline), underscoring the complexity and importance of vertical evolution in the development of antimicrobial resistance. Understanding the dynamics of drug-resistant mutations and their interactions and stress adaptive physiology of bacterial organism is important not only for rational drug design but also for predicting the evolution of antibiotic resistance in clinical settings. In the current study, using four ciprofloxacin susceptible *S. Enteritidis* clinical isolates in combination with stepwise selection, we determined the type and dynamics of the mutations associated with resistance and high-level resistance to ciprofloxacin as well as the stress adaptive response of the *S. Enteritidis* FQ mutant strains.

Among 14 genes, targeting topoisomerase II (*gyrA*, *gyrB*, *parC*, *parE*), the AcrAB-TolC multidrug efflux pump (*acrA*, *acrB*, *tolC*), the efflux pump regulators (*ramR*, *ramA*), porins (*ompF*, *ompC*), extracytoplasmic stress response regulators (*rpoE*, *cpxR*), and lipopolysaccharide component (*lpxA*), the first high-frequency mutation in all the four resistant *S. Enteritidis* strains occurred in the QRDR of *gyrA*, resulting in S83Y and S83F substitutions. Among the population of the resistant strains, another amino acid substitution, S80R, in the *parC* was found only in the A-5 strain, indicating the sporadic (i.e., mutation that occurred in one or two strains) nature of this mutation. As the resistance progressed, the second high-frequency amino acid substitution, D87G, was identified in the *gyrA* among the all-highly resistant strains. Among the group of the highly resistant strains, another sporadic amino acid substitution, G78D, occurred in the *parC* of the A-7 strain, confirming that the *parC* is not the primary target of ciprofloxacin resistance in *S. Enteritidis*.

It has been shown that, in *S. Typhimurium*, the regulator RamA plays a key role in the activation of AcrAB-TolC multidrug efflux pump (Abouzeed et al., 2008; Nikaido et al., 2008). O'Regan et al. (2009) showed that inactivation of AcrAB-TolC leads to an increased susceptibility to ciprofloxacin and other non-quinolone antibiotics, clearly indicating that the AcrAB-TolC efflux pump plays a role in fluoroquinolone resistance and MDR in *S. Enteritidis*. Therefore, we examined the mutations in the coding and non-coding regions of the *ramRA* regulator in all-*S. Enteritidis* resistant and highly resistant mutant strains. The first mutation associated with the *acrAB-tolC* efflux pump was identified as an 11 bp deletion in the *ramRA* intergenic region, affecting the RamR binding site. As the exposure of *S. Enteritidis* to drug concentration increased, deletions in the *ramRA* intergenic regions, affecting the RamR binding site, intensified, resulting in a maximum frequency among the group of highly resistant mutants. To determine the effect of this mutation on antimicrobial susceptibility of *S. Enteritidis*

TABLE 4 | Differences in antimicrobial susceptibility of 5A susceptible strain and its counterparts complemented with an empty pTrc99A and pTrc99A::*ramRA* vectors.

Drug	MIC ($\mu\text{g/mL}$)		
	A-5 Susceptible	A-5 Susceptible /pTrc99A	A-5 Susceptible /pTrc99A:: <i>ramRA</i>
Azithromycin	0.12	0.25	8
Amoxicillin/clavulanic acid	<1/0.5	8/4	>32/16
Ceftiofur	1	1	>8
Tetracycline	<4	<4	32
Ciprofloxacin	0.015	0.08	1

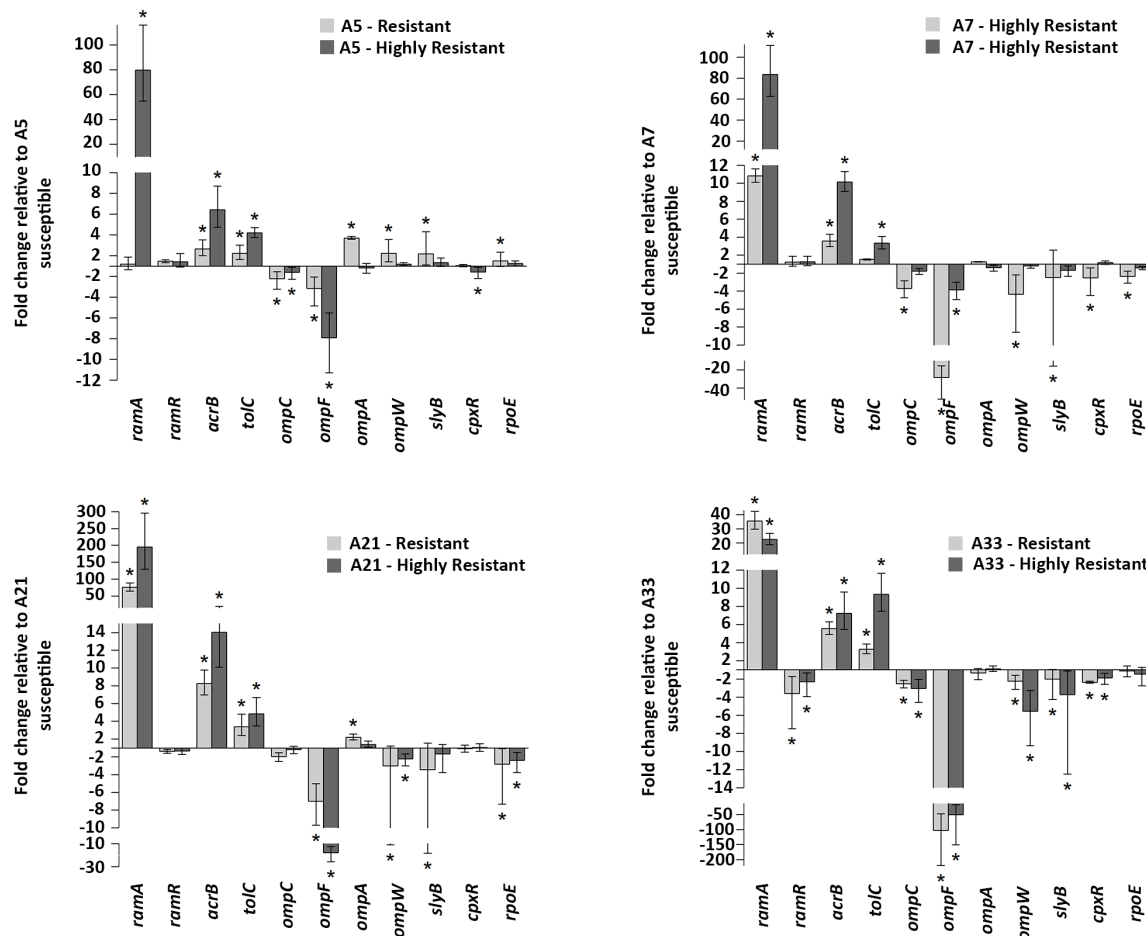


FIGURE 2 | Gene expressions. mRNA expression levels of *acrB*, *tolC* (efflux pump), *ramR*, *ramA* (regulators of the *acrAB-tolC* efflux pump), *ompF*, *ompC*, *ompA*, *ompW*, *slyB* (porins) and *rpoE*, *cpxR* (extracytoplasmic stress response regulators) in all ciprofloxacin susceptible groups. Values on the y axis are relative expression levels (fold change) normalized against levels in the resistant and highly resistant strains. The data correspond to the mean values of three biological replications. Error bars correspond to the standard deviation. Asterisks indicate statistically significant differences (* $p < 0.05$) in *t*-tests.

serovar, we complemented the A-5 parental strain with the recombinant plasmid, which contains the *ramRA* haplotype of its highly resistant A-5 mutant strain. The complemented parental strain showed complete resistance to ceftiofur and amoxicillin/clavulanic acid as well as decreased susceptibility to azithromycin and tetracycline, compared to its counterpart complemented with an empty plasmid. This experimental finding clearly points out that the deletion in the *ramRA* intergenic region, affecting the RamR binding site, plays a role in the development of multidrug resistance in *S. Enteritidis*. It is interesting that the complemented parental strain developed resistance against amoxicillin/clavulanic acid, whereas the highly resistant mutant exhibited susceptibility to the same drug. This discrepancy could be explained by the difference in the fitness landscape of these two organisms. Hartl (2014) showed that there are synergistic and antagonistic interactions among the resistance-conferring mutations, and that the comprehensive measurements of resistance should be based on the sum of these interactions. It is possible that the *ramRA* haplotype in the highly resistant

strain faced antagonistic mutations, whereas these mutations were absent in the complemented parental strain.

To gain an insight into the adaptive response of *S. Enteritidis* to high concentrations of ciprofloxacin, we determined the expression patterns of a wide range of genes in the susceptible, resistant, and highly resistant groups of *S. Enteritidis*. Most notably, the expression of genes, encoding the *acrAB-tolC* efflux pump and its activator *ramA*, were significantly upregulated in almost all the strains of the resistant and highly resistant groups. In contrast, the gene, encoding porin *ompF*, was significantly downregulated in each strain of the resistant and highly resistant groups. Several studies that investigated *ompF* expression in FQs-resistant *Salmonella* illustrated the unclear role of this porin in contribution to FQs resistance (Giraud et al., 2000; O'Regan et al., 2009). Our data shown that the downregulation of *ompF* is a common adaptive response of *S. Enteritidis*, while the downregulation of *ompC* was less profound and more strain-dependent, suggesting its minor role in the development of *Salmonella* FQs resistance. It has been shown

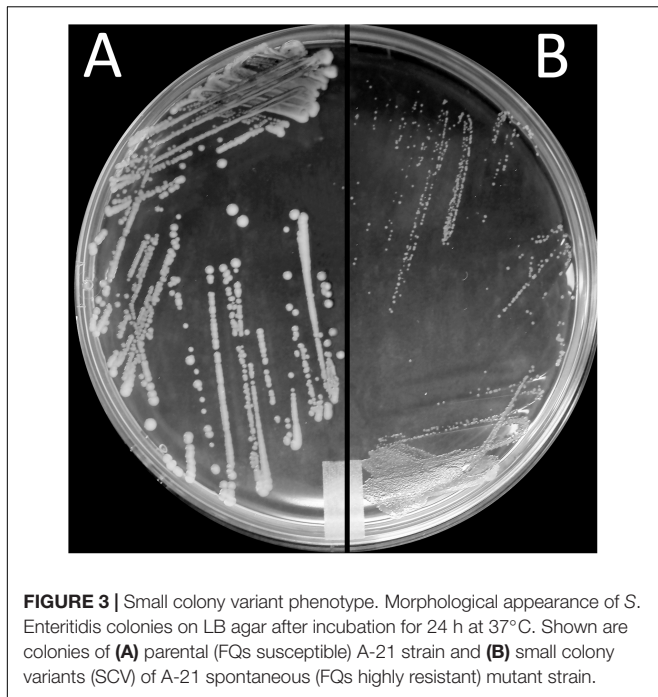


FIGURE 3 | Small colony variant phenotype. Morphological appearance of *S. Enteritidis* colonies on LB agar after incubation for 24 h at 37°C. Shown are colonies of (A) parental (FQs susceptible) A-21 strain and (B) small colony variants (SCV) of A-21 spontaneous (FQs highly resistant) mutant strain.

that mutations in the *marR* regulator result in both an increase in *acrB* expression and a decrease in *ompF* expression (Randall and Woodward, 2002; Hooper and Jacoby, 2015). Our recent study showed that the *rpoE* sigma factor also inhibits the synthesis of outer membrane porins during the antimicrobial treatment of *Escherichia coli* O157 with a polycationic agent (Vidovic et al., 2018), further illustrating that the porin-mediated permeability plays a role in the development of resistance to various antimicrobial agents.

Comparing the expression of the genes and amino acid substitutions, the A-5 resistant strain showed a distinct

characteristic compared to the rest of resistant mutants. Only the A-5 strain, once exposed to the subinhibitory concentration of ciprofloxacin did not up regulate the expression of the *ramA* gene. It is noteworthy to mention that the A-5 resistant strain acquired three amino acid substitutions (S83Y, D87G in GyrA and S80R in ParC), whereas other resistant mutants acquired one amino acid substitution at 83 position of GyrA. It has been documented that the number of QRDR amino acid substitutions has a detrimental effect on the stress adaptive physiology of several bacterial species, including *Staphylococcus aureus* (Horváth et al., 2012), *Klebsiella pneumoniae* (Tóth et al., 2014), and *Escherichia coli* (Johnson et al., 2015). Bacterial strains that acquire multiple QRDR mutations develop high-level resistance against FQs and preserve fitness (Fuzi et al., 2017). In contrast, strains with fewer QRDR mutations must rely on the FQs non-specific mechanisms, primarily on efflux, an energetically demanding mechanism, which has a direct influence on bacterial fitness (Fuzi, 2016). To determine the relationship between the energetically favorable QRDR mutations and bacterial fitness, we performed the growth assays using the A-5 resistant (multiple QRDR mutations) and its parental A-5 susceptible strain as well as the A-21 and A-33 resistant (single QRDR mutation) and their parental strains. Our data showed that the A-5 resistant strain has a similar growth curve as its parental strain, whereas the A-21 and A-33 resistant strains showed altered growth compared to its parental strain. Overall, this study provides evidence that *S. Enteritidis* with multiple QRDR mutations do not activate efflux to reach resistance to ciprofloxacin (4 µg/mL), while a strain with fewer QRDR mutations must activate efflux to reach the same MIC. Further, this stress adaptive response has an important effect on the overall fitness of *S. Enteritidis*.

Finally, this study revealed the existence of a small colony of variants (SCVs) phenotype among the resistant *S. Enteritidis* strains. These highly resistant strains of *S. Enteritidis* are characterized by a significant growth deficiency and small

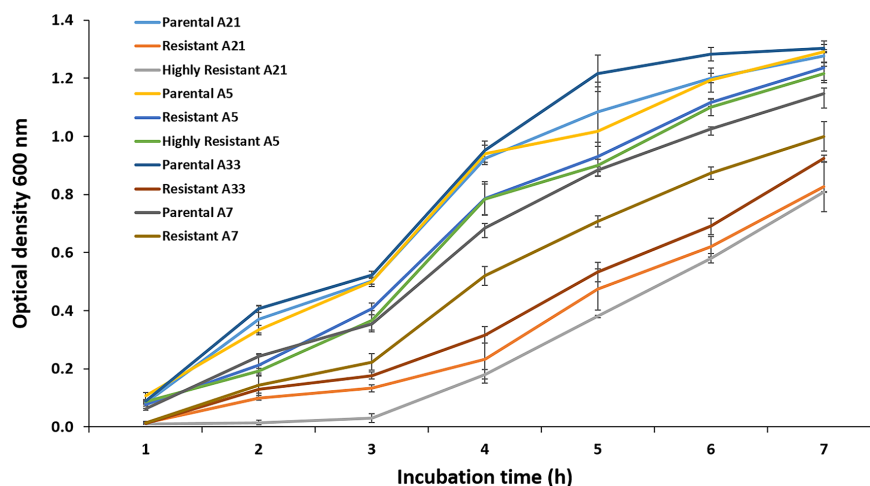


FIGURE 4 | Growth assay. The growth kinetics for the A-5, A-7, A-21, and A-33 parental and their resistant mutants were measured during the exponential growth phase. Also the growth curves were measured for the A-5 and A-21 highly resistant strains. The data correspond to the mean values of three biological replications.

colony size compared to their parental strains. It has been demonstrated that the growth rate of an organism directly correlates to the antimicrobial resistance (Fuentes-Hernandez et al., 2015; Knopp and Andersson, 2018), with the more slowly replicating organisms being more resistant compared to their faster replicating counterparts. O'Regan et al. (2010) showed that in the absence of selective pressure, a spontaneous ciprofloxacin resistant *S. Enteritidis* strain after 20 passages reverted to its parental phenotype (e.g., reversal of all fitness cost except motility). They also showed that with increased fitness of the reverted strain ciprofloxacin susceptibility of this strain significantly increased. This physiological adaptation (i.e., growth rate) most likely represents another mechanism employed by *S. Enteritidis* to confer an extremely high level of resistance to FQs. It is interesting that only the A-5 mutants did not acquire SCV phenotype and showed altered growth compared to their parental strain. These strains preserved their original fitness while developing the high level of FQ resistance further indicating most likely the existence of unique compensatory mutations that maintain the high level of FQ resistance while maintaining the original strain fitness.

In summary, the development of FQs resistance among a population of clinical isolates of *Salmonella* spp. is of a great health concern. This study provides two important novel findings. The first, the deletions in the *ramRA* region, affecting the RamR binding site, and the amino acid substitution at position 87 of the GyrA are mutations significantly associated with the highly resistant *S. Enteritidis* strains, clearly illustrating their importance in conferring a high level of FQs resistance. The second, the deletion in the intergenic region of the *ramRA* operon provides resistance and reduced susceptibility

to several antibiotics, including, ceftiofur, amoxicillin/clavulanic acid, azithromycin and tetracycline. Collectively, our study suggests complex interactions between mutations, bacterial adaptations, and FQs resistance.

AUTHOR CONTRIBUTIONS

SV conceived the study, carried out the experiments, and drafted the manuscript. RA carried out the experiments. AR performed the statistical analysis related to the gene expressions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.00729/full#supplementary-material>

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In vitro Antimicrobial Activity of Robenidine, Ethylenediaminetetraacetic Acid and Polymyxin B Nonapeptide Against Important Human and Veterinary Pathogens

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The emergence and global spread of antimicrobial resistance among bacterial pathogens demand alternative strategies to treat life-threatening infections. Combination drugs and repurposing of old compounds with known safety profiles that are not currently used in human medicine can address the problem of multidrug-resistant infections and promote antimicrobial stewardship in veterinary medicine. In this study, the antimicrobial activity of robenidine alone or in combination with ethylenediaminetetraacetic acid (EDTA) or polymyxin B nonapeptide (PMBN) against Gram-negative bacterial pathogens, including those associated with canine otitis externa and human skin and soft tissue infection, was evaluated *in vitro* using microdilution susceptibility testing and the checkerboard method. Fractional inhibitory concentration indices (FICIs) and dose reduction indices (DRI) of the combinations against tested isolates were determined. Robenidine alone was bactericidal against *Acinetobacter baumannii* [minimum inhibitory concentrations (MIC) mode = 8 µg/ml] and *Acinetobacter calcoaceticus* (MIC mode = 2 µg/ml). Against *Acinetobacter* spp., an additivity/indifference of the combination of robenidine/EDTA (0.53 > FICIs > 1.06) and a synergistic effect of the combination of robenidine/PMBN (0.5 < FICI) were obtained. DRIs of robenidine were significantly increased in the presence of both EDTA and PMBN from 2- to 2048-fold. Robenidine exhibited antimicrobial activity against *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, in the presence of sub-inhibitory concentrations of either EDTA or PMBN. Robenidine also demonstrated potent antibacterial activity against multidrug-resistant Gram-positive pathogens and all Gram-negative pathogens isolated from cases of canine otitis externa in the presence of EDTA. Robenidine did not demonstrate antibiofilm activity against Gram-positive and Gram-negative bacteria. EDTA facilitated biofilm biomass degradation for both Gram-positives

and Gram-negatives. The addition of robenidine to EDTA was not associated with any change in the effect on biofilm biomass degradation. The combination of robenidine with EDTA or PMBN has potential for further exploration and pharmaceutical development, such as incorporation into topical and otic formulations for animal and human use.

Keywords: robenidine, combination, antimicrobial, canine otitis externa, EDTA

INTRODUCTION

The widespread occurrence of multidrug-resistant (MDR) pathogens is problematic in both human and animal medicine (Morehead and Scarbrough, 2018). In particular, ESKAPE pathogens (*Enterococcus* spp., *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) deserve global attention due to the development of MDR (Santajit and Indrawattana, 2016) and increased mortality among patients (Pendleton et al., 2013). The other worrisome factor is the potential of bacteria to form biofilms that are extremely resistant to antimicrobials (Chambers and Deleo, 2009). In the past, resistance could be combated by the development of new drugs active against antimicrobial-resistant bacteria. However, the pharmaceutical industry has reduced its research efforts for the discovery and development of novel antibacterial drugs (Theuretzbacher et al., 2018). Adding to this global issue, the only novel antimicrobial classes that have been introduced in the last 20 years are the lipopeptides (daptomycin), oxazolidinones (linezolid and tedizolid) and the lipoglycopeptides (dalbavancin, oritavancin, and telavancin), which predominantly have a Gram-positive spectrum of activity (Wilcox, 2005; Saravolatz et al., 2009; Zhanel et al., 2012). The lack of novel antimicrobial development has resulted in attempts to safeguard critically important antimicrobials (antimicrobial stewardship) and a search for alternatives to treat MDR infections, including those in animals (Chan et al., 2018a; Hickey et al., 2018).

The use of critically important antimicrobials (WHO, 2017) for veterinary applications may also contribute to the development of antimicrobial resistance. For example, MDR strains of *P. aeruginosa* (MDRPA) and methicillin-resistant strains of coagulase-positive *Staphylococcus* spp. and coagulase-negative *Staphylococcus* spp., are now widespread in veterinary medicine, particularly as a cause of infections such as canine otitis, dermatitis and bovine mastitis (Beck et al., 2012; Abraham et al., 2017; Heward et al., 2018; Khazandi et al., 2018). Otitis externa is one of the most common infectious diseases in dogs, and it can be caused by both Gram-positive and Gram-negative organisms, as well as fungi. It is typically treated by topical administration of antimicrobials, such as aminoglycosides and fluoroquinolones that are critically important for human medicine (Paterson, 2016). Otitis externa treatment failures are often due to the development of antimicrobial resistance in key target pathogens, for example methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) and MDRPA (Martin Barrasa et al., 2000; Heward et al., 2018). Development of antimicrobial resistance in these companion

animal pathogens is a potential public health concern with documented transmission of MDRPA and MRSP occurring between humans and dogs within households (Lozano et al., 2017; Fernandes et al., 2018).

One approach that promotes antimicrobial stewardship and minimizes the likelihood of cross-resistance development and transmission between different host species is the repurposing of existing drugs for new applications. For example, monensin and narasin (polyether ionophores used as anticoccidials in animals, but not in humans), and closantel (a salicylanilide anthelmintic) have both been shown to be active against MRSP and methicillin-resistant *S. aureus* (MRSA) (Rajamuthiah et al., 2015; Chan et al., 2018a,b; Hickey et al., 2018). Robenidine is licensed as an anticoccidial agent and has been used safely worldwide since the early 1970s for control of coccidiosis in poultry and rabbits (Kantor et al., 1970; Bampidis et al., 2019). Recently, our laboratory reported that robenidine had antimicrobial activity against MRSA, vancomycin-resistant enterococci and *Streptococcus pneumoniae*, but no activity against Gram-negative bacteria unless robenidine was tested in combination with sub-inhibitory concentrations of polymyxin B nonapeptide (PMBN) (Abraham et al., 2016). The fact that robenidine only displays activity against Gram-negative organisms in the presence of PMBN is a good indication that robenidine acts on the cytoplasmic membrane of Gram-negative organisms, but is unable to breach the permeability barrier of the outer membrane (OM) (Arzanlou et al., 2017) in the absence of a membrane permeabilizer.

The spectrum of activity of antimicrobial agents can be extended by combining them with adjuvants. Two such agents, ethylenediaminetetraacetic acid (EDTA) and polymyxin B nonapeptide (PMBN), were selected for further investigation in this study. EDTA is a prescription medicine in humans given intravenously or intramuscularly for the treatment of lead poisoning (Selander, 1969), and is a component of many topically applied ointments, eye drops and ear cleaners (Guardabassi et al., 2010). EDTA is a bacteriostatic compound that permeabilizes the outer membrane of Gram-negative bacteria by chelating Ca^{2+} and Mg^{2+} cations (Vaara, 1992). In addition, EDTA has demonstrated antibiofilm activities against existing biofilms as well as preventing biofilm formation (Finnegan and Percival, 2015). PMBN derived from polymyxin B, whilst lacking antibacterial activity (except against *Pseudomonas* spp.), is able to render Gram-negative bacteria more susceptible to antimicrobials by increasing their outer membrane permeability without affecting bacterial cell viability (Schneider et al., 2017). It has been reported that the combination of PMBN with novobiocin or erythromycin

administered intraperitoneally successfully treated mice infected with Gram-negative pathogens (Ofek et al., 1994; Allam et al., 2017).

Our aims in this study were to evaluate the *in vitro* antimicrobial and antibiofilm activities of robenidine either alone or in the presence of EDTA or PMBN against Gram-negative bacteria predominantly associated with otitis externa of animals and skin infections of humans, and assess the activity of the most effective combination/s against field strains of canine otitis externa pathogens including *P. aeruginosa*, *Proteus mirabilis*, *S. pseudintermedius* and beta-haemolytic streptococci. We hypothesized that either EDTA or PMBN would increase the antimicrobial activity of robenidine against Gram-negative bacteria through outer membrane permeabilization.

MATERIALS AND METHODS

Antimicrobial Agents

Analytical grade robenidine was provided by Neoculi Pty Ltd., Burwood, VIC, Australia. The compound was stored in a sealed container in the dark at 4°C at the Infectious Diseases Laboratory, Roseworthy campus, The University of Adelaide. Polymyxin B nonapeptide (PMBN), ampicillin, apramycin, enrofloxacin, and gentamicin were purchased from Sigma-Aldrich (Australia). Stock solutions (25.6 mg/ml of PMBN in DMSO, 12.8 mg/ml of ampicillin in PBS, 12.8 mg/ml of apramycin in DMSO, 3.2 mg/ml of enrofloxacin in 1/2 volume of water to which was added NaOH dropwise to facilitate dissolution and 12.8 mg/ml of gentamicin in Milli-Q water) were prepared and stored in 1 ml aliquots at -80°C. They were defrosted immediately prior to use. EDTA (disodium salt) was purchased from Chem-Supply Pty Ltd., South Australia and was dissolved in Milli-Q water to 200 mM.

Bacterial Strains

Escherichia coli ATCC 25922, *E. coli* ATCC 11229, *P. aeruginosa* ATCC 27853, *P. aeruginosa* PA01, *Pseudomonas putida* ATCC 17428, *P. mirabilis* ATCC 43071, *K. pneumoniae* ATCC 13883, *A. baumannii* ATCC 19606, and *A. baumannii* ATCC 12457 were used for preliminary susceptibility testing and combination experiments. *S. aureus* ATCC 29213 and *S. pneumoniae* ATCC 49619 were used as internal quality controls. A variety of bacterial organisms from both human and canine infections were investigated in this study ($n = 119$ isolates in total). Twenty-eight clinical *Acinetobacter* spp. isolates were obtained from cases of human skin and soft tissue infections, including 18 *Acinetobacter baumannii* and 10 *A. calcoaceticus*, kindly provided by Ms Jan Bell (Institute of Medical and Veterinary Science, South Australia). It is notable that *A. baumannii* ST2 producing OXA-23 have been reported in both humans and animals, representing a possible zoonotic lineage (van der Kolk et al., 2019). Ninety-one clinical isolates were obtained from cases of canine otitis externa, including seven methicillin-susceptible *S. pseudintermedius* (MSSP), 13 multidrug- and methicillin-resistant *S. pseudintermedius* (MRSP) (Saputra et al., 2017),

20 beta-haemolytic *Streptococcus* spp., 30 *P. aeruginosa* (10 of them resistant to gentamicin and 21 *P. mirabilis* isolates). These isolates were obtained from the bacterial collection of the national survey of antimicrobial resistance in animals conducted in Australia. Swab samples from dogs with signs of otitis externa were collected by veterinarians and submitted to government, private or university diagnostic laboratories throughout Australia. After routine bacterial identification and the removal of confidential information, the participating veterinary diagnostic laboratories submitted the bacteria and their clinical information to PC2 Laboratories, Australian Centre for Antimicrobial Resistance Ecology, School of Animal and Veterinary Sciences, University of Adelaide, Roseworthy Campus, Roseworthy, SA, Australia, for further study. Thus, animal ethics approval was not required in this study. These organisms were identified to species level using biochemical testing and MALDI-TOF mass spectrometry (Bruker, Preston, VIC, Australia).

Antimicrobial Susceptibility Testing

Minimum inhibitory concentrations (MIC) were determined for robenidine, EDTA and PMBN in round bottom 96-well microtiter trays (Thermo Fisher Scientific, Australia), using the modified broth micro-dilution method recommended by the Clinical and Laboratory Standards Institute (CLSI, 2015). Testing concentrations were as follows: robenidine- 256–0.25 µg/ml; EDTA- 3800–45 µg/ml; PMBN- 32–0.06 µg/ml. Luria Bertani (LB) broth (Oxoid, Australia) was applied for MIC testing in lieu of cation-adjusted Mueller–Hinton broth as robenidine has been previously shown to chelate calcium ions. Furthermore, a twofold serial dilution of robenidine was performed in 100% DMSO, with 1 µl dispensed to each well due to the hydrophobicity of the compound (Abraham et al., 2016). The MIC for ampicillin or gentamicin against each isolate was determined for each test to serve as an internal quality control. The MICs of isolates were determined by visual reading and using an EnSpire Multimode Plate Reader 2300 at A_{600nm} . MIC₅₀, MIC₉₀, and MIC range for robenidine and EDTA were calculated against clinical isolates of *P. aeruginosa* and *P. mirabilis*, *S. pseudintermedius* and β -haemolytic *Streptococcus* spp., MIC range and MIC mode were calculated for *A. baumannii* and *A. calcoaceticus*.

Minimum Bactericidal Concentration (MBC) Determination

The MBC of robenidine alone or in combination with EDTA or PMBN against Gram-positive and Gram-negative bacteria was determined. Briefly, 10 µl aliquots from each duplicate well from the MIC assays (starting from the MIC for each compound) were inoculated onto a sheep blood agar (SBA) plate and incubated at 37°C. Plates were examined at 24 separate intervals for a period of 2 days, the MBC was recorded as the lowest concentration of each test compound at which a 99.95% colony count reduction was observed on the plate (CLSI, 1999).

Synergy Testing by Checkerboard Microdilution, Isobolograms and Dose Reduction Analysis

To assess the potential activity of robenidine, MICs against a range of Gram-negative ATCC strains as well as clinical isolates of canine otitis externa pathogens were performed in the presence or absence of 23.2–7,500 $\mu\text{g/ml}$ (0.06–20 mM) EDTA and 0.25–128 $\mu\text{g/ml}$ PMBN in a slightly modified standard checkerboard assay as described previously (Hwang et al., 2012). Briefly, antimicrobial stock solutions for robenidine and PMBN were prepared at a concentration of 12.8 mg/ml in DMSO. The antimicrobial stock solution for EDTA was prepared at a concentration of 200 mM in Milli-Q water. Then, a twofold serial dilution of each antimicrobial stock solution was prepared in its appropriate solvent (e.g., DMSO for robenidine and Milli-Q water for EDTA) from wells 12 to 3 (from 12.8 to 0.25 mg/ml for robenidine and PMBN; and 100 to 0.06 mM for EDTA). A 1 μl aliquot of the first compound from each combination was dispensed along the abscissa (from row A to G) of the 96-well microplate, while the second compound was dispensed along the ordinate (from column 12 to column 3) using an electronic multichannel pipette followed by 89 μl of LB broth. Each well of the plate was inoculated with an aliquot of 10 μl bacterial suspension at a concentration of $1\text{--}5 \times 10^6$ colony forming units (CFU) per ml. Subsequently, the plate was incubated at 37°C for 24 h. The fractional inhibitory concentration index (FICI) described the results of the combinations, and was calculated utilizing the following formula:

$$\text{FICI of combination} = \text{FICA} + \text{FICB}$$

FIC A is the MIC of robenidine in the combination/MIC of robenidine alone, FIC B is the MIC of the adjuvant (EDTA or PMBN) in the combination/MIC of the adjuvant alone. The results indicate synergism when the corresponding $\text{FICI} \leq 0.5$, additivity when $0.5 < \text{FICI} \leq 1$, indifference when $1 < \text{FICI} \leq 4$ and antagonism when the $\text{FICI} > 4$. In this study, the FIC for robenidine and PMBN against Gram-negative bacteria in the combination was calculated to be zero (e.g., $1 \div >256 = 0$) when robenidine or PMBN did not show any antibacterial activity alone against Gram-negative bacteria at the highest concentration tested (e.g., 256 $\mu\text{g/ml}$), but antimicrobial activity was observed when the compounds were tested in combination.

The results of the checkerboard experiments are illustrated by isobolograms, as follows: The MIC of drug A is marked on the x-axis of an isobologram and the MIC of drug B on the y-axis, with the line connecting the two marks representing the indifferent line (no interaction) (Tallarida, 2006). The MIC values of the combination located below the indifference line indicate additive ($1 \geq \text{FICI} > 0.5$) or synergistic ($\text{FICI} \leq 0.5$) interactions. Values that are found above the indifferent line indicate indifferent ($1 < \text{FICI} \leq 4$) or antagonistic ($\text{FICI} > 4$) interactions (Hwang et al., 2012).

The dose reduction index (DRI) shows the difference between the effective doses in combination in comparison to its individual dose. DRI was calculated as follows:

$$\text{DRI} = \text{MIC of drug alone} / \text{MIC of drug in combination}$$

Robenidine and PMBN did not show any antimicrobial activity against the majority of Gram-negative bacteria tested, the highest concentration of each compound tested against each isolate was included in the DRI equation as its MIC [e.g., the MIC of robenidine alone against *E. coli* was >256 ($\mu\text{g/ml}$) and its MIC in combination with EDTA was 1 ($\mu\text{g/ml}$); $\text{DRI} = 256/1$].

Dose reduction indices is very important clinically when the dose reduction is associated with a toxicity reduction without changing efficacy (Eid et al., 2012). Commonly, a DRI higher than 1 is considered beneficial.

Time-Dependent Killing Assays

Time kill assays were performed (in duplicate) for the robenidine $\pm$ EDTA assays as described previously (CLSI, 1999) with slight modifications. Briefly, colonies of each bacterium (*P. aeruginosa* ATCC 27853, *P. aeruginosa* PA01, a clinical isolate of *P. aeruginosa* from canine otitis externa, *A. baumannii* ATCC 19606, human clinical isolates of *A. baumannii* B10 and *A. baumannii* B11) from overnight SBA plates were separately emulsified in normal sterile saline and adjusted to $A_{600\text{nm}} = 0.10$ (equivalent to approximately 5×10^7 CFU/ml). Subsequently, the bacterial suspensions were further diluted 1:10 in sterile saline. The robenidine or EDTA were serially diluted in 100% DMSO or Milli-Q water at 100 $\times$ the final desired concentration and a 100 μl aliquot of appropriate concentrations added to each 10 ml preparation. Robenidine or EDTA solution was prepared in 10 ml volumes at MIC and 2 $\times$ MIC concentration in LB broth. After adding inoculum dose to each tube, duplicate cultures were incubated at 37°C, with samples withdrawn at 0, 0.5, 1, 2, 4, and 24 h, serially diluted tenfold and plated on SBA overnight at 37°C for bacterial enumeration. According to CLSI, an antimicrobial agent is considered bactericidal if it causes a $\geq 3 \times \log_{10}$ (99.95%) reduction in CFU/ml after 18–24 h of incubation, and the combination is considered synergistic when it causes a $\geq 2 \times \log_{10}$ reduction in CFU/ml (Tängdén et al., 2014).

Antibiofilm Susceptibility Testing

The minimum biofilm eradication concentration (MBEC) was determined for robenidine and EDTA using the MBECTM High-throughput assay system (MBECTM BioProducts, Innovotech, Canada) consisting of a lid with 96 pegs and a 96-well microtiter plate as previously described (Ceri et al., 2001; Harrison et al., 2010). Briefly, biofilms of *P. aeruginosa* PA01, two clinical isolates of *P. aeruginosa* isolates and two clinical isolates of *S. pseudintermedius* were formed by inoculating 150 μl of 10^7 CFU/ml of each bacterial suspension in the MBECTM device. The inoculated device was aerobically incubated on an orbital shaker at 37°C (OM11, Ratek Instruments Pty Ltd., Australia) for 24 h to produce equivalent (Uniform) biofilms on all pegs. Biofilms of *P. aeruginosa* and *S. pseudintermedius* were exposed to challenge plates

containing a serial concentration of robenidine (from 0.125 to 128 $\mu\text{g/ml}$) or EDTA (1–32 mM) and incubated at 37°C for 24 h. Following antimicrobial challenge, the biofilms were rinsed twice with phosphate buffered saline (pH = 7) and disrupted via sonication (Soniclean, Model 160TD, Australia) for 10 min into the recovery medium. Viable cell counts were determined for recovered cells (colony-forming units per peg) after preparing a serial dilution and plating 10 μl in duplicates of each dilution onto plate count agar. Viable counts were then expressed as a percentage of the mean CFU of growth controls. MBEC was defined as the lowest concentration of antimicrobial agent that eradicates the biofilms recovered from the antimicrobial challenge.

Checkerboard Microdilution Assay for Antibiofilm Activity of Robenidine

A slightly modified standard checkerboard assay was used to determine the activity of robenidine in the presence or absence of 37.2–12,000 $\mu\text{g/ml}$ (1–32 mM) EDTA as described previously (Hwang et al., 2012). Briefly, the MBECTM High-throughput assay system (MBECTM BioProducts, Innovotech, Canada) was used for the preparation of Gram-positive and Gram-negative biofilm producing bacteria as described above for antibiofilm susceptibility testing. The antimicrobial stock solution for EDTA was prepared at a concentration of 128 mM in Milli-Q water and robenidine was prepared at 12.8 mg/ml in DMSO. Then, a twofold serial dilution of each antimicrobial stock solution was prepared in its appropriate solvent from wells 12 to 3 (from 12.8 to 0.25 mg/ml for robenidine and 128 to 1 mM for EDTA). A 2 μl aliquot of robenidine compound from each concentration was dispensed along the abscissa (from row A to H) of the 96-well microplate, while 100 μl of EDTA was dispensed along the ordinate (from column 12 to column 3) using an electronic multichannel pipette followed by 98 μl of LB broth. Subsequently, the plate was incubated at 37°C for 24 h.

In vitro Cytotoxicity Assays

A panel of adherent mammalian cell lines, HaCat (human immortalized keratinocytes), HEK 293 (human embryonic kidney) and MDCK (normal Madin Darby Canine Kidney) were assayed for *in vitro* cytotoxicity of robenidine alone or in combination with EDTA or PMBN. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% PenStrep (100 U/mL Penicillin and 100 $\mu\text{g/ml}$ Streptomycin) at 37°C with 5% CO₂. Cells were serially passaged at ~80% confluence ~ every 4 days. Assays were performed in duplicate in 96 well plates seeded with ~25,000 cells per well. After 24 h, media was removed, washed once with medium without antimicrobials and replaced with fresh media to which robenidine in the presence or absence of EDTA and PMBN were added same concentrations used for antimicrobial susceptibility testing. Briefly, the antimicrobials were prepared by performing a twofold serial dilution at 100 $\times$ of tested concentration in DMSO. Subsequently, a 1 μl aliquot of each concentration was transferred to a sterile

96-well plate containing fresh DMEM with either 10% FBS. After mixing four times, the media aliquots with different concentrations of antimicrobial were transferred to each well of the 96-well plate seeded with cells, using wells containing 1–2% DMSO only as control. To determine the effect of FBS on the cytotoxicity of each compound, DMEM with 40% FBS containing different concentrations of antimicrobial was prepared as described above. After 24 h of exposure, WST-1 reagent (Cell Proliferation Assay reagent, Roche) at a concentration of 10% was added to each well. Absorbance at 450 nm on a Multiskan Ascent 354 Spectrophotometer (Labsystems) was measured after 1 h of incubation. The IC₅₀ value was determined for each compound against each cell line via non-linear regression (three parameters) using GraphPad Prism v6 software.

RESULTS

Antimicrobial Activity of Robenidine Against Gram-Negative Control Strains

Robenidine did not demonstrate any antimicrobial activity against Gram-negative control strains (*E. coli* ATCC 25922, *E. coli* ATCC 11229, *P. aeruginosa* ATCC 27853, *P. mirabilis* ATCC 43071 and *K. pneumoniae* ATCC 13883) at the highest concentrations (256 $\mu\text{g/ml}$) tested except for *A. baumannii* ATCC 19606 (32 $\mu\text{g/ml}$) and *A. baumannii* ATCC 12457 (64 $\mu\text{g/ml}$).

Antimicrobial Activity of Robenidine Against Human Clinical *Acinetobacter* spp.

The MIC results of robenidine against *A. baumannii* and *A. calcoaceticus* isolated from human clinical cases were demonstrated at concentrations ranging from 8 to 64 $\mu\text{g/ml}$ (MIC mode = 8 $\mu\text{g/ml}$) for 18 *A. baumannii* and 1–8 $\mu\text{g/ml}$ (MIC mode = 2 $\mu\text{g/ml}$) for 10 *A. calcoaceticus*. The ratio of MBC/MIC values for both *Acinetobacter* spp. was either 2 $\times$ or 4 $\times$ their MICs.

Combination of Robenidine With EDTA or PMBN Against Gram-Negative Control Strains

The presence of EDTA in combination with robenidine was associated with a notable increase in the potency and spectrum of activity against Gram-negative control strains. The results of MIC and DRI values for the combination of robenidine and EDTA against *E. coli* ATCC 25922, *E. coli* ATCC 11229, *P. aeruginosa* ATCC 27853, *P. mirabilis* ATCC 43071, *K. pneumoniae* ATCC 13883, *A. baumannii* ATCC 19606, and *A. baumannii* ATCC 12457 are presented in **Table 1**. The combination of robenidine and EDTA resulted in a synergistic interaction against the standard isolates of *E. coli* as well as against *P. aeruginosa* ATCC 27853, *P. putida* ATCC 17428, *P. aeruginosa*, and *K. pneumoniae* ATCC 13883. An additive/indifferent interaction was recorded against *P. mirabilis*,

TABLE 1 | The MIC ($\mu\text{g/ml}$) values for robenidine, EDTA and the combination effect of EDTA on the MIC of robenidine for Gram-negative control strains and a human clinical *A. calcoaceticus* isolate.

Isolates	MIC (μg/ml; mM concentrations in parentheses)		Combination Effect (FICI) ^b	DRI ^c	
	Single drug				Combination
	EDTA	ROB ^a			EDTA:ROB
<i>E. coli</i> ATCC 25922	3800 (10)	>256	950 (2.5):4	Synergism (0.25)	4:64
<i>E. coli</i> ATCC 11229	950 (2.5)	>256	228 (0.6):8	Synergism (0.25)	4:32
<i>P. putida</i> ATCC 17428	1900 (5)	>256	950 (2.5):1.25	Synergism (0.5)	2:256
<i>P. aeruginosa</i> PA01	1900 (5)	>256	950 (2.5):1.25	Synergism (0.5)	2:256
<i>P. aeruginosa</i> ATCC 27853	3800 (10)	>256	1900 (5):1.25	Synergism (0.5)	2:256
<i>P. mirabilis</i> ATCC 43071	228 (0.6)	>256	228 (0.6):1.25	Additivity (1)	1:256
<i>K. pneumoniae</i> ATCC 13883	11400 (30)	>256	3800 (10):128	Synergism (0.33)	3:2
<i>A. baumannii</i> ATCC 19606	380 (1)	32	190 (0.5):4	Additivity (0.62)	2:8
<i>A. baumannii</i> ATCC 12457	190 (0.5)	64	95 (0.25):2	Additivity (0.53)	2:32
<i>A. calcoaceticus</i>	228 (0.6)	4	228 (0.6):0.125	Indifference (1.06)	1:32

^aROB, robenidine; ^bthe results indicate synergism when the corresponding FICI ≤ 0.5 ; additivity when $0.5 < \text{FICI} \leq 1$, indifference when $1 < \text{FICI} \leq 4$ and antagonism when the FICI > 4 ; ^cDRI, dose reduction index.

TABLE 2 | The MIC ($\mu\text{g/ml}$) values for robenidine, PMBN and the combination effect of PMBN on the MIC of robenidine for Gram-negative control strains and a human clinical *A. calcoaceticus*.

Isolates	Antimicrobial concentration (μg/ml)		Combination	Combination Effect (FICI) ^c	DRI ^d
	Single drug				
	PMBN ^a	ROB ^b			
			PMBN:ROB		EDTA:ROB
<i>E. coli</i> ATCC 25922	>32	>256	6:8	Synergism (<0.5)	5:32
<i>E. coli</i> ATCC 11229	>32	>256	6:8	Synergism (<0.5)	5:32
<i>P. putida</i> ATCC17428	4	>256	1:1	Synergism (0.25)	4:256
<i>P. aeruginosa</i> ATCC27853	2	>256	0.75:1	Synergism (0.25)	4:256
<i>P. mirabilis</i> ATCC 43071	>32	>256	NA ^e	No effect	NA
<i>K. pneumoniae</i> TCC13883	>32	>256	0.5:4	Synergism (<0.5)	64:64
<i>A. baumannii</i> ATCC19606	>32	32	1:2	Synergism (0.07)	32:16
<i>A. baumannii</i> ATCC 12457	>32	64	1:2	Synergism (0.03)	32:32
<i>A. calcoaceticus</i>	>32	4	1.5:0.5	Synergism (0.12)	21:8

^aPMBN, polymyxin B nonapeptide; ^bROB, robenidine; ^cthe results indicate synergism when the corresponding FICI ≤ 0.5 ; additivity when $0.5 < \text{FICI} \leq 1$, indifference when $1 < \text{FICI} \leq 4$ and antagonism when the FICI > 4 ; ^dDRI, dose reduction index; ^eNA, no antimicrobial activity was recorded.

A. baumannii, and *A. calcoaceticus* control strains. DRIs of robenidine were significantly increased in the presence of EDTA from 2- to 256-fold (**Table 1**).

The results of MIC, FICI, and DRI values for the combination of robenidine and PMBN against *E. coli* ATCC 25922, *E. coli* ATCC 11229, *P. aeruginosa* ATCC 27853, *P. mirabilis* ATCC 43071, *K. pneumoniae* ATCC 13883, *A. baumannii* ATCC 19606, and *A. baumannii* ATCC 12457 are presented in **Table 2**. The combination of robenidine and PMBN resulted in a synergistic interaction against all the isolates tested except *P. mirabilis* ATCC 43071 (**Table 2**). DRIs of robenidine were significantly increased in the presence of PMBN from 8- to 256-fold (**Table 2**).

Isobologram analyses were carried out for the combination of EDTA and robenidine against *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *P. mirabilis* ATCC 43071, and for the combination of PMBN and robenidine against *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853.

Dose-effect curves for drugs with different maxima and the corresponding isobole combination are presented in **Figure 1**. Isoboles of the combination of EDTA and robenidine against *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 indicated synergism. Similarly, isoboles of the combination of PMBN and robenidine against *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 also indicated synergism.

Antimicrobial Activity of Robenidine Against Canine Otitis Externa Pathogens

MIC range, MIC₅₀, MIC₉₀ ($\mu\text{g/ml}$) values of robenidine, gentamicin, apramycin, and ampicillin against quality control strains (*S. aureus* ATCC 29213, *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922) and clinical isolates from otitis externa cases in dogs [*S. pseudintermedius* ($n = 20$), beta-haemolytic streptococci ($n = 20$), *P. mirabilis* ($n = 21$), and *P. aeruginosa* ($n = 30$)] are presented in **Table 3**.

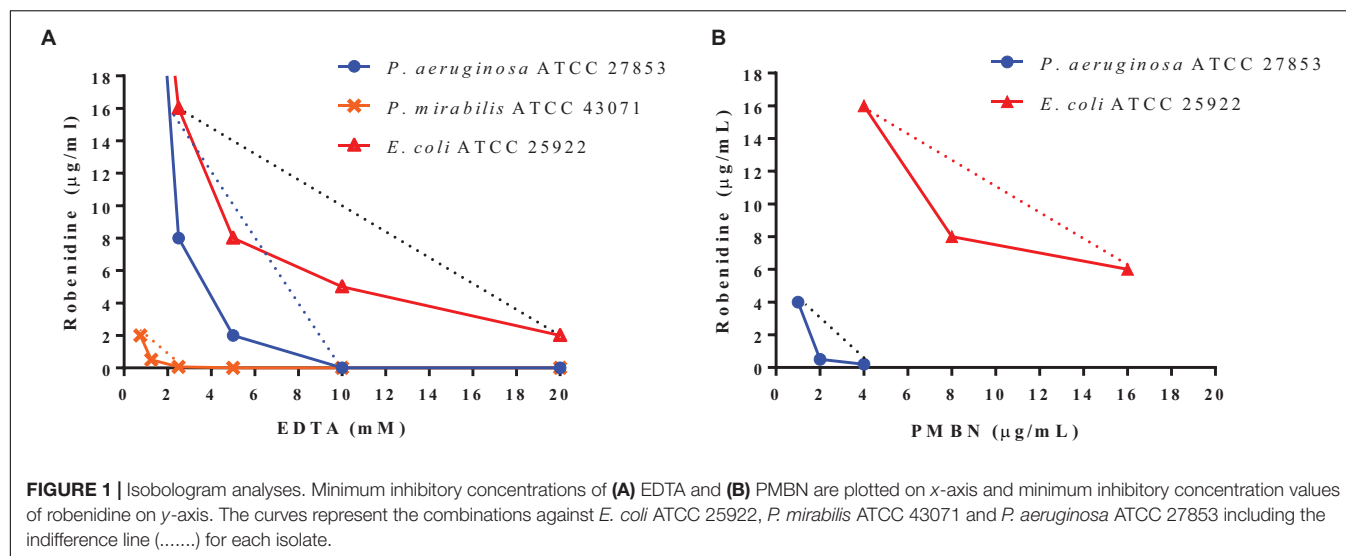


TABLE 3 | The MIC range, MIC₅₀, MIC₉₀ (μg/ml) values of robenidine, gentamicin, apramycin, and ampicillin against control strains, including *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *S. aureus* ATCC 29213, *S. pneumoniae* ATCC 49619 and clinical isolates from otitis externa cases in dogs, including *S. pseudintermedius* (*n* = 20), beta-haemolytic *Streptococci* (*n* = 20), *P. mirabilis* (*n* = 21), and *P. aeruginosa* (*n* = 30).

Value		MIC (μg/ml)			
		Robenidine	Gentamicin	Apramycin	Ampicillin
<i>P. aeruginosa</i> (<i>n</i> = 30)	MIC range	>256	0.25–64	16–64	– ^a
	MIC ₅₀	>256	32	32	–
	MIC ₉₀	>256	64	64	–
<i>P. mirabilis</i> (<i>n</i> = 21)	MIC range	>256	4–> 128	16–64	–
	MIC ₅₀	>256	8	32	–
	MIC ₉₀	>256	32	64	–
<i>S. pseudintermedius</i> (<i>n</i> = 20)	MIC range	1–4	1–64	1–16	0.03–32
	MIC ₅₀	2	2	8	0.125
	MIC ₉₀	2	2	16	8
Beta-haemolytic streptococci (<i>n</i> = 20)	MIC range	4–16	8–16	4–128	0.06–0.5
	MIC ₅₀	8	8	64	0.125
	MIC ₉₀	8	8	128	0.25
Quality control strains					
<i>S. aureus</i> ATCC 29213	MIC	2	0.5	4	1
<i>P. aeruginosa</i> ATCC 27853	MIC	–	2	16	–
<i>E. coli</i> ATCC 25922	MIC	–	0.5	8	4
<i>S. pneumoniae</i> ATCC 49619	MIC	–	–	–	0.125

^aAntimicrobial activity was not tested.

Combination of Robenidine With EDTA Against Canine Otitis Externa Pathogens

Minimum inhibitory concentrations and DRI values for the combination of robenidine and EDTA against 30 *P. aeruginosa*, 21 *P. mirabilis*, 20 *S. pseudintermedius*, and 20 beta-haemolytic streptococci isolated from canine otitis externa cases are shown in Table 4. The clinical isolates of *P. aeruginosa* including 10 antimicrobial-resistant isolates, showed a synergistic interaction with the combination of robenidine and EDTA. An additivity interaction (95.3%) was recorded against clinical isolates of *P. mirabilis* in the combination of robenidine and EDTA.

The DRIs of robenidine for *P. aeruginosa* and *P. mirabilis* isolates increased between 64- and 2048-fold, and the DRIs of EDTA increased two and fourfold. Additionally, additive and indifferent activity of the combination robenidine and EDTA was observed against clinical isolates of MRSP, MSSP and beta-haemolytic streptococci.

Robenidine demonstrated antibacterial activity against Gram-positive bacteria, with MIC values ranging from 1 to 16 μg/ml against *S. pseudintermedius* and beta-haemolytic streptococci, respectively. The lowest level of interaction was recorded for the combination of robenidine and EDTA against *S. pseudintermedius* and beta-haemolytic streptococci. However,

TABLE 4 | The MIC range, MIC₅₀, MIC₉₀ (μg/ml) and DRI values for robenidine, EDTA alone and their combination against 30 *Pseudomonas aeruginosa*, 21 *Proteus mirabilis*, 20 *Staphylococcus pseudintermedius*, and 20 beta-haemolytic streptococci isolated from otitis externa cases in dogs.

Isolates	Value	Antimicrobial concentration (μg/ml; mM concentrations in parentheses)				Combination Effect ^b (percentage)		DRI ^c	
		Single drug		Combination				EDTA	ROB
		EDTA	ROB ^a	EDTA	ROB				
<i>P. aeruginosa</i> [<i>n</i> = 30; MSSP (20), MRSP (10)]	MIC range	750–4500 (2–12)	>256	380–1500 (1–4)	0.125–4	Synergism (100%)		2–3	64–2048
	MIC ₅₀	3000 (8)	>256	1500 (4)	0.25			2	1024
	MIC ₉₀	3000 (8)	>256	1500 (4)	2			2	128
	MBC/MIC	BS ^d	NA ^e	750 (2)	≥2				
<i>P. mirabilis</i> (<i>n</i> = 21)	MIC range	190–750 (0.5–2)	>256	190–380 (0.5–1)	0.125–4	Additivity (95.3%) Synergism (4.7%)		1–2	64–2048
	MIC ₅₀	380 (1)	>256	380 (1)	0.125			2	2048
	MIC ₉₀	750 (2)	>256	380 (1)	0.5			2	512
	MBC/MIC	BS	NA	750 (2)	≥2				
<i>S. pseudintermedius</i> [<i>n</i> = 20; MSSP (7), MRSP (13)]	MIC range	95–380 (0.25–1)	1–4	95 (0.25)	0.25–2	Additivity (100%)		1–4	2–8
	MIC ₅₀	190 (0.5)	2	95 (0.25)	1			2	2
	MIC ₉₀	190 (0.5)	2	95 (0.25)	1			2	2
	MBC/MIC	BS	1.5	750 (2)	2				
Beta-haemolytic streptococci (<i>n</i> = 20)	MIC range	190–750 (0.5–2)	4–16	95–380 (0.25–1)	1–8	Additivity (100%)		1–2	2–8
	MIC ₅₀	380 (1)	8	190 (0.5)	2			2	4
	MIC ₉₀	750 (2)	8	380 (1)	4			1	2
	MBC/MIC	BS	1.75	750 (2)	2				

^aROB, robenidine; ^b the results indicate synergism when the corresponding FICI ≤ 0.5; additivity when 0.5 < FICI ≤ 1, indifference when 1 < FICI ≤ 4; ^cDRI, dose reduction index; ^dBS, bacteriostatic compound; ^eNA, no antimicrobial activity was recorded.

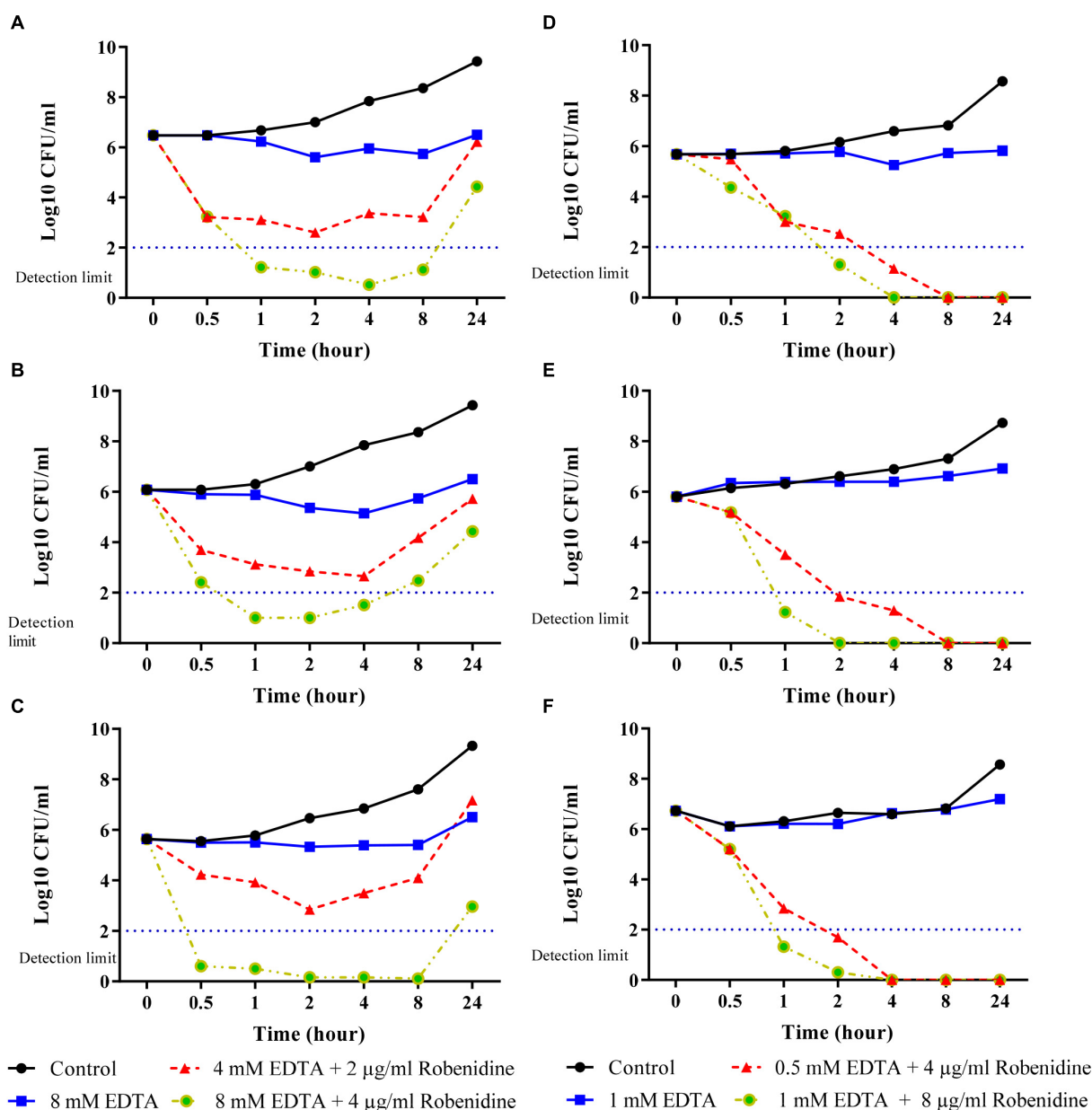


FIGURE 2 | Time kill curves of EDTA and the combination of robenidine and EDTA against (A) *Pseudomonas aeruginosa* ATCC 27853, (B) *P. aeruginosa* PA01, (C) a clinical isolate of *P. aeruginosa* from dog (D) *Acinetobacter baumannii* ATCC 19606, (E) a clinical isolate of *A. baumannii* B10 from human and (F) a clinical isolate of *A. baumannii* B11 from human. Control represents bacteria incubated in the absence of EDTA and robenidine. Bactericidal activity of robenidine in the combination with EDTA was defined as a reduction in the numbers of viable bacteria of $\geq 3 \log_{10}$ CFU/ml at any incubation time tested.

the dose reduction for beta-haemolytic streptococci ranged from 2- to 16-fold for robenidine and twofold for EDTA.

Time Kill Kinetics of Drug Combinations Against *P. aeruginosa* and *A. baumannii*

Time kill curves for robenidine in the presence of EDTA at the concentration of MIC₉₀ (2 μg/ml robenidine + 1,500 μg/ml or 4 mM of EDTA) and 2× MIC₉₀ (4 μg/ml robenidine + 3,000 μg/ml or 8 mM of EDTA)

were obtained for *P. aeruginosa* ATCC 27853, *P. aeruginosa* PA01 and a clinical isolate of *P. aeruginosa* from a canine otitis externa case are presented in Figures 2A–C. The combination of robenidine and EDTA at MIC₉₀ significantly reduced the colony count of *P. aeruginosa* isolates (about 3 log₁₀) over 0.5, 1, 2, and 4 h with a synergistic effect in comparison to the control growth and EDTA alone. However, at 24 h, bacterial regrowth was observed to almost the same level as the sample treated with EDTA alone. Further reductions of the bacteria (greater than 5 log₁₀ CFU/ml reduction) at 0.5 h were recorded when

the EDTA concentration increased from MIC₉₀ (3,000 µg/ml or 4 mM) to 2× MIC₉₀ (3,000 µg/ml or 8 mM) in comparison to control and EDTA alone. A minimum of a 5 log₁₀ reduction was still evident at 4 h incubation, however, after 24 h the numbers of bacteria present had increased. However, this reduction (approximately 5 log₁₀ reduction) remained consistent in comparison to growth control.

Time kill curves for robenidine in the presence of EDTA at the concentration of MIC (4 µg/ml robenidine + 188 µg/ml or 0.5 mM of EDTA) and 2× MIC (8 µg/ml robenidine + 376 µg/ml or 1 mM of EDTA) for *A. baumannii* ATCC 19606, two human clinical isolates of *A. baumannii* (B10 and B11) from canine otitis externa are presented in **Figures 2D–F**. The combination of robenidine and EDTA at both MIC and 2× MIC significantly reduced the colony counts of *A. baumannii* ATCC 19606 and two clinical isolates of *A. baumannii* over 1 and 2 h with a synergistic effect in comparison to the control growth and EDTA alone. After 8 and 4 h, bacteria were eliminated for tested isolates in both MIC and 2× MIC, respectively.

Antibiofilm Activity of Robenidine Alone and in the Presence of EDTA

Preformed biofilms of *P. aeruginosa* PA01, two clinical isolates of *P. aeruginosa* and two clinical isolates of *S. pseudintermedius* were tested against robenidine and EDTA to determine their activities. Robenidine at concentration of up to 128 µg/ml did not show any antibiofilm activity against *P. aeruginosa* and *S. pseudintermedius* isolates in comparison to enrofloxacin as a positive control (**Figures 3A,B**). However, 1 mM concentration of EDTA demonstrated a significantly effect in disrupting the 24 h preformed biofilms in comparison to growth control against both Gram-positive and Gram-negative bacteria (**Figure 3C**). EDTA was more effective against the biofilms when the concentration of EDTA increased to 16 mM. However, the presence of robenidine in combination with EDTA was not associated with any change in the antibiofilm activity of EDTA against both Gram-positive and Gram-negative bacteria. The results of the antibiofilm activity of the EDTA in the presence of robenidine against *P. aeruginosa* PA01 are shown in **Figure 3D**.

Robenidine Cytotoxicity to Mammalian Cell Lines

The cytotoxicity profile of robenidine in the presence and absence of EDTA and PMBN was evaluated in a panel of different cultured mammalian cells using the WST-1 Cell Proliferation Assay reagent (Roche). The results of the *in vitro* cytotoxicity measurements show IC₅₀ values of 12 µg/ml for robenidine, IC₅₀ values of 3.4 mM for EDTA, while PMBN gave IC₅₀ values of > 32 µg/ml against all the cell lines tested (**Table 5**). We found that the *in vitro* cytotoxicity measurements show IC₅₀ values of 12 µg/ml for robenidine in the presence of either 3.4 mM for EDTA or 32 µg/ml for PMBN (**Table 5**). Real-time cell viability measurements using HaCaT and HEK 239 cell lines also confirmed no measurable effect on cell viability for robenidine at either 12 µg/ml up to 24 h post-treatment alone or in the

presence of either 3.4 mM for EDTA or 32 µg/ml for PMBN. Real-time cell viability showed that the combination of 8 µg/ml robenidine with 4 mM EDTA was not toxic during the first 12 h of assays. Importantly, the toxicity of robenidine alone or in the presence of either EDTA or PMBN was significantly reduced from 12 µg/ml to higher than 32 µg/ml for all tested cell lines when the amount of FBS was increased from 10 to 40% (**Table 5**).

DISCUSSION

Bacterial pathogens have developed numerous resistance strategies against antimicrobial agents used in both humans and animals. A major challenge in successful treatment of bacterial infections is the emergence and rapid global spread of multidrug-resistant clones that are refractory to current antimicrobial therapy. To address this problem, we have examined and repurposed robenidine as a new class of antibacterial agent. To evaluate the potential of robenidine as an antibacterial agent, we previously assessed its potency, metabolic stability, pharmacokinetic and safety profiles, in a mouse PK study and a series of *in vitro* efficacy and cell toxicity studies (Abraham et al., 2016; Ogunniyi et al., 2017). We identified that robenidine had a predominantly Gram-positive spectrum of activity, and that the site of action was likely to be the cytoplasmic membrane (Ogunniyi et al., 2017) hence this compound should potentially have an antimicrobial effect on Gram-negative organisms. The Gram-positive selective activity of robenidine is most likely to be a result of the inability of this compound to traverse the outer membrane of Gram-negative organisms (Arzanlou et al., 2017). In the present study, we extended our analyses by assessing *in vitro* efficacy against a range of clinical human and animal Gram-negative bacterial isolates in the presence or absence of sub-inhibitory concentrations of EDTA and PMBN.

We found that robenidine showed antimicrobial activity against *Acinetobacter* spp. even in the absence of OM permeabilisation, and its MICs were reduced 8- to 32-fold in the presence of EDTA and PMBN. This result is quite surprising as the permeability of the OM of *A. baumannii* is estimated to be only 1–8% that of *E. coli* as *A. baumannii* lacks the general, non-specific trimeric porins found in *E. coli* (Nikaido, 2003; Zgurskaya et al., 2015). The general architecture of the OM between *A. baumannii* and other Gram-negative bacteria is the same, however, lipid A in *A. baumannii* is acylated with C12 and C14 fatty acids, compared with C10 and C12 fatty acids in *E. coli* (Zgurskaya et al., 2015). As a result, the hydrophobic core of *A. baumannii* is expected to be thicker and lipid A should occupy a larger area per lipid. These features are likely to make the OM of *A. baumannii* more hydrophobic and could be responsible for the susceptibility of this organism to amphiphilic antimicrobials such as novobiocin and tetracycline (Krishnamoorthy et al., 2017). Similarly, robenidine is an amphiphilic molecule and the same differences in the OM of *A. baumannii* could increase its susceptibility to robenidine.

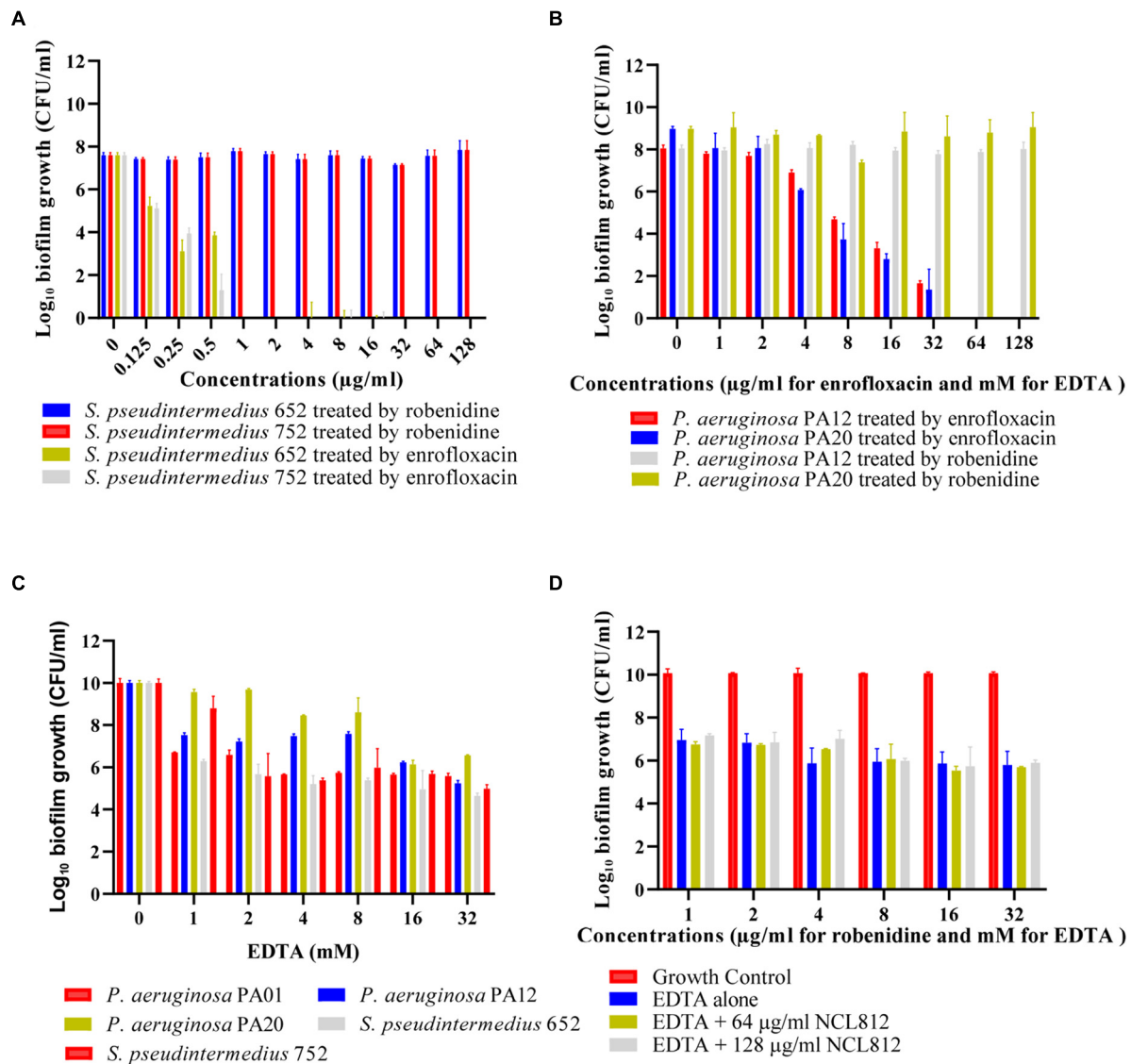


FIGURE 3 | Antibiofilm activity of robenidine, EDTA, the combination of robenidine and EDTA and enrofloxacin as a control against *P. aeruginosa* PA01, clinical isolates of *P. aeruginosa* (PA12 and PA20) and *S. pseudintermedius* (652 and 752) from dogs. **(A)** antibiofilm activity of robenidine against *S. pseudintermedius* isolates (652 and 752), **(B)** antibiofilm activity of robenidine against *P. aeruginosa* PA01 and clinical isolate of *P. aeruginosa*, **(C)** antibiofilm activity of EDTA against *P. aeruginosa* PA01, two clinical isolates of *P. aeruginosa* (PA12 and PA20) and *S. pseudintermedius* (652 and 752), and **(D)** antibiofilm activity of the EDTA in the presence of robenidine against *P. aeruginosa* PA01.

Given these encouraging results for antibacterial activity against *Acinetobacter* spp., the safety and efficacy of robenidine could be further explored in animal models of *Acinetobacter* infection (Paluchowska et al., 2017; Gorla et al., 2018) prior to further clinical development. In addition, using an appropriate formulation can improve the potency and safety of robenidine as a novel treatment for infections caused by *A. baumannii* (Paluchowska et al., 2017; Gorla et al., 2018) and *A. baumannii-calcoaceticus* complex (Clark et al., 2016; Ozvatan et al., 2016), which are reported to be emerging pathogens worldwide (Gales et al., 2001). Our results show that EDTA would be a suitable adjuvant for topical delivery but not systemic use due

to the high concentrations of Ca^{2+} and Mg^{2+} in blood, while PMBN does not have this limitation and could be included as a possible adjuvant in both topical and systemic *Acinetobacter* infection models.

Robenidine in the presence of sub-inhibitory concentrations of EDTA or PMBN also displayed improved antibacterial activity against a variety of ESKAPE isolates (*S. aureus*, *E. coli*, *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa*). In the case of PMBN, it resulted in a 4- to 256-fold increase in the susceptibility of tested Gram-negative ATCC strains of ESKAPE pathogens in combination with robenidine, inhibiting growth at robenidine concentrations as low as 0.125 μg/ml, whilst the MIC

TABLE 5 | IC₅₀ data for robenidine, EDTA, PMBN and robenidine in the combination with either EDTA or PMBN against the HaCaT, HEK 293, and MDCK cell lines in the presence of 10 or 40% FBS in DMEM.

Agent	IC ₅₀ values (μg/ml for robenidine and PMBN; mM for EDTA)									
	ROB ^a		EDTA		PMBN		ROB:EDTA		ROB:PMBN	
FBS	10% ^b	40% ^c	10%	40%	10%	40%	10%	40%	10%	40%
HaCaT	14	>32	3.8	3.8	>32	>32	12:3	>32:3	12:>32	>32:>32
HEK 293	12	>32	3.4	3.4	>32	>32	12:3	>32:3	12:>32	>32:>32
MDCK	12	>32	3.4	3.4	>32	>32	12:3	>32:3	12:>32	>32:>32

^aROB, robenidine, ^bDMEM with 10% FBS used for cytotoxicity, ^cDMEM with 40% FBS used for cytotoxicity.

of PMBN was reduced 4- to 64-fold when used in combination for *P. aeruginosa*. Our cytotoxicity results showed that robenidine (IC₅₀ = 12 μg/ml) was not toxic at the MIC₉₀ (0.5–4 μg/ml) of the tested pathogens, with IC₅₀/MIC ratio ranging from sixfold (Gram-negative pathogens) to threefold (Gram-positive pathogens) in the presence of EDTA. The MIC (0.5–8 μg/ml) obtained for robenidine in the presence of PMBN against Gram-negative pathogens was not toxic against all tested cell lines, with IC₅₀/MIC ratio ranging from approximately 2- to 24-fold. In this study, we found that toxicity of robenidine was significantly reduced in the presence of serum, possibly due to the interaction between robenidine and serum. This serum impact was observed on the MIC values of robenidine with 10% serum (fourfold increase) and 50% serum (no antimicrobial activity), which was reported in a previous study (Abraham et al., 2016). This suggests the probable high level of serum protein binding with robenidine may significantly reduce its toxicity and robenidine would be likely to be safe when applied as a topical or otic treatment. However, testing in animal models would be required to confirm efficacy and safety. In addition, the use of EDTA in topical treatments containing robenidine also is expected to be safe. EDTA-tromethamine solution consisting of 250 mM EDTA and 50 mM tromethamine has previously been used for the treatment of otitis externa, dermatitis and cystitis without any toxicity or other side effects observed (Farca et al., 1997). Given the substantial reduction in MICs and toxicity of robenidine in the presence of either EDTA or PMBN, the *in vivo* activity of these combinations for topical and systemic treatment of ESKAPE pathogen infections could be evaluated in mouse models of infection.

We found that robenidine has no activity against biofilms formed by Gram-positive or Gram-negative bacteria. However, in this study EDTA demonstrated antibiofilm activity against both Gram-positive and Gram-negative species at a concentration of 1 mM that is in agreement with previous studies (Al-Bakri et al., 2009; Finnegan and Percival, 2015). Our results demonstrate that the presence of robenidine does not affect the antibiofilm activity of EDTA. Many pathogens are able to form biofilms making them less susceptible to various classes of antimicrobials (Chambers and Deleo, 2009). There is an urgent need for antimicrobials that can either kill planktonic cells or eradicate biofilms. Together, our results show that the combination of EDTA and robenidine is a suitable antimicrobial

combination with activity against both Gram-positive and Gram-negative species and their biofilm formation.

Commercially available otic products typically contain antifungal, antibiotic and anti-inflammatory agents, such as Surotan® (polymyxin B-miconazole-prednisolone), Aurizon® (marbofloxacin-clotrimazole-dexamethasone) and Otomax® (gentamicin-clotrimazole-betamethasone) (Rougier et al., 2005; Rigaut et al., 2011). These otic products share antimicrobial agents used in human medicine, increasing the likelihood of cross-resistance development and transmission between different host species. In addition, the response to these otic products varies due to the emergence of antimicrobial resistance in canine otic pathogens. Polymyxin B resistance was reported in 100% of *S. pseudintermedius* and *Proteus* spp. and 7% of *P. aeruginosa* from cases of canine otitis externa in Australia (Bugden, 2013) and between 9.6 and 27% of canine otitis/pyoderma isolates were resistant to marbofloxacin (Rubin et al., 2008; Arais et al., 2016). Resistance to gentamicin was found in 43.3% *P. aeruginosa* otitis isolates (Mekic et al., 2011). It is notable that there is no study that demonstrates an otic product with 100% cure rate. For instance, cure rates of 58.3% for Aurizon® and 41.2% for Surotan® were observed in one study (Rougier et al., 2005). We found that the new combination of robenidine and EDTA has potential for development as a topical treatment of canine otitis externa with mixed bacterial infections. In our study, EDTA acted as an adjuvant that potentiates the activity of robenidine against Gram-negative bacteria with additional inhibitory activity against biofilm-forming bacteria. The use of an antimicrobial and an antimicrobial adjuvant as a two-drug combination antimicrobial therapy such as robenidine and EDTA has the benefit of reducing the onset of resistance development compared to monotherapy (Worthington and Melander, 2013). Recently, we reported that EDTA has anti-fungal activity against *Malassezia pachydermatis* isolated from canine otitis externa (Chan et al., 2018c) which is an advantage to the use of combination therapy of robenidine and EDTA for canine otitis externa. This combination is an approach to promote antimicrobial stewardship by eliminating the likelihood of cross-resistance development and transmission of resistance determinants of public health significance between dogs and humans.

In our study, robenidine demonstrated noteworthy activity against thirteen multidrug- and methicillin-resistant *S. pseudintermedius* and 20 β-haemolytic streptococci isolates

from clinical cases of canine otitis externa. This is in agreement with our previous study that reported robenidine was effective against clinical MRSA and *S. pneumoniae* strains at concentrations ranging from 1–2 µg/ml and 2–8 µg/ml, respectively (Abraham et al., 2016; Oggunniyi et al., 2017). The finding that robenidine in the presence of EDTA demonstrated antibacterial activity against the Gram-negative canine otitis externa pathogens, *P. aeruginosa* and *P. mirabilis* is in agreement with our previous findings for robenidine tested against two strains each of *E. coli* and *P. aeruginosa* in the presence of PMBN (Abraham et al., 2016). However, our results showed that low concentrations of robenidine in combination with PMBN or EDTA improved potency and spectrum of activity, specifically targeting Gram-negative pathogens. These results suggest that in addition to having excellent activity against Gram-positive organisms, robenidine in combination with EDTA or PMBN has potential as a broad-spectrum topical treatment, particularly against pathogens that have become resistant to multiple classes of currently registered antimicrobial agents.

CONCLUSION

The results of our study demonstrate that robenidine is not suitable as a sole antimicrobial agent for the treatment of Gram-negative pathogen infections due to the lack of activity against the majority of Gram-negative isolates except for *A. baumannii* and *A. calcoaceticus*. However, we demonstrated *in vitro* efficacy against all selected Gram-negative organisms when robenidine was tested in combination with EDTA or PMBN, including against multidrug-resistant strains. Therefore, robenidine may be an appropriate candidate as a component of a combination preparation for the treatment of otitis externa in dogs. This study provides proof of concept of drug repurposing in the field of veterinary otology and would represent a good example of antimicrobial stewardship when the compound is ultimately developed and used clinically

in dogs. Finally, the additive and synergistic effects of robenidine in combination with EDTA or PMBN provide an important and novel development pathways for treatment of additional antimicrobial-resistant Gram-negative pathogens in animals and humans.

AUTHOR CONTRIBUTIONS

MK contributed to the study design, MIC and combination testing, kill time, biofilm assay, analyzed results, and wrote the preliminary manuscript. HP contributed towards kill time assay, MIC testing, biofilm assay, and data analysis. WC performed testing on the robenidine and EDTA combination, biofilm assay, and data analysis. JS participated in cell cytotoxicity assays. AO contributed to data analysis and manuscript editing. HV and PH contributed to interpretation, analysis, and discussion. AM and SG contributed to discussion, writing and editing. SP conceived the study's design, and contributed to the writing and editing, and provided financial support for the study. DT contributed to study design, and participated in writing, editing, and discussion, and provided financial support for the study. All authors read and approved the submitted version of the manuscript, in addition to contributing to manuscript revision.

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Evaluating the Efficacies of Carbapenem/ β -Lactamase Inhibitors Against Carbapenem-Resistant Gram-Negative Bacteria *in vitro* and *in vivo*

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Background: Carbapenem-resistant Gram-negative bacteria are a major clinical concern as they cause virtually untreatable infections since carbapenems are among the last-resort antimicrobial agents. β -Lactamases implicated in carbapenem resistance include KPC, NDM, and OXA-type carbapenemases. Antimicrobial combination therapy is the current treatment approach against carbapenem resistance in order to limit the excessive use of colistin; however, its advantages over monotherapy remain debatable. An alternative treatment strategy would be the use of carbapenem/ β -lactamase inhibitor (β LI) combinations. In this study, we assessed the *in vitro* and *in vivo* phenotypic and molecular efficacies of three β LI when combined with different carbapenems against carbapenem-resistant Gram-negative clinical isolates. The chosen β LI were (1) Avibactam, against OXA-type carbapenemases, (2) calcium-EDTA, against NDM-1, and (3) Relebactam, against KPC-2.

Methods: Six *Acinetobacter baumannii* clinical isolates were screened for *bla*_{OXA-23-like}, *bla*_{OXA-24/40}, *bla*_{OXA-51-like}, *bla*_{OXA-58}, and *bla*_{OXA-143-like}, and eight *Enterobacteriaceae* clinical isolates were screened for *bla*_{OXA-48}, *bla*_{NDM-1}, and *bla*_{KPC-2}. The minimal inhibitory concentrations of Imipenem (IPM), Ertapenem (ETP), and Meropenem (MEM) with corresponding β LI for each isolate were determined. The efficacy of the most suitable *in vitro* treatment option against each of *bla*_{OXA-48}, *bla*_{NDM-1}, and *bla*_{KPC-2} was assessed via survival studies in a BALB/c murine infection model. Finally, RT-qPCR was performed to assess the molecular response of the genes of resistance to the carbapenem/ β LI combinations used under both *in vitro* and *in vivo* settings.

Results: Combining MEM, IPM, and ETP with the corresponding β LI restored the isolates' susceptibilities to those antimicrobial agents in 66.7%, 57.1%, and 30.8% of the samples, respectively. Survival studies in mice revealed 100% survival rates when MEM was combined with either Avibactam or Relebactam against *bla*_{OXA-48}

and *bla*_{KPC-2}, respectively. RT-qPCR demonstrated the consistent overexpression of *bla*_{OXA-48} upon treatment, without hindering Avibactam's activity, while *bla*_{NDM-1} and *bla*_{KPC-2} experienced variable expression levels upon treatment under *in vitro* and *in vivo* settings despite their effective phenotypic results.

Conclusion: New carbapenem/ β LI combinations may be viable alternatives to antimicrobial combination therapy as they displayed high efficacy *in vitro* and *in vivo*. Meropenem/Avibactam and Meropenem/Relebactam should be tested on larger sample sizes with different carbapenemases before progressing further in its preclinical development.

Keywords: OXA-48, NDM-1, KPC, carbapenem, Avibactam, Relebactam, calcium-EDTA, antimicrobial resistance

INTRODUCTION

Carbapenem resistant Gram-negative bacteria have been gradually increasing in prevalence in recent years. In the United States, the latest CDC Antibiotic Resistance Threat Report indicates that Carbapenem-Resistant *Enterobacteriaceae* (CREs) are responsible for 9,000 annual nosocomial infections, with a 6.67% mortality rate; a potentially underestimated percentage due to different definitions of CRE infections (Livorsi et al., 2018). The same report also estimates 7,300 annual multidrug-resistant (MDR) *Acinetobacter baumannii* infections; with a 6.85% mortality rate. In Lebanon, the most recent nation-wide survey indicates that around 2% of *Enterobacteriaceae* isolates identified over the past few years were Imipenem-resistant, while that percentage was much higher among *Acinetobacter* spp. at 82.4% (Chamoun et al., 2016). At the American University of Beirut Medical Center (AUBMC), the prevalence of CREs has doubled since 2015, reaching 11%, while carbapenem resistance among *A. baumannii* isolates has remained high beyond 75% during the same time period (Araj and Zaatari, 2015, 2018).

Carbapenem resistance can manifest through several mechanisms. Notably, the combined effect of extended-spectrum β -lactamases (ESBLs) or AmpC-type enzymes production, coupled with increased efflux pump activity and porin loss (Baroud et al., 2013). However, the main mechanism of resistance to carbapenems is through the expression of chromosomal or plasmid-mediated carbapenem-hydrolyzing β -lactamases such as *Klebsiella pneumoniae* carbapenemases (KPC), OXA-type carbapenemases, and New Delhi metallo- β -lactamases (Lapuebla et al., 2015) (Meletis, 2016). KPC and OXA-type carbapenemases are families of Ambler Class A and Class D serine β -lactamases, respectively, that contain a serine moiety in their active sites (Sahuquillo-Arce et al., 2015). Among the KPC family, KPC-2 and KPC-3 are the most commonly encountered between the 20-plus variant KPCs (Djahmi et al., 2014; Sahuquillo-Arce et al., 2015; Satlin et al., 2017). The OXA-type carbapenemases are grouped into nine clusters with 1, 2, 3, and 4 being associated with *A. baumannii*, and include the subfamilies OXA-23, OXA-51, OXA-24/40, and OXA-58, while cluster 6, being associated with *Enterobacteriaceae*, comprises the subfamily OXA-48 (Woodford et al., 2006; Queenan and Bush, 2007). On the other hand, NDM is a family of Ambler Class B metallo- β -lactamases that

contain a divalent cation in their active site (Sahuquillo-Arce et al., 2015) with NDM-1 being the most prominent member (Nordmann et al., 2011).

Treating carbapenem-resistant Gram-negative bacteria poses a major clinical challenge as carbapenems are among the last-resort antimicrobial agents to be used, and CREs along with MDR-*A. baumannii* can cause terminal infections ranging from upper and lower respiratory, wound, bloodstream and cerebrospinal fluid infections in the case of *A. baumannii* (Queenan et al., 2012), to complicated intra-abdominal infections, sepsis, and meningitis, in the case of CREs (Murray et al., 2016; Yu and Chuang, 2016). The current recommendation to treat carbapenem-resistant Gram-negative infections involves the use of antimicrobial combination therapy (The Medical Letter, 2013). This approach is mostly guided by the lack of new classes of antimicrobial agents that can overcome such resistance since it is usually compounded with fluoroquinolone as well as aminoglycoside resistances within the same isolate (Meletis, 2016). Consequently, nephrotoxic antimicrobial agents such as polymyxins have to be combined with tetracyclines, such as tigecycline (Meletis, 2016). However, the efficacy of antimicrobial combination therapy in comparison to monotherapy has been a topic of debate in the literature. One study concluded that combination therapy improved the survival rates of bloodstream infection patients and decreased their mortality rates by 20.2% ($p = 0.02$) when compared to monotherapy (Tumbarello et al., 2012). Another study conducted on 205 patients infected with KPC-producing *K. pneumoniae* determined that combination therapy decreased patient mortality rate from 40 to 19.4% when a carbapenem is used in addition to other antimicrobials (Daikos et al., 2014). However, there exists sources of bias in combination therapy reports since a lot of studies include both carbapenem-resistant and carbapenem-susceptible isolates, not to mention that they disregard empirical treatment that the patient might have taken prior to being enrolled in the study (Paul et al., 2014). Additionally, certain studies report that the use of carbapenems as part of a double or triple therapy is recommended when the MIC needed against the isolate is ≤ 8 μ g/ml (Tzouveleakis et al., 2012; Daikos et al., 2014) whereas other studies assign that breakpoint at ≤ 4 μ g/ml (Miyakis et al., 2011; Tängdén, 2014). Finally, combination therapy increases the cost of treatment (Kmeid et al., 2013) and exposes the bacteria to several antimicrobials that it might develop resistance to.

As such, the utility of combination therapy remains off-label and largely biased. Therefore, an alternative treatment approach to carbapenem-resistant bacterial infections could be the genetically guided use of β -lactam/ β -lactamase inhibitors (β LI)s, namely, carbapenems/ β LI combinations.

Three β LI were selectively chosen to target specific mechanisms of carbapenem resistance: Avibactam against OXA-type carbapenemases, Relebactam against KPC, and calcium-EDTA against NDM. First, Avibactam is a non- β -lactam-based β LI that reversibly inactivates serine carbapenemases through the covalent acylation of the β -lactamase followed by a slow deacylation step that restores the inhibitor's core chemical structure (Ehmann et al., 2012). Avibactam is United States FDA-approved in combination with Ceftazidime (U.S. Food and Drug Administration [FDA], 2015) and is marketed as a treatment option against hospital-acquired and ventilator-associated bacterial pneumonias, and complicated intra-abdominal and urinary tract infections (Allergan, 2018). Secondly, Relebactam is also a non- β -lactam-based β LI that targets serine carbapenemases (Hirsch et al., 2012); however, it is combined with Imipenem/Cilastatin to target Imipenem-resistant bacteria (Livermore et al., 2013; Lapuebla et al., 2015; Lob et al., 2017; Karlowsky et al., 2018). Finally, calcium disodium EDTA is a divalent metal-chelating agent that is United States FDA-approved to treat acute and chronic lead poisoning (U.S. Food and Drug Administration [FDA], 2009); however, it has been described in the literature to have the capacity to chelate the divalent cations found in the active sites of metallo- β -lactamases and has shown *in vivo* efficacy against *Pseudomonas aeruginosa* and *Escherichia coli* when combined with β -lactams (Aoki et al., 2010; Yoshizumi et al., 2013).

In this study, we first aim to assess the *in vitro* and *in vivo* efficacies of carbapenems in combination with the β LI Avibactam, Relebactam, and calcium-EDTA when targeting OXA-type carbapenemases, KPC-2, and NDM-1, respectively, and then investigate the molecular response of those genes of resistance against the carbapenem/ β LI combinations.

MATERIALS AND METHODS

Isolate Collection

The Department of Pathology and Laboratory Medicine at the American University of Beirut Medical Center (AUBMC), Beirut, Lebanon provided all of the clinical bacterial isolates included in this study with the exception of one *Salmonella* spp. isolate that was provided by the Centers for Disease Control and Prevention (CDC), Atlanta, GA, United States. A total of 14 isolates were used, including: six *A. baumannii*, five *K. pneumoniae*, two *E. coli*, and one *Salmonella* spp. isolates. In addition to those isolates, three samples of presumptive carbapenem-resistant *Pseudomonas aeruginosa* were provided. All isolates were collected as part of routine medical sampling; thus, did not require Institutional Review Board (IRB) approval nor patient consent. The labeling of each isolate can be found in Table 1.

Detection of Carbapenem Resistance Genes

Total genomic DNA of each of the collected isolates was extracted from an overnight culture using the QIAamp® DNA Mini Kit (QIAGEN, Germany) according to manufacturer's instructions. Polymerase chain reaction (PCR) using TopTaq™ DNA Polymerase (QIAGEN, Germany) was then utilized to amplify and detect several β -lactamase-encoding genes that are implicated in carbapenem resistance. For the *Enterobacteriaceae* isolates, *bla*_{OXA-48}, *bla*_{NDM-1}, and *bla*_{KPC-2} genes were tested. For the *A. baumannii* isolates, *bla*_{NDM-1}, *bla*_{OXA-23-like}, *bla*_{OXA-24/40}, *bla*_{OXA-51-like}, *bla*_{OXA-58}, and *bla*_{OXA-143-like} genes were tested. For the *P. aeruginosa* isolates, *bla*_{NDM-1} was tested. The list of PCR primer sequences along with their target genes amplicon sizes are available in Table 2.

Determination of Minimal Inhibitory Concentrations

For each of the *Enterobacteriaceae* and *A. baumannii* isolates included in this study, the minimal inhibitory concentrations (MICs) of Imipenem (as Imipenem/Cilastatin, Tienam®, Merck & Co., Inc., Whitehouse Station, NJ, United States), Ertapenem (Invanz®, Merck & Co., Inc., Whitehouse Station, NJ, United States), and Meropenem (Meronem®, AstraZeneca, Wilmington, DE, United States) were determined via antimicrobial broth microdilution in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute [CLSI], 2012). *Escherichia coli* (ATCC® 25922™) was used as a quality control strain (Clinical and Laboratory Standards Institute [CLSI], 2018c).

Assessment of the *in vitro* Efficacy of the Carbapenem/ β -Lactamase Inhibitor Combinations

Following initial MIC determination, the *in vitro* efficacies of the carbapenem/ β LI combinations was assessed by adding fixed concentrations of the inhibitors to the experimental wells of a standard antimicrobial broth microdilution assay; thus, testing for Imipenem/ β LI, Ertapenem/ β LI, and Meropenem/ β LI. The procedure followed in this assay adhered to CLSI guidelines; however, minor modifications to broth volumes were made in order to accommodate for the presence of the β LI while keeping the concentrations of the carbapenems and bacterial suspensions in accordance with CLSI recommendations (Clinical and Laboratory Standards Institute [CLSI], 2012).

For isolates harboring OXA-type carbapenemases, Avibactam (MedChem Express, Monmouth Junction, NJ, United States) was used as the β LI at a fixed concentration of 4 μ g/mL (Livermore et al., 2011; Aktas et al., 2012; Sader et al., 2015). Concerning the isolates that harbored *bla*_{NDM-1}, ethylenediaminetetraacetic acid calcium disodium salt (calcium-EDTA) (Sigma®, St. Louis, MO, United States) was used as the β LI at a fixed concentration of 32 μ g/mL (Aoki et al., 2010; Yoshizumi et al., 2013). As for the isolate that harbored *bla*_{KPC-2}, Relebactam (MedChem Express,

TABLE 1 | Labels of each isolate included in the study along with their detected genes of carbapenem resistance.

Bacterial species	Isolate label	Genes of carbapenem resistance							
		<i>bla</i> _{OXA-48}	<i>bla</i> _{NDM-1}	<i>bla</i> _{KPC-2}	<i>bla</i> _{OXA-23-like}	<i>bla</i> _{OXA-24/40}	<i>bla</i> _{OXA-51-like}	<i>bla</i> _{OXA-58}	<i>bla</i> _{OXA-143-like}
<i>Escherichia coli</i>	IMP 53	+	—	—					
	IMP 57	+	—	—					
<i>Klebsiella pneumoniae</i>	IMP 197	+	—	—					
	IMP 215	+	—	—					
	IMP 223	+	—	—					
	IMP 216	—	+	—					
	IMP 217	—	+	—					
<i>Salmonella</i> spp.	KPC	—	—	+					
<i>Acinetobacter baumannii</i>	ACN 2090		—		+	—	+	+	—
	ACN 2209		—		+	—	+	—	—
	ACN 2273		—		+	—	+	—	—
	ACN 2285		—		+	—	+	—	+
	ACN 2493		—		+	—	+	—	—
	ACN 3630		—		+	—	+	+	+
<i>Pseudomonas aeruginosa</i>	PSA 41		—						
	PSA 44		—						
	PSA 45		—						

+, denotes the presence of the gene, – denotes the absence of the gene.

TABLE 2 | List of target genes along with their PCR primer sequences and amplicon sizes.

	Target gene	PCR primer sequence (5'→3')	Amplicon (bp)	References
PCR primers	<i>bla</i> _{OXA-48}	F: TTGGTGGCATCGATTATCGG R: GAGCACTTCTTTGTGATGGC	744	Salloum et al., 2015
	<i>bla</i> _{NDM-1}	F: GGAACTGGCGACCAACG R: ATGCGGGCCGTATGAGTGA	678	Salloum et al., 2015
	<i>bla</i> _{OXA-23-like}	F: GATCGGATTGGAGAACCAGA R: ATTTCTGACCGCATTTCCAT	501	Woodford et al., 2006
	<i>bla</i> _{OXA-24/40}	F: GGTTAGTTGGCCCCCTTAAA R: AGTTGAGCGAAAAGGGGATT	246	Woodford et al., 2006
	<i>bla</i> _{OXA-51-like}	F: TAATGCTTTGATCGGCCTTG R: TGGATTGCACTTCATCTTGG	353	Woodford et al., 2006
	<i>bla</i> _{OXA-58}	F: AAGTATTGGGGCTTGTGCTG R: CCCCTCTGCGCTCTACATAC	599	Woodford et al., 2006
	<i>bla</i> _{OXA-143-like}	F: TGGCACTTTCAGCAGTTCCT R: TAATCTTGAGGGGGCCAACC	149	Higgins et al., 2010
	<i>bla</i> _{KPC-2}	F: GCAGCGGCAGCAGTTTGTGATT R: GTAGACGGCCAACACAATAGGTGC	184	Salloum et al., 2015
RT-qPCR primers	<i>bla</i> _{OXA-48}	F: TTCGGCCACGGAGCAAATCAG R: GATGTGGGCATATCCATATTCATCGCA	240	Salloum et al., 2015
	<i>bla</i> _{NDM-1}	F: TTGGCGATCTGGTTTTC R: GGTTGATCTCCTG CTTGA	195	Salloum et al., 2015
	<i>bla</i> _{KPC-2}	F: GCAGCGGCAGCAGTTTGTGATT R: GTAGACGGCCAACACAATAGGTGC	184	Salloum et al., 2015
	<i>rpoB</i>	F: TCGAAACGCCTGAAGGTC R: TTGGAGTTCGCCTGAGC	184	Salloum et al., 2015

Monmouth Junction, NJ, United States) was used as the β LI at a fixed concentration of 4 μ g/mL (Snydman et al., 2016).

In addition, each isolate was tested against its corresponding β LI at their aforementioned fixed concentrations without the addition of carbapenems in order to rule out any anti-bacterial activity exhibited by the inhibitors on the tested isolates.

The MICs of Imipenem (IPM), Ertapenem (ETP), and Meropenem (MEM) for all tested isolates were interpreted according to the CLSI M100 guideline (Clinical and Laboratory Standards Institute [CLSI], 2018a,b). MIC breakpoints for carbapenems in combinations with the β LI used in this study are currently unavailable for *Enterobacteriaceae* and *A. baumannii*.

As such, the MIC breakpoints for Ceftazidime/Avibactam (CAZ/AVI) were used to interpret the MIC results of the carbapenem/Avibactam combinations and the MIC breakpoints for IPM, ETP, and MEM alone were used to interpret the results of their combinations with Relebactam (REL) and Ca-EDTA.

As a quality control strain, *Escherichia coli* (ATCC® 35218™) was used according to CLSI recommendations for β -lactam/ β LI combination testing (Clinical and Laboratory Standards Institute [CLSI], 2018c).

Assessment of the *in vivo* Efficacy of Meropenem/ β -Lactamase Inhibitor Combinations

The most efficacious *in vitro* treatment options that restored antimicrobial susceptibility of three isolates: *E. coli* IMP 57, *K. pneumoniae* IMP 216, and *Salmonella* spp. KPC, of which each harbored *bla*_{OXA-48}, *bla*_{NDM-1}, and *bla*_{KPC-2}, respectively, were further investigated in animal experimentation models.

The animals involved in this study were purchased from the Animal Care Facility at the American University of Beirut. The protocols adopted in these experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at AUB under approval #17-08-432.

A total of 150 BALB/c male mice, 6–8 weeks old, weighing 20–40 g, were used in these sets of experiments. The mice were allowed to consume food and water *ad libitum* throughout the experimentation period and at the end of each set of experiments, all surviving mice were humanely euthanized.

Determination of the Median Lethal Dose in a BALB/c Murine Infection Model

The procedure followed in determining the LD₅₀ of each of the isolates *E. coli* IMP 57, *K. pneumoniae* IMP 216, and *Salmonella* spp. KPC involved in the animal experimentations relied on an earlier protocol (Nowotny, 1979) with an extended monitoring period. Briefly, for each of the three tested bacterial isolates, 20 mice were divided into five groups of four mice. Each

group of mice were intraperitoneally injected with increasing concentrations of the tested isolate, starting with 10⁴ CFU up to 10⁸ CFU. Following infection, the mice were daily monitored over a 1-week period for their survival, weight, physical appearance, and behavioral changes.

At the end of the monitoring period, the LD₅₀ was calculated using the Spearman-Kärber method (Nowotny, 1979).

Investigation of Survival Rates in a BALB/c Murine Infection Model

For each of the bacterial isolates *E. coli* IMP 57, *K. pneumoniae* IMP 216, and *Salmonella* spp. KPC, 30 mice were divided into five groups of six mice. The experimental setup was designed over a 7-day period (Table 3):

1. Group 1 acted as a positive control and received a 1-time intraperitoneal bacterial dose of $3 \times \text{LD}_{50}$ suspended in tryptic soy broth (TSB) on day 1 without receiving any subsequent treatment throughout the experiment.
2. Groups 2–4 received a 1-time intraperitoneal bacterial dose of $3 \times \text{LD}_{50}$ suspended in TSB on day 1 followed by intraperitoneal treatment courses once, daily, starting 1-h post-infection on day 1 and continued days 2–7. Treatment for Group 2 was Meropenem only, for Group 3 was Meropenem/ β LI, and for Group 4 was the corresponding β LI only.
3. Group 5 acted as a negative control and received a 1-time intraperitoneal injection of sterile TSB as a blank on day 1 without any subsequent injections throughout the experiment.

Meropenem (MEM) was used as the antimicrobial agent of choice in these sets of experiments as it was the most effective carbapenem *in vitro*. MEM was combined with Avibactam (AVI), calcium-EDTA (Ca-EDTA), and Relebactam (REL) against *E. coli* IMP 57, *K. pneumoniae* IMP 216 and *Salmonella* spp. KPC, respectively.

TABLE 3 | Mice groups and injections used in survival experimentation.

Days		Group 1 (P.C.)	Group 2 (MEM)	Group 3 (MEM + β LI)	Group 4 (β LI)	Group 5 (N.C.)
Day 1	<i>t</i> = 0 hr	Bacterial injection	Bacterial injection	Bacterial injection	Bacterial injection	TSB
	<i>t</i> = 1 hr	–	Administer Meropenem	Administer Meropenem + β -lactamase inhibitor	Administer β -lactamase inhibitor	–
Day 2						
Day 3						
Day 4						
Day 5						
Day 6						
Day 7						

The β -lactamase inhibitor (β LI) Avibactam was used against *E. coli* IMP 57 harboring *bla*_{OXA-48}. The β LI calcium-EDTA was used against *K. pneumoniae* IMP 216 harboring *bla*_{NDM-1}. The β LI Relebactam was used against *Salmonella* spp. KPC harboring *bla*_{KPC-2}. TSB is tryptic soy broth; used as the injection solution for bacteria in Groups 1–4 and as a sterile blank in Group 5.

The required dose of MEM against the tested isolates was determined according to an earlier protocol (Rahal et al., 2011b) and was 1.6 mg/kg for *E. coli* IMP 57, 1.75 mg/kg for *Salmonella* spp. KPC, and 0.115 mg/kg for *K. pneumoniae* IMP 216.

As for the doses of the β LI, AVI was administered at a 1:4 ratio with the antimicrobial agents (Endimiani et al., 2011; Levasseur et al., 2014), while Ca-EDTA and REL were each administered at an 8:1 ratio with the antimicrobial agents (Yoshizumi et al., 2013; Powles et al., 2018).

All mice were daily monitored for their survival, weight, physical appearance, and behavioral changes. Test subjects that expired prior to the end of the monitoring period had their blood cultured and API® 20E (bioMérieux, Marcy l'Etoile, France) performed on the colonies retrieved in order to confirm that the cause of death was the administered agent (Salloum et al., 2015).

Assessment of the Molecular Response to the Carbapenem/ β -Lactamase Inhibitor Combinations

Reverse transcription real-time polymerase chain reaction (RT-qPCR) was used to quantitate the expression levels of *bla*_{OXA-48}, *bla*_{NDM-1}, and *bla*_{KPC-2} in the tested isolates. The relative normalized expressions of the target genes were calculated using the Livak $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008).

Under *in vitro* Conditions

Bacterial suspensions of *E. coli* IMP 57, *K. pneumoniae* IMP 216, and *Salmonella* spp. KPC were collected for RT-qPCR following their incubations with Meropenem alone as well as in combination with their corresponding β LI at MICs. An untreated sample of each bacterial isolates was used as a positive control and the *rpoB* gene was used as a reference housekeeping gene (Salloum et al., 2015).

Under *in vivo* Conditions

For each of *E. coli* IMP 57, *K. pneumoniae* IMP 216, and *Salmonella* spp. KPC, 15 BALB/c male mice were divided into five groups of three mice and followed the same IACUC-approved infection and treatment protocols used in the survival studies above. However, mice from Groups 1–3 were then scarified via cardiac puncture under general anesthesia 4 h post-treatment, and their blood was collected for RT-qPCR. All blood samples were centrifuged at $1,500 \times g$ for 30 min and the separated plasma was retrieved for bacterial RNA extraction (Rasheed, 2016).

For both *in vitro* and *in vivo* settings, the illustra™ RNAspin Mini Kit (GE Healthcare UK Limited, Buckinghamshire, United Kingdom) was used to extract the RNA of each of the tested isolates, the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, United States) was used to synthesize complementary DNA of the extracted RNA templates, and the iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, United States) was used for qPCR. All kits were utilized according to their manufacturers' instructions. The real-time PCR primer sequences along with their amplicon sizes are available in Table 2.

Statistical Analysis

The logrank (Mantel-Cox) test was utilized in the survival studies analysis to calculate the statistical significance while the unpaired Student's *t*-test was used in the quantitative PCR analysis, in which *p*-values ≤ 0.05 were considered statistically significant.

RESULTS

Detection of Carbapenem Resistance Genes

Following PCR amplification, it was observed that all collected isolates harbored at least 1 of the carbapenem resistance genes they were tested for. Consequently, *bla*_{OXA-48} was detected in five of the eight *Enterobacteriaceae* isolates (62.5%), *bla*_{NDM-1} was detected in 2 (25%), while *bla*_{KPC-2} was only detected in one *Enterobacteriaceae* isolate (12.5%). As for *bla*_{OXA-23-like} and *bla*_{OXA-51-like}, they were amplified in all tested *A. baumannii* isolates (100%), while each of *bla*_{OXA-58} and *bla*_{OXA-143-like} were amplified in two of the six *A. baumannii* isolates (33.3%), and neither *bla*_{OXA-24/40} nor *bla*_{NDM-1} were detected in any of the tested *A. baumannii*. Concerning *bla*_{NDM-1} in *P. aeruginosa*, none of the tested isolates harbored the gene of resistance; thus, the *P. aeruginosa* isolates were not considered further in experimentation. A summary of the identified genes is available in Table 1.

Efficacy of the Carbapenem/ β -Lactamase Inhibitor Combinations *in vitro*

Among the isolates that harbor *bla*_{OXA-48}, IPM/AVI and MEM/AVI managed to restore carbapenem susceptibility to 100% of them while ETP/AVI restored carbapenem susceptibility to 60% of them. Similarly, among the isolates that harbor *bla*_{NDM-1}, only testing with IPM/Ca-EDTA and MEM/Ca-EDTA resulted in 100% susceptibility, while for the isolate that harbored *bla*_{KPC-2}, combining any of the carbapenems with REL restored carbapenem susceptibility. On the other hand, the combinations of any of the carbapenems with AVI were unsuccessful at restoring carbapenem susceptibility among the *A. baumannii* isolates that mainly harbored *bla*_{OXA-23-like} and *bla*_{OXA-51-like}; however, they did manage to lower their MIC values by twofold in the case of ETP/AVI and at least eightfold in the cases of IPM/AVI and MEM/AVI. Minimal inhibitory concentration results of all isolates are available in Table 4 and Figure 1.

Finally, none of the inhibitors used in this study were solely successful at inhibiting the growth of any isolate; thus, confirming that they do not exhibit antibacterial activities themselves.

Efficacy of Meropenem/ β -Lactamase Inhibitor Combinations *in vivo*

The median lethal dose of the tested isolates was determined as follows: 1.78×10^8 CFU for *E. coli* IMP 57, 3.16×10^7 CFU for *K. pneumoniae* IMP 216, and 3.16×10^8 CFU for *Salmonella* spp. KPC. Recorded average mice weights are available in Supplementary Figure S1.

TABLE 4 | MICs of Imipenem (IPM), Ertapenem (ETP), and Meropenem (MEM) with and without the β -lactamase inhibitors (β LI) against the tested isolates.

Gene of resistance	Isolate ID	MIC (μ g/mL)					
		IPM	IPM + β LI	ETP	ETP + β LI	MEM	MEM + β LI
<i>bla_{OXA-48}</i>	IMP 53	32	0.25	128	2	16	0.03125
	IMP 57	64	1	>256	16	64	0.25
	IMP 197	>256	1	>256	16	128	1
	IMP 215	128	0.5	>256	8	128	0.125
	IMP 223	16	0.25	16	0.25	2	0.03125
<i>bla_{NDM-1}</i>	IMP 216	>256	0.5	>256	2	>256	0.5
	IMP 217	8	1	1	1	1	0.03125
<i>bla_{KPC-2}</i>	KPC	4	1	4	0.03125	4	0.03125
<i>bla_{OXA-23-like}</i> , <i>bla_{OXA-51-like}</i>	ACN 2090	>256	32	>256	>256	128	32
	ACN 2209	>256	32	>256	>256	128	32
	ACN 2273	>256	8	>256	>256	128	8
	ACN 2285	>256	32	>256	>256	128	32
	ACN 2493	>256	32	>256	>256	128	32
	ACN 3630	>256	8	>256	>256	128	8

The β LI for *bla_{OXA-48}*, *bla_{OXA-23-like}*, and *bla_{OXA-51-like}* is Avibactam. The β LI for *bla_{NDM-1}* is Ca-EDTA. The β LI for *bla_{KPC-2}* is Relebactam.

Concerning the survival rate of the BALB/c mice upon infection with the tested isolates and treatment with Meropenem monotherapy in comparison to Meropenem/ β LI combinations, the group receiving Meropenem/Avibactam against *E. coli* IMP 57 experienced a 100% survival rate ($p < 0.0001$) when compared to their positive control group (16.7% survival) as well as the group receiving Meropenem monotherapy (0% survival) and the group receiving Avibactam alone (0% survival) (**Figure 2**). Similarly, the group treated with Meropenem/Relebactam against *Salmonella* spp. KPC experienced a 100% survival rate ($p < 0.0001$) in comparison to their positive control group (0% survival) in addition to the group treated with Meropenem monotherapy (0% survival) and the group treated with Relebactam alone (0% survival) (**Figure 2**). However, the group receiving Meropenem/calcium-EDTA against *K. pneumoniae* IMP 216 experienced a 16.7% survival rate ($p = 0.0009$), identical to that of the group receiving Meropenem monotherapy, but higher than the positive control (0% survival) as well as the group receiving calcium-EDTA alone (0% survival) (**Figure 2**).

The average weights of the different mice groups against each of the tested bacterial isolates during the survival studies are available in **Supplementary Figure S2**.

The Molecular Response to Meropenem/ β -Lactamase Inhibitor Combinations

bla_{OXA-48}

Quantifying the *in vitro* relative normalized expression levels of *bla_{OXA-48}* in *E. coli* IMP 57 following the addition of Meropenem only and Meropenem/Avibactam in comparison to the positive control indicated a sixfold increase ($p = 0.0024$) in *bla_{OXA-48}* expression when Meropenem was added, and a 10-fold increase ($p = 0.00072$) when Meropenem/Avibactam were

added. Moreover, there was a statistically significant difference ($p = 0.028$) in expression levels when comparing Meropenem to Meropenem/Avibactam (**Figure 3**).

Concerning the *in vivo* relative normalized expression levels of *bla_{OXA-48}* in *E. coli* IMP 57, the treatment with Meropenem only and Meropenem/Avibactam in comparison to the positive control indicated a threefold increase ($p = 0.0292$) in *bla_{OXA-48}* expression when Meropenem was administered, and a fourfold increase ($p = 0.0361$) when Meropenem/Avibactam were administered (**Figure 3**).

bla_{KPC-2}

Measuring the *in vitro* relative normalized expression levels of *bla_{KPC-2}* in *Salmonella* spp. KPC following the addition of Meropenem only and Meropenem/Relebactam, when compared to the positive control, indicated a fivefold increase ($p = 0.008$) in *bla_{KPC-2}* expression when Meropenem was added, and a fourfold increase ($p = 0.05$) when Meropenem/Relebactam were added (**Figure 3**).

With respect to the *in vivo* relative normalized expression levels of *bla_{KPC-2}* in *Salmonella* spp. KPC, the treatment with Meropenem only and Meropenem/Relebactam, when compared to the positive control, indicated a twofold increase in *bla_{KPC-2}* expression when either Meropenem alone ($p = 0.43$) or Meropenem/Relebactam ($p = 0.29$) were administered (**Figure 3**).

bla_{NDM-1}

Quantifying the *in vitro* relative normalized expression levels of *bla_{NDM-1}* in *K. pneumoniae* IMP 216 following the addition of Meropenem only and Meropenem/calcium-EDTA when compared to the positive control indicated a fourfold increase ($p = 0.134$) in *bla_{NDM-1}* expression when Meropenem was added, but a significant eightfold decrease ($p = 0.029$) when Meropenem/calcium-EDTA were added. Moreover,

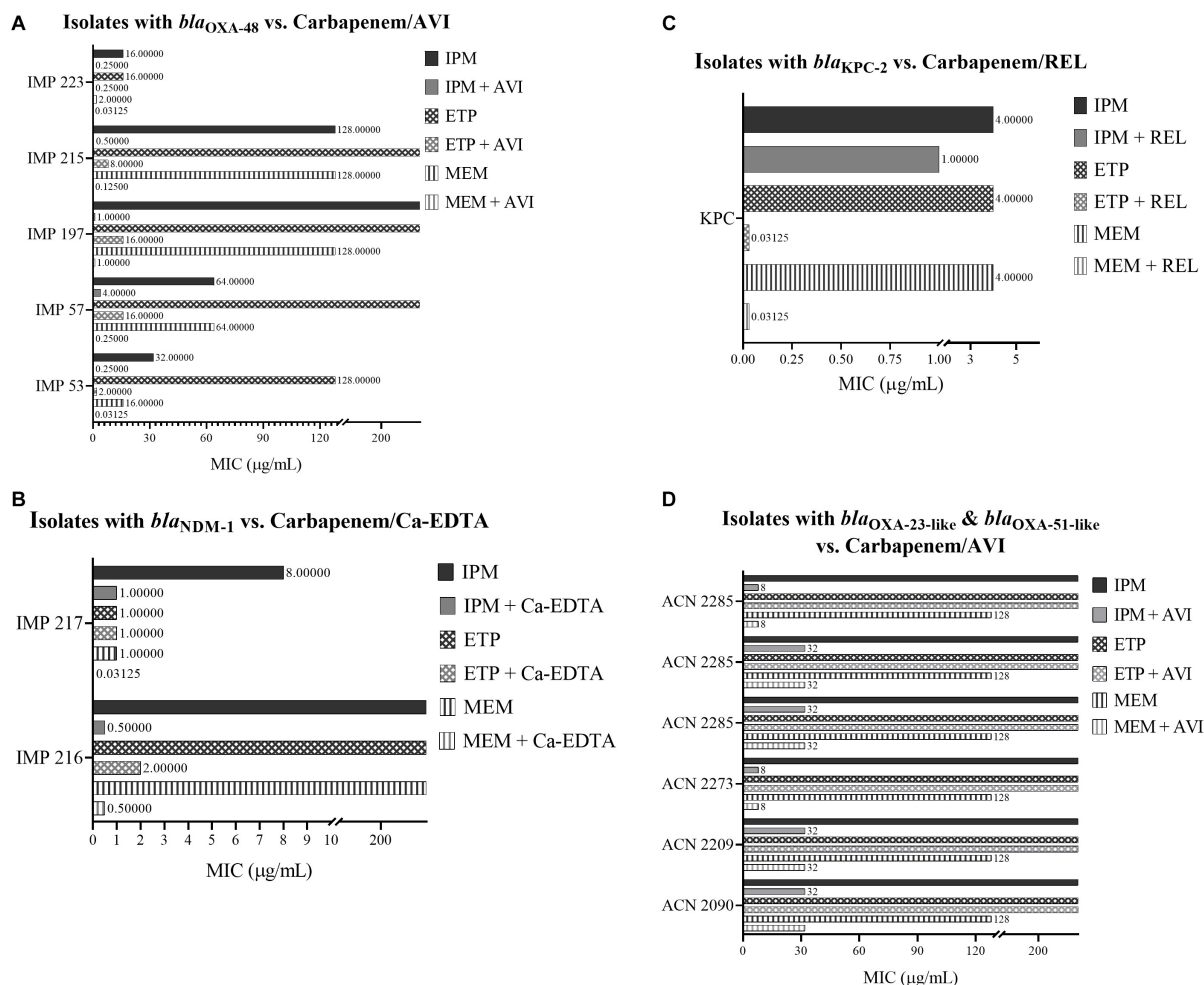


FIGURE 1 | (A) MIC of carbapenems with and without Avibactam against *Enterobacteriaceae* isolates that harbor *bla*_{OXA-48}. **(B)** MIC of carbapenems with and without Ca-EDTA against *Enterobacteriaceae* isolates that harbor *bla*_{NDM-1}. **(C)** MIC of carbapenems with and without Relebactam against a *Salmonella* spp. isolate that harbors *bla*_{KPC-2}. **(D)** MIC of carbapenems with and without Avibactam against *A. baumannii* isolates that mainly harbor *bla*_{OXA-23-like} and *bla*_{OXA-51-like}.

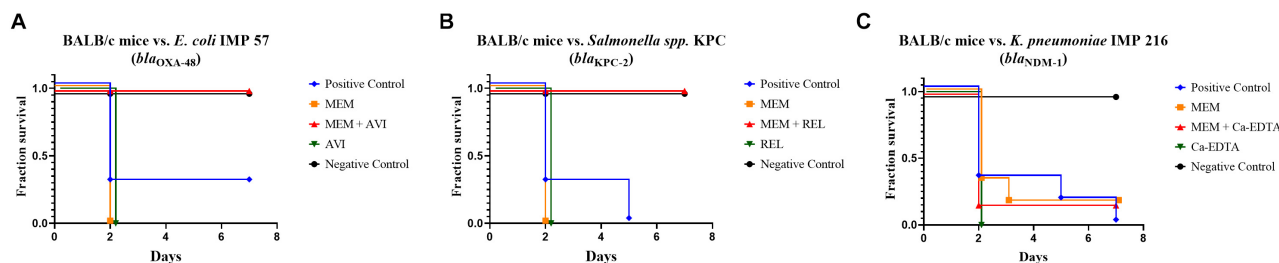


FIGURE 2 | (A) Kaplan-Meier plot showing the survival rates of treated and untreated BALB/c mice infected with *E. coli* IMP 57. **(B)** Kaplan-Meier plot showing the survival rates of treated and untreated BALB/c mice infected with *Salmonella* spp. KPC. **(C)** Kaplan-Meier plot showing the survival rates of treated and untreated BALB/c mice infected with *K. pneumoniae* IMP 216.

there was a statistically significant difference ($p = 0.021$) in expression levels when comparing Meropenem to Meropenem/calcium-EDTA (Figure 3).

Quantifying the *in vivo* relative normalized expression levels of *bla*_{NDM-1} in *K. pneumoniae* IMP 216 following the

treatment with Meropenem only and Meropenem/calcium-EDTA when compared to the positive control indicated 2.3-fold and 3-fold decreases in *bla*_{NDM-1} expression when Meropenem and Meropenem/Ca-EDTA were administered, respectively (Figure 3).

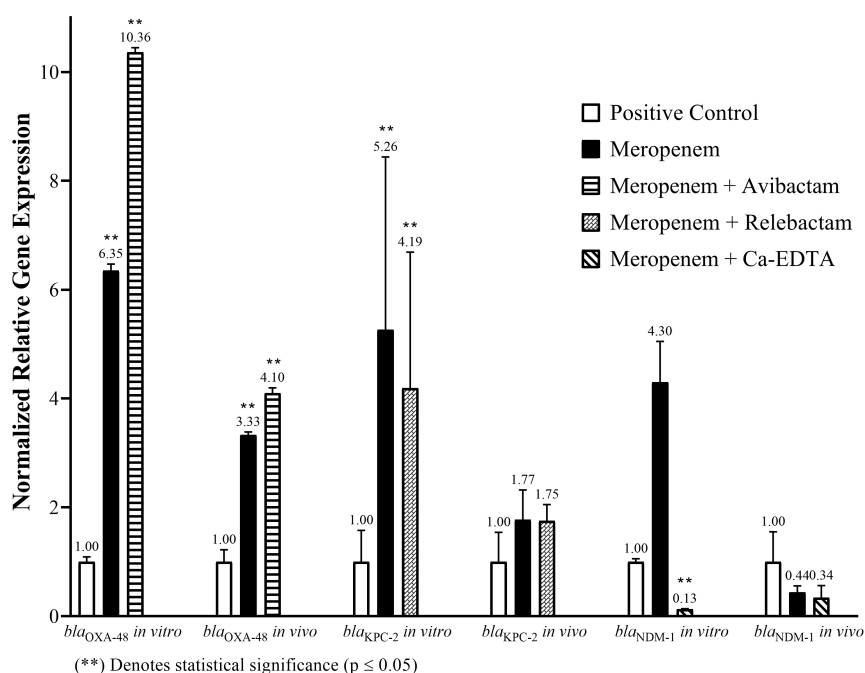


FIGURE 3 | The *in vitro* and *in vivo* normalized relative gene expression levels of *bla*_{OXA-48} in *E. coli* IMP 57, *bla*_{KPC-2} in *Salmonella* spp. KPC, and *bla*_{NDM-1} in *K. pneumoniae* IMP 216 following the addition of Meropenem alone or in combination with Avibactam, Relebactam, and calcium-EDTA, respectively.

DISCUSSION

Antimicrobial resistance is a public health threat with major repercussions. Bacteria can rapidly develop resistance to new antimicrobial agents a few years after they become available for commercial use (Centers for Disease Control and Prevention [CDC], 2013). Carbapenem-resistant *Enterobacteriaceae* (CRE) and multidrug resistant *Acinetobacter baumannii* (MDR-A. *baumannii*) rank among the highest priority pathogens for research and drug discovery according to the (World Health Organization [WHO], 2017). Similarly, CREs are classified as an urgent health hazard, while MDR-A. *baumannii* is classified as a serious health hazard according to the (Centers for Disease Control and Prevention [CDC], 2013). Evidently, providing new and alternative solutions to treat carbapenem-resistant bacterial infections is a critical need. Although combination therapy has proven to be useful, its benefits over monotherapy remain debatable. Therefore, carbapenems/ β LI combinations were chosen as potential alternative therapeutic solutions.

Evaluating Avibactam

When assessing the *in vitro* capacity of carbapenem/Avibactam combinations against CREs and MDR-A. *baumannii*, the addition of the β LI to carbapenems successfully restored most of the tested isolates' susceptibility to that class of antimicrobial agents.

The majority of the literature reports the combination of Ceftazidime/Avibactam against antimicrobial-resistant isolates; however, Aktas et al. (2012) have found that Imipenem/Avibactam managed to restore the susceptibility

of 26 *Enterobacteriaceae* isolates with OXA-48. The findings reported in this study coincide with Aktas et al. (2012) regarding Imipenem/Avibactam; however, it was observed that the addition of Meropenem/Avibactam displayed considerably lower MIC values than the former combination (Table 4); thus, highlighting Meropenem/Avibactam as the more efficacious carbapenem/ β LI combination against the tested *bla*_{OXA-48}-positive *Enterobacteriaceae* isolates. On the other hand, Ertapenem was not as effective as Imipenem or Meropenem when combined with Avibactam against OXA-48 as it only managed to restore the susceptibility of three *Enterobacteriaceae* isolates. Finally, none of the carbapenem/Avibactam combinations used in this study managed to restore the susceptibility of any of the *A. baumannii* isolates that mainly harbored *bla*_{OXA-23-like} and *bla*_{OXA-51-like} (Table 1), which is consistent with the reported literature (Thaden et al., 2016).

Concerning the *in vivo* experiments, assessing the survival rate of murine infection models against *E. coli* IMP 57 that harbors *bla*_{OXA-48} and attempting to treat the animals with Meropenem/Avibactam has not been documented in the literature yet. As such, experimental design and dosage determinations were guided by earlier studies with similar target parameters (Rahal et al., 2011a; Levasseur et al., 2014; Salloum et al., 2015). The *in vitro* and *in vivo* results observed were compatible with minimal discrepancy. The group of mice that was infected with *E. coli* IMP 57 and treated with Meropenem/Avibactam showed a 100% survival rate, while that of the groups that received Meropenem or Avibactam monotherapy experienced a 0% survival rate (Figure 2). These findings prove the efficacy of Meropenem/Avibactam against

OXA-48 among *Enterobacteriaceae*, and that the concentration of Meropenem to Avibactam at a 4:1 ratio is effective. Concerning the positive control group that was infected with *E. coli* IMP 57 without receiving any treatment, the survival of one mouse might have been due to a technical error during the intraperitoneal injection of the bacterial inoculum (Steward et al., 1968).

At the molecular level, both *in vitro* and *in vivo* results showed similar trends in relative *bla*_{OXA-48} expression, signifying the consistency of Meropenem/Avibactam activity despite the differences in environments. Meropenem appears to have induced the expression of *bla*_{OXA-48}; however, its statistically significant overexpression upon the addition of Meropenem/Avibactam could be due to a synergistic relationship between Meropenem and Avibactam since it has been previously proven that Avibactam does not induce the production of β -lactamases by itself (Miossec et al., 2013), especially at concentrations below 32 μ g/mL (Livermore et al., 2017); thus, had there not been synergism between them, the level of *bla*_{OXA-48} expression upon the addition of Meropenem/Avibactam would be similar to that following the addition of Meropenem alone. Additional data supporting the possibility of having synergism between Avibactam and Meropenem is their high affinity to the same penicillin-binding protein 2 (PBP2) in *E. coli* (Davies et al., 2008; Asli et al., 2016). It is worthy to note that regardless of the overexpressed carbapenemase, a low concentration of Avibactam was sufficient to inhibit the enzyme and permit the activity of Meropenem. It is possible that such a low concentration of Avibactam was sufficient due to the reversible inhibition property that it displays (Ehmann et al., 2012).

Evaluating Relebactam

The *in vitro* testing of REL in combination with carbapenems has demonstrated its effectiveness against the tested *Salmonella* spp. KPC isolate as its susceptibility to IPM, ETP, and MEM was restored. These findings are in line with a previous study that reported IPM/REL as an effective combination against 78.5% Imipenem-non-susceptible non-*Proteaeae Enterobacteriaceae* (Karlowsky et al., 2018). Furthermore, carbapenems/Relebactam showed targeted potency against KPC-2 and that also complements an earlier study that reported Imipenem/Relebactam restoring the susceptibility of 97% of KPC-producing *K. pneumoniae* isolates (Lapuebla et al., 2015). In addition, the safety and efficacy of Imipenem/Cilastatin/Relebactam versus Imipenem/Cilastatin alone have been tested in clinical trials among patients with complicated intra-abdominal infections (cIAI) (Lucasti et al., 2016) and complicated urinary tract infections (cUTI) (Sims et al., 2017). Both of those trials concluded that treating patients suffering from cIAI or cUTI using IPM/REL resulted in high rates of favorable microbiological and clinical responses at the end of treatment although IPM/REL was non-inferior to IPM alone. Despite the majority of the literature reporting Imipenem/Relebactam combinations, the results presented here provide Ertapenem and Meropenem as more efficacious alternatives as they displayed lower MICs, reaching 0.03125 μ g/mL, upon their combination with Relebactam (Table 4).

With regards to the *in vivo* murine survival studies, the 100% survival rate of the mice treated with Meropenem/Relebactam against the *bla*_{KPC-2}-positive bacterial isolate in comparison to the 0% survival rates of their control groups matched the *in vitro* results and mimicked those of Meropenem/Avibactam against *bla*_{OXA-48} (Figure 2), as this has also not yet been documented in the literature. These findings support the potency of the Meropenem/Relebactam combination against the tested *Salmonella* spp. KPC isolate *in vivo* as well as the efficacy of the 8:1 dosing ratio of Relebactam to Meropenem and the route of antimicrobial administration.

At the molecular level, Meropenem seemed to have induced the overexpression of *bla*_{KPC-2} *in vitro* regardless of the potentiating effect of Relebactam, as the latter is not a β -lactamase inducer by itself (Livermore et al., 2017); however, the insignificant decrease in gene expression levels due to the addition of Meropenem/Relebactam as compared to Meropenem alone could be due to the inhibitory effect that Relebactam exerted on KPC-2 without it having a compensatory mechanism that is similar to the one observed in *bla*_{OXA-48}. On the other hand, the near-identical expression levels of *bla*_{KPC-2} *in vivo* upon the treatment with Meropenem monotherapy in comparison to Meropenem/Relebactam does not correlate with its *in vitro* gene expression observations despite displaying potency in the murine infection model. That discrepancy might be due to the change of environment between *in vitro* and *in vivo* settings, which could have altered the behavior of *bla*_{KPC-2} in response to treatment; however, that conclusion requires further investigation.

Evaluating Calcium-EDTA

The *in vitro* assessment of Ca-EDTA in combination with carbapenems displayed high susceptibility rates amongst the tested isolates that harbor *bla*_{NDM-1} (Table 4). These results validate an earlier study that investigated the efficacy of Imipenem and Meropenem in combination with Ca-EDTA against NDM-1-positive *K. pneumoniae* and *E. coli* isolates (Yoshizumi et al., 2013); however, in contrast to that study, the Imipenem and Meropenem combinations with Ca-EDTA successfully lowered MIC values by a maximum of 512-fold, to reach 0.03125 μ g/mL for Meropenem/Ca-EDTA, whereas those in Yoshizumi et al. (2013) were lowered by 256-fold at most, reaching 1 μ g/mL. Finally, the combination of Ca-EDTA with Ertapenem was not as successful as with the other two carbapenems since it failed to render any of the tested isolates susceptible although it did lower the MIC of one isolate by at least 128-fold. These finding highlight Meropenem/Ca-EDTA as the more efficacious combination against the tested isolates.

Concerning the *in vivo* murine survival studies, Meropenem/Ca-EDTA did not demonstrate any added efficacy when compared to Meropenem monotherapy as the mice from both groups achieved identical survival rates of 16.7% whereas the positive control and Ca-EDTA monotherapy groups each resulted in 0% survival. A similar acute lethal septicemia experiment involving an NDM-1-positive *E. coli* isolate was performed by Yoshizumi et al. (2013); however, bacterial burden was assessed in that study and it was concluded that

Imipenem/Ca-EDTA significantly reduced the bacterial burden in the blood and liver of neutropenic mice. In this study, the Meropenem/Ca-EDTA combination might have failed to demonstrate additional potency due to a potentially insufficient dose or an inappropriate intraperitoneal route of administration as Ca-EDTA might have chelated non-specific divalent cations found in the mice's bodies.

With regards to the gene expression levels, the addition of Meropenem *in vitro* did not seem to significantly alter the expression of *bla_{NDM-1}*; however, supplementing Ca-EDTA to Meropenem managed to significantly suppress the expression of the gene by approximately eightfold. These findings directly explain the observed decrease in MIC levels as Meropenem/Ca-EDTA successfully inhibited the carbapenemase and restored the isolate's susceptibility. On the other hand, the use of Meropenem/Ca-EDTA as a treatment option in the murine infection model did not cause a significant difference in the expression levels of *bla_{NDM-1}* as compared to the positive control, but instead resulted in a gene expression level that is similar to the one due to Meropenem monotherapy. This latter observation supports the inefficacy of Meropenem/Ca-EDTA that was observed in the survival studies, as Ca-EDTA does not appear to have exerted its inhibitory effect on NDM-1 *in vivo*, leaving Meropenem to act on its own, which resulted in similar gene expression levels under both treatment conditions.

CONCLUSION

Utilizing carbapenems, namely Meropenem, with the novel β -lactamase inhibitors Avibactam, Relebactam, and Ca-EDTA has proven to be capable of restoring carbapenem susceptibility among bacterial isolates that express the highly clinically relevant carbapenemases OXA-48, KPC-2, and NDM-1. Taken together, the *in vitro*, *in vivo*, and gene expression data encourage further investigating Meropenem/Avibactam and Meropenem/Relebactam as potential targeted therapeutic options against OXA-48 and KPC, respectively. However, a multi-faceted approach with a larger sample size and greater genetic diversity is required for both phenotypic and genotypic testing before proceeding into further preclinical and clinical development.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

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ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Institutional Animal Care and Use Committee at the American University of Beirut. The protocol was approved by the Institutional Animal Care and Use Committee at the American University of Beirut under approval #17-08-432.

AUTHOR CONTRIBUTIONS

BH designed and executed the experiments, analyzed the data, and wrote and edited the manuscript. SR designed the experiments, analyzed the data, and reviewed and edited the manuscript. AAF provided scientific feedback, analyzed the data, and reviewed and edited the manuscript. GA performed phenotypic bacteriological testing, supplied the bacterial isolates, provided scientific feedback, and reviewed and edited the manuscript. GM conceived the study, designed the experiments, analyzed the data, and edited the overall 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/fmicb.2019.00933/full#supplementary-material>

FIGURE S1 | (A) Recorded average mice weights in their different groups during LD₅₀ determination of *E. coli* IMP 57. **(B)** Recorded average mice weights in their different groups during LD₅₀ determination of *K. pneumoniae* IMP 216. **(C)** Recorded average mice weights in their different groups during LD₅₀ determination of *Salmonella* spp. KPC.

FIGURE S2 | (A) Recorded average mice weights in their different groups during survival studies against *E. coli* IMP 57. **(B)** Recorded average mice weights in their different groups during survival studies against *K. pneumoniae* IMP 216. **(C)** Recorded average mice weights in their different groups during survival studies against *Salmonella* spp. KPC.

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YhjX Regulates the Growth of *Escherichia coli* in the Presence of a Subinhibitory Concentration of Gentamicin and Mediates the Adaptive Resistance to Gentamicin

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The mechanisms of adaptive resistance of *Escherichia coli* to aminoglycosides remain unclear. Our RNA-Seq study found that expression of *yhjX* was markedly upregulated during initial exposure to subinhibitory concentrations of gentamicin. The expression of *yhjX* was then downregulated dramatically during a second exposure to gentamicin compared to the first exposure. *YhjX* encodes a putative transporter of the major facilitator superfamily, which is known to be the sole target of the YpdA/YpdB two-component system, the expression of which is highly and specifically induced by pyruvate. To investigate the effect of *yhjX* on the adaptive resistance of *E. coli*, in the present study, we constructed *yhjX* deletion and complemented strains of *E. coli* ATCC25922. Changes in extracellular pyruvate levels of wide-type and *yhjX* mutant were measured to determine whether *YhjX* functions as a pyruvate transporter. The results showed that *yhjX* deletion improved the growth of *E. coli* in medium containing subinhibitory concentrations of gentamicin. The *yhjX* deletion mutant did not exhibit adaptive resistance to subinhibitory concentrations of gentamicin. *YhjX* might not function as a pyruvate efflux pump in *E. coli* but was associated with the decrease following a sharp increase in the extracellular pyruvate level. Our findings indicate that *yhjX* regulates the growth of *E. coli* in the presence of a subinhibitory concentration of gentamicin and mediates the adaptive resistance to gentamicin.

Keywords: *Escherichia coli*, adaptive resistance, gentamicin, *YhjX*, bacterial growth

INTRODUCTION

Aminoglycosides are commonly used to treat clinical infections because of the excellent effects of these compounds against Gram-negative bacteria. However, adaptive resistance to aminoglycosides has been observed *in vitro* and *in vivo* in aerobic and facultative Gram-negative bacilli (Karlowsky et al., 1997b), which might limit the efficacy of these antibiotics in the treatment of clinical infections.

Adaptive resistance to aminoglycosides refers to reduced antimicrobial killing in originally susceptible bacterial populations after initial exposure to aminoglycosides (Karlowsky et al., 1997a; Xiong et al., 1997). Adaptive resistance to aminoglycosides has been reported mostly with *Pseudomonas aeruginosa* but also with *Escherichia coli*, *Staphylococcus aureus*, and other bacteria (Karlowsky et al., 1997b; Motta et al., 2015; Uemura et al., 2017).

Aberrant expression of efflux pumps in the membrane and reduced cellular uptake of aminoglycosides have been commonly considered to be involved in adaptive resistance to aminoglycosides (Hocquet et al., 2003; Skiada et al., 2011). However, the underlying molecular basis of adaptive resistance to aminoglycosides remains unclear. Therefore, it is essential that we gain a better understanding of the causes and mechanisms of adaptive resistance to aminoglycosides.

Escherichia coli is a leading pathogen that usually causes infections in the urinary tract and intestines (Katouli, 2010). Although there have been some studies investigating the mechanisms of adaptive resistance of *P. aeruginosa* to aminoglycosides (Gilleland et al., 1989; Daikos et al., 1991; Barclay et al., 1996; Xiong et al., 1997), few studies have examined the adaptive resistance of *E. coli* to aminoglycosides. Previous studies have shown that pretreatment with subinhibitory levels of kanamycin resulted in resistance to subsequent treatment with aminoglycosides in *E. coli* (Sidhu et al., 2012; Xiacong et al., 2013). In our initial study, we also found that pretreatment with a subinhibitory concentration of gentamicin, another aminoglycoside, induced adaptive resistance to gentamicin in *E. coli* ATCC25922. To investigate the mechanisms involved in this process, we conducted transcriptome sequencing of *E. coli* after pretreatment with subinhibitory concentration of gentamicin. The results of RNA sequencing showed that the expression of *yhjX*, a gene encoding a putative transporter of the major facilitator superfamily, increased 20.65 times compared to that in untreated cells, which was the greatest increase among the upregulated genes. The expression of *yhjX* was then downregulated dramatically during the second exposure to gentamicin compared to the first exposure. This phenomenon suggested that *yhjX* might be involved in the occurrence of adaptive resistance to gentamicin. It encodes a putative major facilitator superfamily transporter with 12 predicted transmembrane helices (Pao et al., 1998). It has been reported that *yhjX* is the sole target of the YpdA/YpdB two-component system, which is strongly and specifically induced by pyruvate (Fried et al., 2013). To investigate the role of *yhjX* in the adaptive resistance of *E. coli* to sub-MIC gentamicin, in this study, we confirmed the changes in expression of *yhjX* in *E. coli* after initial

and second exposure to gentamicin and constructed a *yhjX* knockout strain and the corresponding complemented strain. We found that the *yhjX* mutant grew better when exposed to sub-MIC gentamicin initially but less well during the second exposure to gentamicin. It has also been found that when glucuronate or gluconate is present as the primary carbon source, the extracellular pyruvate level increases and *yhjX* expression is induced. Although YhjX protein is annotated as a “putative pyruvate transporter¹”, this function in *E. coli* has not yet been proven. We suspected that YhjX might be a pyruvate efflux pump that contributes to the slow growth in the presence of gentamicin. To prove this hypothesis, the extracellular pyruvate levels were also measured. However, the extracellular pyruvate levels of the *yhjX*-deleted mutant did not decrease but increased instead in Muller-Hinton broth (MHB) supplemented with gentamicin and M9 minimal medium supplemented with glucuronate compared with those of the wild-type. Our findings demonstrate that *yhjX* regulates the growth of *E. coli* in the presence of a subinhibitory concentration of gentamicin and mediates the adaptive resistance to gentamicin. The protein encoded by *yhjX* is not a pyruvate efflux pump in *E. coli*, and further studies are necessary to investigate the mode of transport and specific substrate of YhjX.

MATERIALS AND METHODS

Bacterial Strain and Determination of the MIC of Gentamicin

Escherichia coli strain ATCC25922 was used as the wild-type strain for this study. The MIC of gentamicin was determined using the broth microdilution method recommended by CLSI (Clinical and Laboratory Standards Institute) 2009. Overnight cultures were grown in MHB (Oxoid, United Kingdom, cat:CM0405) at 37°C and diluted to yield an inoculum of approximately 1×10^8 CFU (colony-forming units)/ml. Then, 50 μ l of gentamicin (0.5–128 μ g/ml) was dispensed into each well of a microtiter plate, and 50 μ l of a 10^5 CFU/ml bacterial suspension was added to each well. The plate was incubated at 37°C for 24 h. The MIC was identified as the lowest concentration of gentamicin at which visible growth was inhibited. Each experiment was replicated three times.

Determination of Adaptive Resistance by Growth Curve Analysis

A single colony of *E. coli* ATCC 25922 was inoculated in 5 ml of MHB and incubated overnight at 37°C with shaking at 200 rpm. The overnight bacterial culture was diluted 1:20 in fresh MHB pretreated with 1 μ g/ml (1/2 MIC) gentamicin at 200 rpm for 1 h at 37°C. The pretreated culture was then centrifuged at 10,000 rpm for 3 min at room temperature, and the pellet was washed 3 times with fresh media and then resuspended in MHB. The bacterial suspension was adjusted to a final OD₆₀₀ of 0.2 (as detected by a Bio-Rad spectrophotometer). Simultaneously, the non-pretreated cultures were also centrifuged and resuspended

¹<https://www.ncbi.nlm.nih.gov/gene/948066>

as described above. The bacterial suspensions were diluted 1:1 with MHB containing gentamicin. The final concentration of gentamicin in each suspension was 1 µg/ml (1/2 MIC). Then, 100 µl of each suspension containing gentamicin was added to a 96-well plate and placed in a microplate reader (Molecular Devices, SpectraMax M2e) for monitoring of bacterial growth at 600 nm. Fifty microliters of sterile paraffin oil was added into each well to avoid fluid evaporation. Readings were taken every 30 min for 24 h by the microplate reader. Each experiment was performed in triplicate.

RNA Extraction, Sequencing and Analysis

Overnight cultures were diluted 1:20 and then either treated with 1/2 MIC gentamicin or left untreated and incubated at 37°C for 1 h. The cells were harvested for RNA isolation. Total RNA was isolated using the RNeasy Protect Mini Kit (Qiagen, Hilden, Germany, cat.: 74134) according to the manufacturer's instructions. RNA sequencing (RNA-Seq) was performed by Shanghai Bohao Co., Ltd., using an Illumina HiSeq 2500 (Illumina) as previously described (Li et al., 2016). RNA integrity and purity were analyzed using an Agilent 2100 bioanalyzer. The transcriptome sequencing data were aligned with the genome and plasmid sequences of *E. coli* ATCC 25922 (GenBank: CP009072.1 and CP009073.1) in the NCBI database. The relative gene expression levels were estimated by RPKM (reads per kilobase of exon sequence per million mapped reads) for normalization of gene expression (Heo et al., 2014).

Quantitative Real-Time PCR

Quantitative real-time PCR (qRT-PCR) was performed to verify the results of RNA-Seq. The RNA was converted to cDNA (Takara, Dalian, China, cat.: RR047A) by reverse transcription. One microliter of cDNA was amplified (Takara, cat.: RR820A) using an ABI 7500 real-time PCR system. *YhjX* and its regulator gene *ypdB*, *fliN* (flagellar motor switch protein), *cpxP* (inhibitor of the cpx response periplasmic adaptor protein), *glcA* (type II citrate synthase), *aceE* (pyruvate dehydrogenase), *sdhC* and *sdhD* (succinate dehydrogenase cytochrome b556 small membrane subunits) were selected for real-time PCR studies. The GAPDH gene was used as the housekeeping reference gene (Lee et al., 2010). The primers used for real-time PCR quantification of the expression of each gene are listed in Table 1. The fold change was calculated using the $2^{-\Delta\Delta C_t}$ method and is presented as the fold change in the expression of pretreatment groups relative to that of the control group (no drug treatment). In addition, the fold change of most highly upregulated gene in RNA-Seq, namely, *yhjX*, and its regulator *ypdB* were also detected in cells that were re-exposed to a sub-MIC of gentamicin.

Construction of the Δ *yhjX* Mutant and Complemented Strain

The Δ *yhjX* mutant was constructed using the suicide T-vector pLP12 carrying a counterselectable marker, *vmi480* (Luo et al., 2015). Briefly, a *yhjX* gene fusion fragment was amplified by PCR, ligated with pLP12 and subsequently transformed into

E. coli β 2163. The resulting plasmids were introduced into *E. coli* ATCC25922 via conjugation with *E. coli* β 2163. After two rounds of selection, the mutant with the *yhjX* gene deleted was validated by PCR using primers corresponding to sequences upstream and downstream of the deletion and by subsequent sequencing. The *yhjX* knock-out mutant was transformed with the pBAD30:*yhjX* plasmid (carrying ampicillin resistance marker gene *ampR*) to obtain the *yhjX*-complemented strain. The complemented strain was cultured in medium containing 100 µg/ml ampicillin. L-arabinose (0.2%) was added to the medium to induce the expression of *yhjX* in the complemented strain.

Determination of the MICs of Gentamicin and Other Antibiotics Against the ATCC 25922 Wild-Type, Δ *yhjX* Mutant and Complemented Strains

The MICs of gentamicin and other antibiotics, including cefuroxime, cefotiam, ceftazidime, ciprofloxacin, and imipenem, against the ATCC 25922 wild-type, Δ *yhjX* mutant and complemented strains were determined using the broth microdilution method as previously mentioned.

Growth Curve of the *E. coli* Wild-Type, Δ *yhjX* Mutant and Complemented Strains in the Presence and Absence of a Subinhibitory Concentration of Gentamicin

Overnight cultures of the *E. coli* ATCC25922 wild-type, Δ *yhjX* mutant and complemented strains were diluted in fresh MHB to a final OD₆₀₀ of 0.2. The suspensions were diluted 1:1 with MHB with or without gentamicin in a 96-well plate. The treated cells were grown in the presence of 1/2 MIC gentamicin, and the control was treated with MHB. Then, 50 µl of sterile paraffin oil was added into each well to avoid fluid evaporation. The 96-well plate was then placed into a microplate reader for OD₆₀₀ measurements every 0.5 h at 37°C for 24 h. The experiment was replicated three times.

Determination of Adaptive Resistance of the *E. coli* Wild-Type, Δ *yhjX* Mutant and Complemented Strains by Growth Curve Analysis

The same method used for the adaptive resistance experiment above was used to examine the *E. coli* wild-type, Δ *yhjX* mutant and complemented strains.

Measurement of Extracellular Pyruvate Levels in *E. coli* Δ *yhjX* Mutant and Wild-Type Cultures and Relative Expression of *yhjX* in *E. coli* Grown in Different Media

Overnight cultures of the ATCC 25922 wild-type and Δ *yhjX* mutant strains were diluted with fresh media (MHB with or

TABLE 1 | Primers used for qRT-PCR.

Gene	Forward primer	Reverse primer	References
<i>yhjX</i>	ATGTATGTGATTGGTGTAGCGAAAG	CAGAAAGGTTGGCGATGGA	
<i>ypdB</i>	CATTACCGGGATGCTGCAAA	GGTCATTTCTCGTGCGCTT	
<i>flhN</i>	GGACGATCTGTGGGCTGAA	GACATCACCACCGCCAAA	
<i>cpxP</i>	GGCATCCGGGTGAAGAACTT	AACCTATGCCGTGGAACATATGG	Price and Raivio, 2009
<i>gltA</i>	AACCTATGCCGTGGAACATATGG	TGTTTTCCAGCTCCATAGCC	Martinez et al., 2010
<i>aceE</i>	ACGTACCGGCTGACGACTAC	CTTATCGATTTCGCCACGTT	Lin et al., 2005
<i>sdhC</i>	AGATTTTGGCGGAGCGTTT	ATTATCATGTGGGGCATCCT'	
<i>sdhD</i>	CGGTCAACACCTGCCACAT	TGGATTGGTTTCTTCGCCTCT	
<i>GAPDH</i>	ACTTACGAGCAGATCAAAGC	AGTTTCACGAAGTTGTCGTT	

without $1/2$ MIC gentamicin, M9 minimal medium containing 0.4% glucuronate and M9 minimal medium containing 0.4% glucose) to a final OD₆₀₀ of 0.2. The levels of pyruvate in fresh culture and in supernatants of *E. coli* cultures were determined before inoculation, 30 min after inoculation and 60 min after inoculation using a pyruvate colorimetric/fluorometric assay kit (BioVision). Each experiment was replicated three times. The experimental values were calculated from a standard curve.

Quantitative RT-PCR was also performed to compare the expression levels of *yhjX* in the wild-type *E. coli* strain in different media and to analyze the relationship between *yhjX* expression levels and extracellular pyruvate concentrations. The *yhjX* expression in *E. coli* growing in M9 containing glucose at the 30 min time point was set as the control.

RESULTS

Adaptive Resistance Detected in *E. coli* After Pretreatment With a Subinhibitory Concentration of Gentamicin

The MIC of *E. coli* 25922 against gentamicin was 2.0 μ g/ml. As shown in **Figure 1A**, in comparison to the control (exposed to gentamicin for the first time), *E. coli* pretreated with $1/2$ MIC gentamicin for a short duration (1 h) exhibited low growth during the early phase (8 h of re-exposure to gentamicin), suggesting a postantibiotic effect (PAE) caused by gentamicin. However, the growth rate of the pretreated cells markedly increased during the late phase (from 10 to 24 h, $p = 0.027$ at the 24-h time point). This result suggested that the adaptive resistance of *E. coli* could be induced by initial exposure and re-exposure to subinhibitory concentrations of gentamicin.

Comparative Transcriptomic Analysis of *E. coli* ATCC 25922 Exposed to a Sub-MIC of Gentamicin

To screen out genes that may be involved in the development of adaptive resistance, RNA-Seq of untreated *E. coli* and *E. coli* treated with a sub-MIC of gentamicin was performed. We selected genes that showed a twofold change in expression after treatment with sub-MIC gentamicin compared with the expression in control cells that were not exposed to gentamicin.

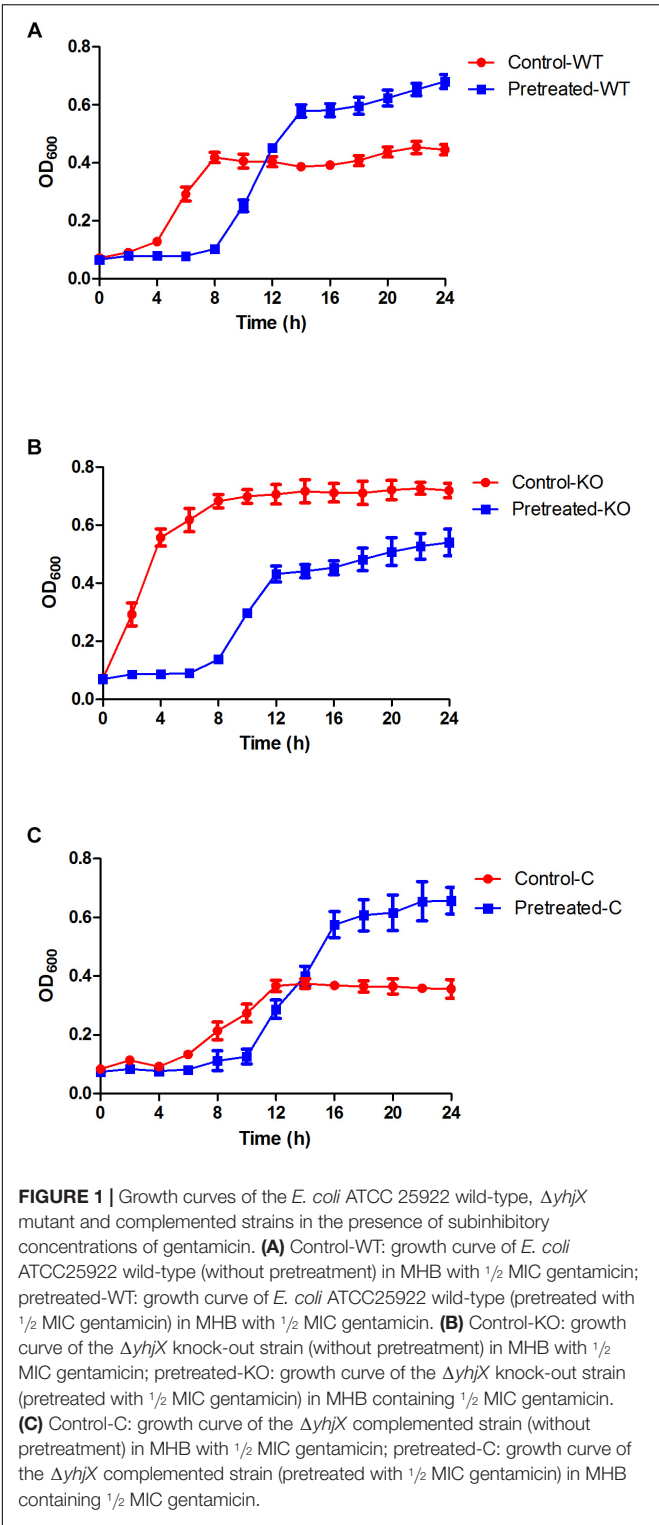
In response to gentamicin, the expression levels of 235 genes were upregulated (**Supplementary Table S1**), and the levels of 349 genes were downregulated (**Supplementary Table S2**). The roles of differentially regulated genes were assigned according to the KEGG database². The results were verified using qRT-PCR (**Table 2**).

As shown in **Figure 2**, differentially regulated genes were mainly enriched in the categories membrane and transporter, ribosome and translation, stress response, motility, TCA (tricarboxylic acid) cycle, glycolysis/gluconeogenesis and other carbohydrate metabolism processes, protein and amino acid metabolism, transcription, DNA binding and recombination, nucleic acid metabolism, oxidation–reduction process and hypothetical proteins with unknown functions. Ninety-seven genes with membrane and transporter functions were differentially expressed; 36 of these genes were upregulated, and 61 were downregulated. *YhjX*, a gene encoding a putative transporter of the major facilitator superfamily, was upregulated with a 20.65-fold change in expression, which was the highest fold change among all the genes. Sixty-six genes involved in ribosome and translation were upregulated, and 7 such genes were downregulated. A total of 56 genes involved in the TCA cycle, glycolysis/gluconeogenesis and other carbohydrate metabolism were also differentially expressed. Twenty-eight genes involved in stress response and 22 genes involved in motility were differentially regulated.

YhjX Was Highly Activated When Exposed to Subinhibitory Concentrations of Gentamicin

YpdB protein has been proven to be the regulator of *yhjX*, functioning by binding to two direct repeats of a motif in the *yhjX* promoter. We therefore performed qRT-PCR to detect the differential expression of *yhjX* and *ypdB* in *E. coli* initially exposed to and then re-exposed to subinhibitory concentrations of gentamicin. An untreated strain was set as the control group. As shown in **Figure 3**, compared to the untreated cells, *yhjX* of *E. coli* was 43.6- and 7.6-fold upregulated after first exposure and second exposure to $1/2$ MIC gentamicin, respectively. However, the expression level of *ypdB* was unchanged after both initial exposure and re-exposure to gentamicin.

²<https://www.kegg.jp/kegg/pathway.html>



YhjX Deletion Did Not Influence the MICs of Gentamicin and Other Antibiotics

To understand whether the expression of *yhjX* affects the sensitivity of *E. coli* to antibiotics, we determined the MICs of gentamicin and other antibiotics, including cefuroxime, cefotiam,

TABLE 2 | Validation of RNA-Seq results using qRT-PCR.

Gene	Description	RNA-Seq	qRT-PCR
		Fold change	
<i>yhjX</i>	Membrane protein	20.65	46.57
<i>fliN</i>	Flagellar motor switch protein	2.18	5.04
<i>cpxP</i>	Inhibitor of the cpx response periplasmic adaptor protein	11.26	23.74
<i>gltA</i>	Type II citrate synthase	0.32	0.24
<i>aceE</i>	Pyruvate dehydrogenase	0.49	0.34
<i>sdhC</i>	Succinate dehydrogenase cytochrome b556 small membrane subunit	0.43	0.31
<i>sdhD</i>	Succinate dehydrogenase cytochrome b556 small membrane subunit	0.47	0.25
<i>ypdB</i>	Regulator of <i>yhjX</i>	0.75	0.89

ceftazidime, ciprofloxacin, and imipenem, against the ATCC 25922 wild-type, $\Delta yhjX$ mutant and complemented strains. The MICs of gentamicin, cefuroxime, cefotiam, ceftazidime, and imipenem against ATCC 25922 wild-type were 2, 4, 0.25, 0.25, and 0.25 $\mu\text{g/ml}$, respectively. Ciprofloxacin showed the strongest antibacterial effect against ATCC 25922 (MIC ≤ 0.0625). The MICs of all the tested antibiotics against the $\Delta yhjX$ mutant were the same as those against the wild-type, suggesting that *yhjX* deletion did not influence the MICs of gentamicin and other antibiotics. The MICs of cefotiam and ceftazidime against the complemented strain increased to 1 $\mu\text{g/ml}$ from the original value of 0.25 $\mu\text{g/ml}$ against the wild-type. Except for the increase in the MICs of cefuroxime and ceftazidime, there was no change in the MICs of the antibiotics against the complemented strain. However, as the vector in the complemented strain carries the ampicillin resistance gene *ampR*, the changes in the MICs of cefuroxime and ceftazidime might not be associated with the overexpression of *yhjX*.

YhjX Deletion Improved the Growth of *E. coli* in Medium Containing a Subinhibitory Concentration of Gentamicin

When cultured in MH medium, the growth curves of the $\Delta yhjX$ mutant and complemented strain were very similar to that of the wild-type *E. coli*. Although a change in MIC was not observed among these strains, the $\Delta yhjX$ mutant exhibited a higher growth rate in the $1/2$ MIC gentamicin-containing medium than the wild-type and complemented strains (Figure 3). As shown in Figure 3, both the wild-type and complemented strains cultured in $1/2$ MIC gentamicin reached stationary phase at 18 h, while the $\Delta yhjX$ mutant remained in the late exponential growth phase.

The $\Delta yhjX$ Mutant Did Not Exhibit Adaptive Resistance to a Subinhibitory Concentration of Gentamicin

After pretreatment with $1/2$ MIC gentamicin for 1 h, the $\Delta yhjX$ mutant was washed and re-exposed to $1/2$ MIC gentamicin but

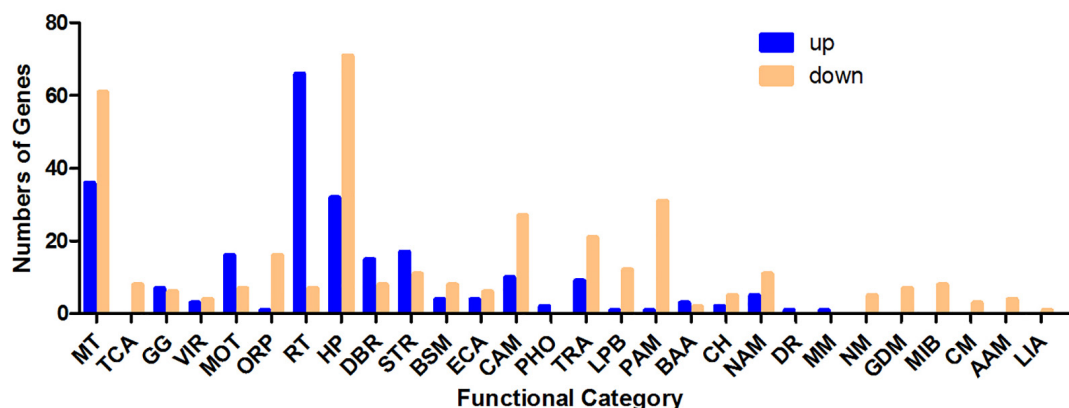


FIGURE 2 | Number of genes (classified by functional category) upregulated and downregulated in response to sub-MIC gentamicin in the RNA-Seq data. MT, membrane and transporter; TCA, TCA cycle; GG, glycolysis/gluconeogenesis. VIR, virulence; MOT, motility; ORP, oxidation–reduction process; RT, ribosome and translation; HP, hypothetical protein; DBR, DNA binding and recombination; STR, stress response; BSM, biosynthesis of secondary metabolites; ECA, electron carrier activity; CAM, other carbohydrate metabolism processes; PHO, phosphorylation; TRA, transcription; LPB, lipid biosynthesis; PAM, protein and amino acid metabolism; BAA, biosynthesis of amino acids; CH, cell shape; NAM, nucleic acid metabolism; DR, DNA replication; MM, methane metabolism; NM, nitrogen metabolism; GDM, glyoxylate and dicarboxylate metabolism; MIB, metal ion binding; CM, carnitine metabolic process; AAM, ascorbate and aldarate metabolism; LIA, lysozyme inhibitor activity.

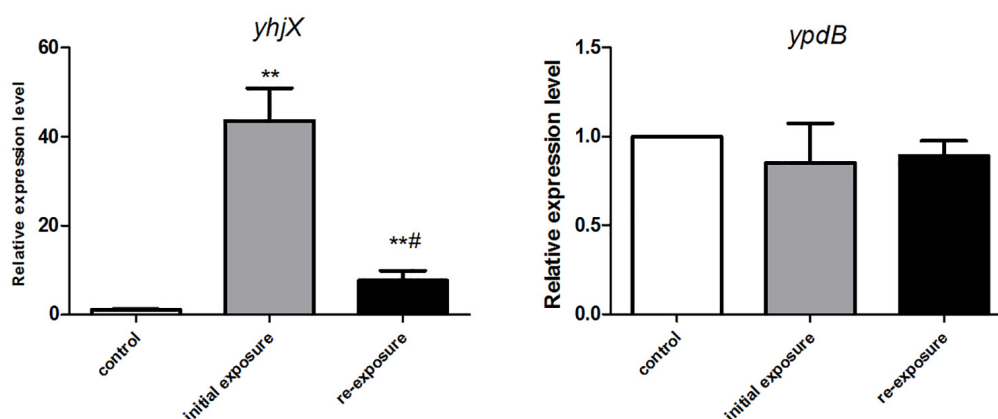


FIGURE 3 | Changes in gene expression of *yhjX* and *ypdB* in *E. coli* ATCC25922 wild-type after initial exposure and re-exposure to $1/2$ MIC gentamicin. ** indicates a statistically significant difference ($P < 0.01$) compared to the control (without treatment); # indicates a statistically significant difference ($P < 0.01$) compared to initial exposure to gentamicin.

showed lower growth than the mutant without pretreatment (Figures 1B,C). This result indicated that the *yhjX*-deleted mutant did not exhibit adaptive resistance to subinhibitory concentrations of gentamicin, unlike the wild-type strain.

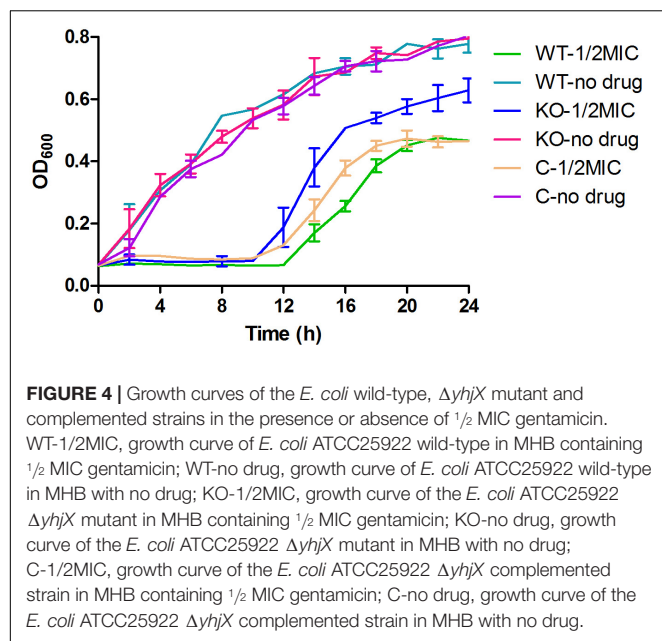
YhjX Was Not a Pyruvate Efflux Pump but Was Associated With the Decrease Following an Increase in Extracellular Pyruvate Levels

A previous study demonstrated that extracellular pyruvate stimulated the induction of *yhjX*. *YhjX* induction was observed in LB medium and M9 minimal medium with gluconate or glucuronate (Fried et al., 2013). When glucose was the sole C source, extracellular pyruvate levels did not increase, and the

expression levels of *yhjX* remained low. To verify these results and compare the expression of *yhjX* in different media, the extracellular concentrations of pyruvate were determined, and relative *yhjX* expression was detected by qRT-PCR.

As shown in Figure 5A, the expression levels of *yhjX* in *E. coli* grown in MH medium, MH medium with gentamicin and M9 minimal medium showed a higher fold change at both 30 and 60 min than the expression levels in the control (grown in M9 minimal medium with glucose). The expression level of *yhjX* in *E. coli* grown in MH medium with gentamicin was dramatically upregulated compared to that in other media.

Figure 5B shows that the basal concentrations of pyruvate in MH medium and MH plus gentamicin were more than 3 times higher than those in M9 medium containing gluconate or glucose. A sharp increase in extracellular pyruvate levels at

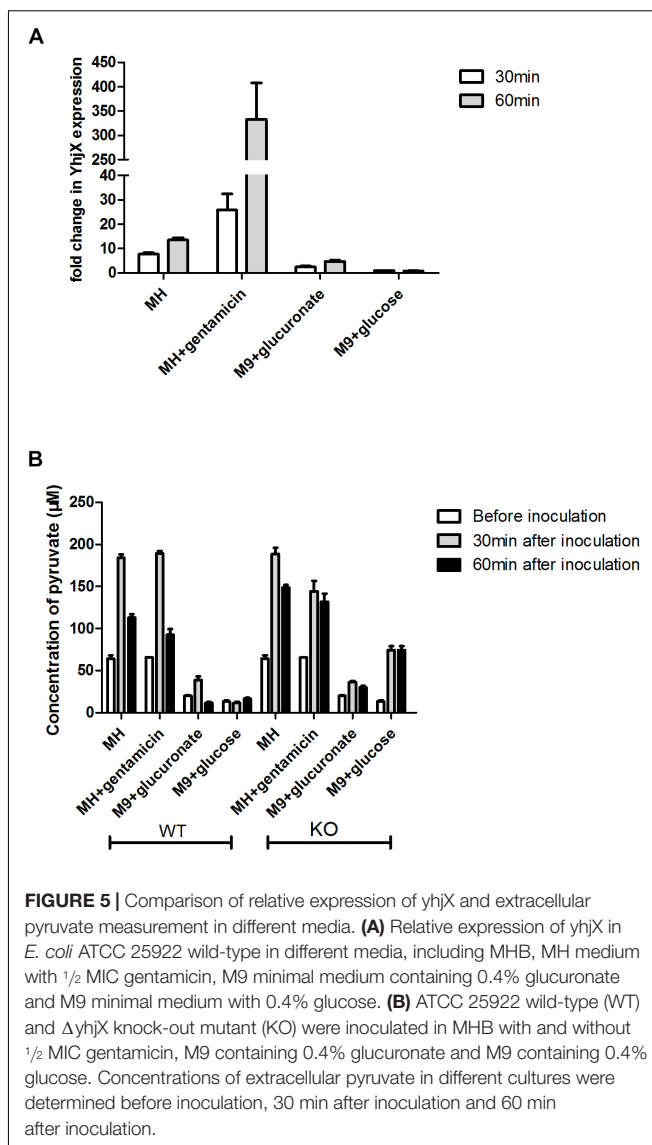


30 min and a subsequent more than 40% decrease at 60 min could be detected when *E. coli* wild-type was grown in MH medium, MHB containing 0.4% gentamicin and M9 containing 0.4% glucuronate. There were no significant changes in pyruvate concentration at different time points for the wild-type grown in M9 medium plus glucose. In contrast, for the *yhjX* mutant, no significant decrease ($p > 0.05$) was detected at 60 min after inoculation (compared to the level of pyruvate at 30 min) in any of the media. In addition, the extracellular levels of pyruvate of the *yhjX* knock-out mutant were higher at 60 min after inoculation than those of the wild-type strain grown in all four media. These results suggested that YhjX might not play the role of pyruvate efflux pump in *E. coli* but was associated with the decrease following a sharp increase in the extracellular pyruvate level.

DISCUSSION

In vitro, animal and clinical studies have shown that the development of marked adaptive resistance of Gram-negative bacteria to aminoglycosides occurs within 1–2 h of the first dose. Adaptive resistance to aminoglycosides seems to be caused not by a genetic mutational change but rather by a protective phenotypic alteration of bacteria. It has been reported that exposure of bacteria to sublethal concentrations of antibiotics can lead to increased efflux pump expression, providing adaptive antibiotic resistance (Sidhu et al., 2012). However, the mechanisms of adaptive resistance of Gram-negative bacteria to aminoglycosides remain unclear.

Adaptive resistance could be defined as reduced antimicrobial susceptibility in bacteria after initial exposure to antibiotics. This study confirmed that the adaptive resistance of *E. coli* ATCC 25922 could be induced by initial exposure to subinhibitory



concentrations of gentamicin. To improve the understanding of the molecular mechanisms involved in adaptive resistance in *E. coli*, we performed whole-transcriptome profiling of ATCC 25922 using RNA-Seq. RNA-Seq data (**Supplementary Tables S1, S2**) showed that a high number of genes associated with membrane and transporter functions were strongly regulated, suggesting that these genes might play key roles in the gentamicin tolerance of *E. coli*. Sixty-six genes associated with ribosome and translation were upregulated, and 7 such genes were downregulated. We hypothesize that the changes in the expression of these genes are associated with the mechanism of action of gentamicin, which inhibits protein synthesis by binding to the 30S subunit of the ribosome. A total of 56 genes involved in the TCA cycle, glycolysis/gluconeogenesis and other carbohydrate metabolism processes were also differentially expressed. Notably, sub-MICs of gentamicin clearly inhibit the TCA cycle of *E. coli* by downregulating 8 genes involved in the

TCA cycle. These genes include the pyruvate dehydrogenase-encoding genes DR76_21695 and *aceE*; type II citrate synthase-encoding gene *gltA*; *sdhD* and *sdhC*, encoding succinate dehydrogenase cytochrome b556 small membrane subunits; *sucC*, encoding succinyl-CoA synthetase subunit beta; the isocitrate dehydrogenase-encoding gene DR76_19495; and the fumarate hydratase class I-encoding gene DR76_16820. These genes encode the key citric acid cycle enzymes that contribute to energy production and metabolism. We speculate that TCA cycle inhibition and thereby the scarcity of energy sources caused by gentamicin might be the reason underlying the altered expression of genes involved in glycolysis/gluconeogenesis and other carbohydrate metabolism pathways. RNA-Seq also showed that 22 genes associated with bacterial motility were differentially regulated. Among these genes, 16 genes associated with flagella and fimbriae were upregulated. Our previous study proved that pretreatment with subinhibitory concentrations of gentamicin inhibits the swarming motility of *E. coli* ATCC 25922 (Zhuang et al., 2016), suggesting that high expression of these flagella- and fimbriae-associated genes was more likely a stress-related feedback of *E. coli* in response to the limited energy supply. Twenty-eight stress response genes were also differentially regulated by treatment with a sub-MIC of gentamicin. These genes may play a role in protecting *E. coli* from damage caused by environmental stress.

Our transcriptome sequencing data showed that *yhjX*, encoding a putative protein that is a transporter of the major facilitator superfamily (Behr et al., 2014), was the most highly upregulated gene during the first exposure to a subinhibitory concentration of gentamicin. The expression of *yhjX* in *E. coli* was then confirmed by qRT-PCR. The results showed that *yhjX* expression was upregulated 46.6- and 7.2-fold in during initial exposure and re-exposure to $1/2$ MIC gentamicin, respectively, compared to that in untreated cells. These data suggest that *yhjX* is a sensitive gentamicin response-related gene in *E. coli*.

The changes in expression of *yhjX* between the first and second exposures to gentamicin seem to be associated with the growth status of *E. coli* in the presence of gentamicin. To investigate the involvement of the *yhjX* gene in the growth of *E. coli* and the tolerance of the cells to gentamicin, we constructed the *yhjX*-deleted strain and the complemented strain. We observed that there was no difference in growth rate among the wild-type, *yhjX*-deleted and complemented strains cultured in MH medium. In addition, *yhjX* deletion did not influence the MICs of gentamicin or other antibiotics, including cefuroxime, cefotiam, ceftazidime, ciprofloxacin, and imipenem. However, when cultured in MH medium containing $1/2$ MIC gentamicin, the *yhjX*-deleted strain showed a higher growth rate than the wild-type and complemented strains, suggesting that *yhjX* contributes to bacterial tolerance to a subinhibitory concentration of gentamicin.

A previous study reported that *yhjX* of *E. coli* was highly activated in the presence of 1,4-butanediol; however, overexpression of the *yhjX* gene did not result in any improvement in 1,4-BDO tolerance (Szmidt-Middleton et al., 2013). In contrast, *yhjX* deletion improved the growth of *E. coli* strains in the control defined medium but not in 1,4-BDO

(Szmidt-Middleton et al., 2013). Behr et al. (2014) reported that shortage of certain C sources increases extracellular pyruvate release and thereby triggers the expression of *yhjX* (Fried et al., 2013). However, whether *yhjX* inversely plays a role in pyruvate efflux and thereby decreases the growth of *E. coli* remains unclear. Our qRT-PCR data showed that relative to the control (grown in M9 minimal medium with glucose), the expression levels of *yhjX* in *E. coli* grown in MH medium, MH medium with gentamicin or M9 minimal medium were higher at both 30 and 60 min. A sharp increase in extracellular pyruvate levels at 30 min and a subsequent decrease at 60 min could be detected when wild-type *E. coli* was grown in MH medium, MHB containing 0.4% gentamicin or M9 containing 0.4% glucuronate. However, for the $\Delta yhjX$ knock-out mutant, the extracellular levels of pyruvate did not decrease significantly at 60 min after inoculation (compared to the level of pyruvate at 30 min) in any of the media. The above results suggested that *yhjX* might not encode a pyruvate efflux pump in *E. coli* but was associated with the decrease following a sharp increase in the extracellular pyruvate level. This finding was in consistent with the study reported by Fried et al. (2013). In addition, although *yhjX* expression was greatly induced in gentamicin-containing MH medium, we did not detect a marked change in extracellular pyruvate levels compared to the levels in MH medium without gentamicin. This result implies that pyruvate is not the only factor that triggers the induction of *yhjX* expression. Because the subinhibitory concentration of gentamicin influenced the metabolic pathway of *E. coli* by downregulating the TCA cycle enzymes, we hypothesize that the presence of a subinhibitory concentration of gentamicin induces *yhjX* expression by interfering with the nutrient and energy metabolism pathways of *E. coli*. *YhjX* was also predicted to be involved in the exchange carboxylic acids based on sequence similarity to the oxalate:formate antiporter OxIT in *Oxalobacter formigenes* (Pao et al., 1998; Keseler et al., 2009). In *O. formigenes*, oxalate is imported by OxIT to synthesize acetyl-CoA for further energy production. *YhjX* functioning as an oxalate transporter like OxIT might explain the higher extracellular pyruvate levels of the $\Delta yhjX$ mutant. Further studies are needed to investigate the specific function of *YhjX* and the mode of action of this protein.

To determine whether *yhjX* is associated with adaptive resistance to gentamicin, we monitored the growth of the $\Delta yhjX$ mutant during the second exposure to $1/2$ MIC gentamicin. Unlike the *E. coli* wild-type strain and complemented strain, the $\Delta yhjX$ mutant during the second exposure to gentamicin showed a lower growth rate than the control (the $\Delta yhjX$ mutant during the first exposure to gentamicin), indicating that no adaptive resistance to gentamicin was induced in the $\Delta yhjX$ mutant. The complemented strain regained the adaptive resistance when exposed to gentamicin for a second time. These results imply that *yhjX* mediates the adaptive resistance of *E. coli* to subinhibitory concentrations of gentamicin.

YhjX is suggested to be the only target gene regulated by the YpdA/YpdB system of *E. coli* (Fried et al., 2013; Behr et al., 2017). *YhjX* expression is dependent on the specific binding of $6 \times \text{His-ypdB}$ with the *yhjX* promoter (Fried et al., 2013). In this study, no change in the expression of ypdB in the

presence of 1/2 MIC gentamicin was observed by qRT-PCR or transcriptome sequencing (data not shown), suggesting that the induction of *yhjX* expression by gentamicin might be mediated by post-transcriptional regulation of *ypdB*.

It has been reported that *yhjX* contributes to nutrient scavenging before cells enter the stationary phase. It seems that *yhjX* limits bacterial growth under specific stress conditions via the control of nutrient consumption. Taken together, our results indicate that *yhjX* facilitates a sensitive bacterial response to environmental stress. This protein also functions as a regulator of bacterial growth and metabolism in nutrient-limited or energy-scarce conditions. *YhjX* expression is highly upregulated by specific stress conditions (such as the TCA cycle-inhibited condition in the presence of gentamicin), which in turn reduces the growth and metabolism of bacteria. This response of bacteria may aid the long-term survival of the cells in a nutrient- or energy-limited environment. When the *yhjX* gene is deleted, bacteria lose the ability to regulate growth and metabolism under environmental stress. Therefore, the growth and metabolism of *yhjX*-deficient bacteria are accelerated under environmental stress, and simultaneously, the long-term tolerance to the stress condition is also impaired. This finding may explain the impaired ability of adaptive resistance of the *E. coli* $\Delta yhjX$ mutant during the second exposure to gentamicin. Further study is needed to investigate the underlying molecular mechanisms of the involvement of *yhjX* in bacterial growth and adaptive resistance. In addition, this study needs to be extended to the tests on clinical strains, not just limited to a standard strain.

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CONCLUSION

The function of *yhjX* in *E. coli* is complex, which may be associated with the regulation of bacterial growth under specific stress conditions. It also mediates the adaptive resistance of *E. coli* to subinhibitory concentrations of gentamicin.

AUTHOR CONTRIBUTIONS

SZ and YZ completed the majority of this study and contributed equally. SZ wrote the first draft. XJZ, FY, and HFL helped with the gene knockout experiments. HYL, XGZ, JW, HZ, YH, and SL assisted with the bacteria experiments. GN assisted with the other experiments. QP designed the experiments, provided funding, and revised the manuscript.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest Statement: 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 *in vitro*, *ex vivo*, and *in vivo* Efficacy of the Antimicrobial Peptide DPK-060 Used for Topical Treatment

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Antimicrobial peptides, also known as host defense peptides, have recently emerged as a promising new category of therapeutic agents for the treatment of infectious diseases. This study evaluated the preclinical *in vitro*, *ex vivo*, and *in vivo* antimicrobial activity, as well as the potential to cause skin irritation, of human kininogen-derived antimicrobial peptide DPK-060 in different formulations designed for topical delivery. We found that DPK-060 formulated in acetate buffer or poloxamer gel caused a marked reduction of bacterial counts of *Staphylococcus aureus in vitro* (minimum microbicidal concentration <5 µg/ml). We also found that DPK-060 in poloxamer gel significantly suppressed microbial survival in an *ex vivo* wound infection model using pig skin and in an *in vivo* mouse model of surgical site infection (≥99 or ≥94% reduction in bacterial counts was achieved with 1% DPK-060 at 4 h post-treatment, respectively). Encapsulation of DPK-060 in different types of lipid nanocapsules or cubosomes did not improve the bactericidal potential of the peptide under the applied test conditions. No reduction in cell viability was observed in response to administration of DPK-060 in any of the formulations tested. In conclusion, the present study confirms that DPK-060 has the potential to be an effective and safe drug candidate for the topical treatment of microbial infections; however, adsorption of the peptide to nanocarriers failed to show any additional benefits.

Keywords: antimicrobial peptides, DPK-060, skin infections, lipid nanocapsules, cubosomes

INTRODUCTION

The rapidly increasing resistance toward conventional antibiotics has accelerated research efforts to identify new and non-conventional anti-infective therapies (Czaplewski et al., 2016). One category of the recently emerged novel drug candidates for the treatment of infectious disease are antimicrobial peptides (AMPs), also known as host defense peptides (Mahlapuu et al., 2016). AMPs are low mass and generally positively charged peptides, which display a large structural and functional diversity. Most AMPs have the ability to kill microbial pathogens directly, whereas others act indirectly via immunomodulatory actions (Ejell et al., 2011). Importantly, AMPs

are generally considered to be less prone to microbial resistance compared with conventional antibiotics (Andersson et al., 2016). A number of AMPs have already been introduced into the market, and additional AMPs are currently being tested in clinical trials (Fox, 2013). In spite of these encouraging examples, there is still a considerable discrepancy between the extensive range of AMPs claimed as potent drug candidates in the patents or related scientific literature and the relatively few reported outcomes of the clinical trials (Kosikowska and Lesner, 2016).

The implementation of innovative formulation strategies is one of the factors, which is expected to accelerate the translation of preclinical candidate AMPs into successful clinical products. Notably, the use of nanocarriers for the delivery of AMPs has recently emerged as one area of interest, since nanoparticles provide unique advantages due to their large surface area for adsorption/encapsulation of AMPs and prevention of self-aggregation of the peptides (Eckert, 2011; Sandreschi et al., 2016; Nordström and Malmsten, 2017).

One of the AMPs, where intense preclinical and clinical research has been conducted in the past, is DPK-060 (also known as GKH17-WWW), which was developed for the treatment of skin infections. DPK-060 is a chemically synthesized peptide structurally derived from human protein kininogen, where three tryptophan residues have been added to the C-terminal end of the endogenous 17-amino acid sequence (Schmidtchen et al., 2009). In comparison to its endogenous analog, this addition enhances the ability of DPK-060 to withstand enzymatic degradation by infection-affiliated proteases, without any signs of aggravated cytotoxicity (Schmidtchen et al., 2009). DPK-060 exhibits a potent broad-spectrum antimicrobial activity *in vitro* against both Gram-positive and Gram-negative bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA; Boge et al., 2017; Nordström et al., 2018). The safety and efficacy of 1% DPK-060 in a polyethylene glycol (PEG)-based ointment has been studied in a clinical phase II trial in the treatment of skin infections in atopic dermatitis patients. The results of this trial revealed that DPK-060 1% ointment was well tolerated by the subjects and significantly reduced the microbial density in eczematous lesions after 14 days with twice daily application compared with placebo (ClinicalTrials.gov Identifier: NCT01522391; EudraCT: 2007-007103-32).

This study applies new formulation strategies including different classes of nanocarriers to formulate DPK-060 for topical delivery, and characterizes these different dose formulations in terms of preclinical *in vitro*, *ex vivo*, and *in vivo* antimicrobial activity (see **Figure 1** for a schematic overview of the study).

MATERIALS AND METHODS

Peptide and Antibiotics

DPK-060 peptide (GKHKNKGKNGKHNGWKWWW; molecular weight 2.5 kDa, net charge +8.5 at pH 5.5, random coil secondary structure) was produced using Fmoc solid

phase technology at Bachem AG, Bubendorf, Switzerland. The peptide was identified by electrospray ionization mass spectrometry and the purity was assessed by HPLC. The purity of the peptide used in all studies was 98.5%. Bactroban, 2% ointment (GlaxoSmithKline, Brentford, UK) was used as control antibiotics in *in vivo* studies.

Formulations

DPK-060 was first associated with the nanocarriers, whereupon the DPK-060-loaded nanocarriers were dispersed into an *in situ* gelling poloxamer formulation (**Table 1**). The nanocarriers were characterized in terms of size (Z-average particle diameter) and polydispersity index by dynamic light scattering prior to use (Boge et al., 2016; Umerska et al., 2016b). The chemical stability of the peptide in all formulations investigated has been verified (assay by HPLC >95%).

Poloxamer Gel Formulation

Poloxamer 407 (Kolliphor® P407; kindly provided by BASF, Ludwigshafen, Germany) and methyl paraben (Fluka, Buchs, Switzerland) were dissolved in 20 mM acetate buffer pH 5.5 in an ice bath for 1–2 h until the polymer was completely dissolved (23 wt% poloxamer 407 and 0.03 wt% methyl paraben). For formulations containing DPK-060 without a nanocarrier, DPK-060 was first dissolved in acetate buffer and then added to the polymer solution while stirring. The final concentrations of poloxamer 407 and methyl paraben were 17 wt% and 0.02 wt%, respectively; the final concentrations of DPK-060 were 0.25 wt%, 0.5 wt%, and 1.0 wt%, corresponding to approximately 1.0 mM, 2.0 mM, and 4.0 mM, respectively.

Formulations With Lipid Nanocapsules

Lipid nanocapsules (LNCs) were prepared as described previously (Valcourt et al., 2016; Umerska et al., 2017). LNCs were composed of lecithin (Lipoid® S75-3; kindly provided by Lipoid GmbH, Ludwigshafen, Germany), medium chain triglycerides (Labrafac® WL1349; kindly provided by Gattefossé S.A., Saint-Priest, France), and macrogol 15 hydroxystearate (Kolliphor® HS 15; BASF, Ludwigshafen, Germany). Briefly, lecithin (75 mg), triglycerides (846 mg), macrogol 15 hydroxystearate (1,028 mg), sodium chloride (90 mg), and 20 mM acetate buffer pH 5.5 (3 ml) were mixed and homogenized under magnetic stirring. The resulting emulsion was then heated to 90°C and cooled to 60°C 2 times, and then heated to 90°C and cooled to 78°C, to obtain reversible emulsion phase inversions. After the temperature of 78°C (the phase inversion temperature) had been reached, an irreversible shock was induced by a rapid dilution with 12.5 ml 4°C purified water to reach room temperature. For monolaurin (ML)-LNCs, lecithin was replaced by monolaurin (Sigma Aldrich, Lyon, France); monolaurin (300 mg), triglycerides (930 mg), macrogol 15 hydroxystearate (770 mg), sodium chloride (90 mg), and 20 mM acetate buffer pH 5.5 (3 ml) were mixed. The resulting emulsion was then heated to 60°C and cooled to 20°C 2 times, and then heated to 60°C and cooled to 37°C. After the temperature of 37°C (the phase inversion temperature) had been reached, the dispersion was rapidly diluted with 20°C purified water to a

Abbreviations: AMP, antimicrobial peptide; LCNP, liquid crystalline nanoparticle; LNC, lipid nanocapsule; ML-LNC, monolaurin lipid nanocapsule; MMC, minimum microbicidal concentration.

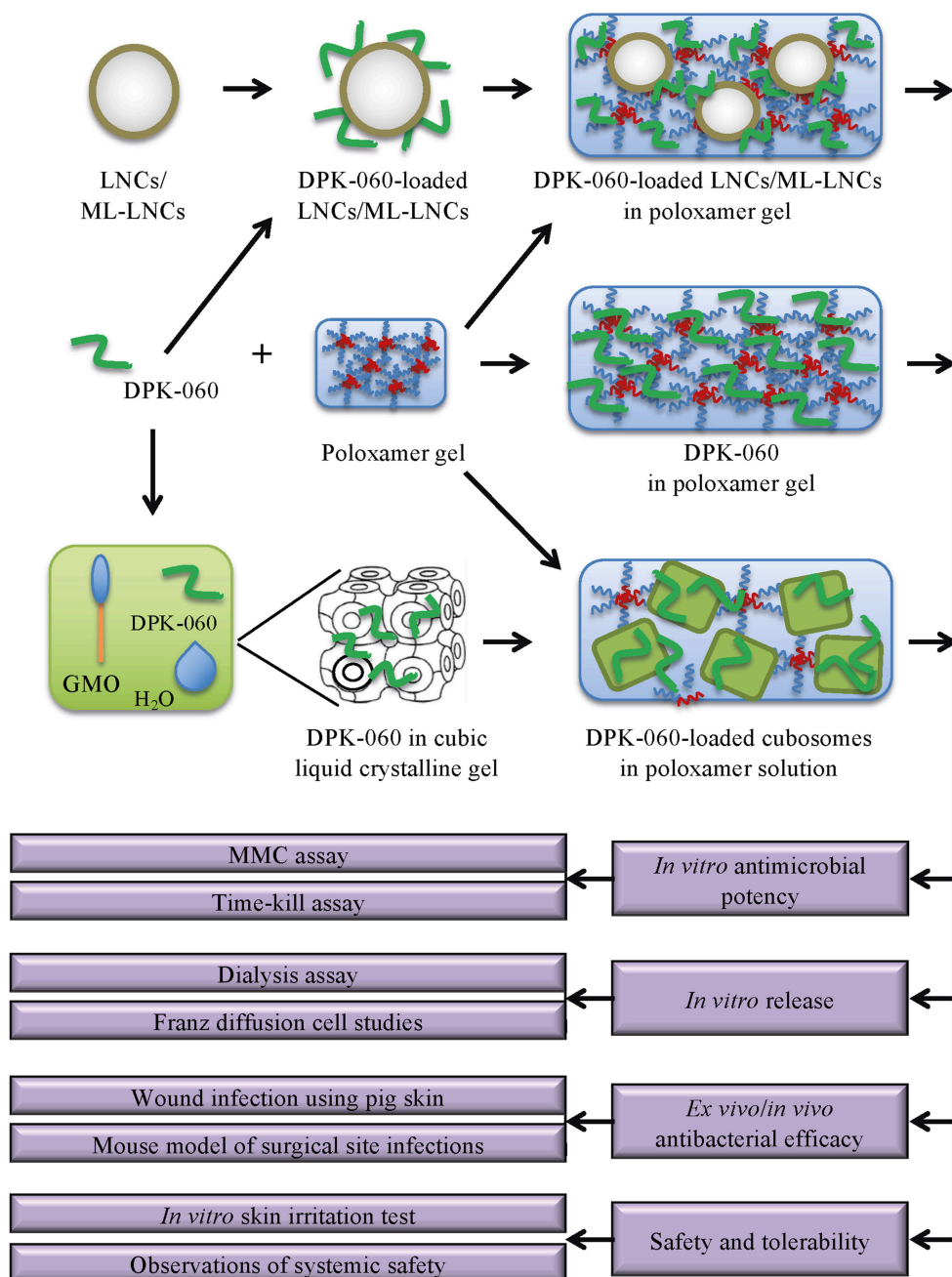


FIGURE 1 | Schematic presentation of the experimental design of the study. LNC/ML-LNC particles were constructed and DPK-060 was adsorbed to the particle surface; DPK060-loaded LNCs/ML-LNCs were then added to the poloxamer gel. A cubic liquid crystalline gel was formed by mixing glycerol monooleate with DPK-060; cubosomes were then prepared by dispersing the cubic gel in poloxamer solution. In most experiments, DPK-060 dissolved in poloxamer gel was included as a reference group. The *in vitro/ex vivo/in vivo* antimicrobial efficacy of the formulations was characterized, and the release profile and skin irritancy were assessed. GMO, glycerol monooleate.

volume of 10 ml. The particle size of the LNCs and ML-LNCs was in the range of 50–80 nm and 30–45 nm, respectively, with a polydispersity index <0.1.

To prepare peptide-loaded LNCs/ML-LNCs, the LNCs/ML-LNC dispersions were mixed with 160 mg/g (16

wt%) DPK-060 in acetate buffer at a ratio of 3:1 LNCs/ML-LNCs:DPK-060 solution, for 2–3 h [sufficient for peptide molecules to achieve a dynamic equilibrium between the LNCs and the surrounding medium (Umerska et al., 2016b)], resulting in a peptide concentration of 40 mg/g (4 wt%).

TABLE 1 | Formulation properties.

Formulation	Poloxamer 407 (wt%)	Nanocarrier	Nanocarrier components	Particle size, nanocarrier (nm)	Peptide loading
Poloxamer gel	17	–	–	–	–
LNCs in poloxamer gel	17	LNC	Lecithin, medium-chain triglycerides, macrogol 15 hydroxystearate	50–80	Adsorption to nanocarrier
ML-LNCs in poloxamer gel	17	ML-LNC	Monolaurin, medium-chain triglycerides, macrogol 15 hydroxystearate	30–45	Adsorption to nanocarrier
Cubosomes in poloxamer solution	3	Cubosomes	Glycerol monooleate	200–300	Loading during nanocarrier formation

Finally, the DPK-060-loaded LNCs/ML-LNCs were added to the poloxamer gel in a 1:3 ratio while stirring at slow speed (300–500 rpm) in an ice bath for 60 min, which resulted in a homogenous viscous liquid. The final concentration of poloxamer 407 was 17 wt%; the final concentrations of DPK-060 were 0.25 wt%, 0.5 wt%, and 1.0 wt%, corresponding to approximately 1.0 mM, 2.0 mM, and 4.0 mM, respectively. The final concentrations of LNCs/ML-LNCs were five-fold higher than the DPK-060-concentrations, i.e., 1.25 wt%, 2.5 wt%, and 5.0 wt%.

The adsorption efficiencies of DPK-060 in LNCs and ML-LNCs were $33.8 \pm 3.5\%$ and $33 \pm 5\%$ – $42 \pm 2\%$, respectively, (manuscript by Matougui et al., in preparation; Rozenbaum et al., 2019).

Formulations With Cubosomes

Liquid crystalline nanoparticles (LCNPs) with cubic phase structure, i.e., cubosomes, were prepared as described previously (Boge et al., 2016). In brief, molten glycerol monooleate (Capmul-90 EP/NF; kindly provided by Abitec Corp., Columbus, OH) at 50°C was mixed with DPK-060 (at 28, 56, or 111 mg/ml) in acetate buffer (20 mM pH 5.5) at 22°C to reach a final concentration of 70:30 wt% glycerol monooleate:buffer with DPK-060 and was then allowed to equilibrate for 2 days at 22°C to form a cubic gel. Typical weights of prepared liquid crystalline gels were 1.7 g. Cubosomes were formed by first dispersing 1.5 g liquid crystalline gel in 3.5 g 20 mM acetate buffer pH 5.5 containing 3 wt% poloxamer 407 for 1 min using a Ultra-Turrax high-shear mixer (IKA T25, Staufen, Germany) with a diameter of 8 mm at 15,000 rpm, followed by sonication using a Vibracell Probe Sonicator (Sonics and Materials, Inc., Newtown, CT) with a 6 mm probe operating in pulse mode (3 s sonication followed by 7 s break) over a period of 10 min. This resulted in cubosomes in the size range of 200–300 nm with a polydispersity index about 0.3. The preparation of cubosomes using the shearing and sonication was shown not to affect the chemical stability of DPK-060 as investigated by HPLC. The cubic structures of the particles were verified by small angle x-ray scattering (Boge et al., 2016). The final concentration of poloxamer 407 was 3% (higher concentrations influence the phase properties of the LCNPs); the final concentrations of DPK-060 were 0.25, 0.5 and 1.0 wt% (corresponding to approximately 1.0, 2.0, and 4.0 mM, respectively); the final concentration of cubosomes was 30 wt%

in all formulations. Methyl paraben (0.02 wt%) was added to the final dispersions.

Loading of DPK-060 into the cubic liquid crystalline gel followed by dispersion, as was used in this study, has been previously shown to result in a high peptide entrapment of 50–70%, whereas post-loading of cubosomes results in only 10–20% of DPK-060 being associated with the particles at the condition of low ionic strength (Boge et al., 2016, 2017). Therefore, the method of DPK-060 loading into the cubic liquid crystalline gel, which results in highest peptide encapsulation efficiency, was selected in this study.

Assessment of Antimicrobial Activity *in vitro*

Minimum Microbicidal Concentration Assay

The microbicidal effect of DPK-060 in different formulations was assessed against *Staphylococcus aureus* (ATCC 29213; American Type Culture Collection, Manassas, VA) using a minimum microbicidal concentration (MMC) assay as described (Björn et al., 2016). The MMC assay was performed in 100× diluted brain-heart infusion broth (BHI_{dil}; Håversen et al., 2000, 2010; Kondori et al., 2011). The minimal peptide concentration causing >99.6% reduction of microorganisms was defined as the MMC.

Time-Kill Assay

Time-kill assay against *S. aureus* (ATCC 29213) was performed as previously described (Boge et al., 2017) incubating 20 µl of a bacterial suspension ($\sim 1.8 \times 10^7$ CFU/ml) with 0.25 ml of each test item and 1.48 ml of BHI_{dil}.

In vitro Release

Release of DPK-060 from the different formulations was monitored through dialysis as described previously (Boge et al., 2017) using a Float-A-Lyzer® G2 dialysis device with 100 kDa molecular weight cut-off (Spectrum Laboratories Inc., Rancho Domingues, CA). The dialysis experiments were performed at 0, 7, and 14 days of storage of the formulations at room temperature. The samples were allowed to dialyze in 20 mM acetate buffer pH 5.5 for 24 h at room temperature, whereupon a 450 µl aliquot was removed from the receptor side/dialysate and diluted with 50 µl formic acid. The concentration of DPK-060 was then analyzed in duplicate samples by UHPLC using gradient elution and UV detection at 214 nm (Boge et al., 2016). The

samples were analyzed on an Acquity UHPLC system (Waters corp, Milford, MA). The injection volume was 5 μ l and the sampler was kept at 5°C. The mobile phases were; A: acetonitrile and purified water with 0.1% TFA and 50 mM NaCl 5/95 and B: acetonitrile and purified water with 0.1% TFA and 50 mM NaCl 70/30. The separation was performed by gradient elution starting from 26% phase B (kept constant for 1.5 min) increased to 35% phase B in 11 min (kept constant for 3 min), followed by a wash step at 100% phase B (1.1 min) and an equilibration step at 26% phase B (3.4 min). The flow rate was 0.25 ml/min and the column was kept at 55°C. DPK-060 was detected at 214 nm and quantified by peak area, quantitative dilutions, and external calibration in the range of 0.01–0.25 mg/ml.

The release of DPK-060 from the poloxamer gel formulation with and without LNCs was also investigated by Franz diffusion cell studies. Aluminum oxide membranes (Anodisc, 25 mm, pore size 0.02 μ m, Whatman, GE Healthcare, Buckinghamshire, UK) were mounted in Franz diffusion cells (PermeGear Inc., Hellertown, PA) with a 0.64 mm² surface area and receptor volume of 5 ml. 10 mM phosphate buffer pH 7.4, 0.8% NaCl was used as receptor medium and allowed to equilibrate at 32°C for 30 min prior to the application of 100 μ l formulation to the donor compartment. Aliquots of 200 μ l were collected over the period of 36 h and analyzed by UHPLC as described above.

***In vitro* EpiDerm Skin Irritation Test**

The effect of DPK-060 in different formulations on cell viability was determined in compliance with OECD Test Guideline 439 by using the EpiDerm Skin Irritation Test (EPI-200-SIT; MatTek In Vitro Life Science Laboratories, Bratislava, Slovakia) according to manufacturer's instruction.

***Ex vivo* Wound Infection Model Using Pig Skin**

The ability of DPK-060 in different formulations to reduce colonization of *S. aureus* (ATCC 29213) was evaluated in an *ex vivo* wound infection model using pig skin as previously described (Björn et al., 2016).

Mouse Model of Surgical Site Infection

The injury, infection with *S. aureus* (ATCC 29213), and sample collection/analysis for surgical site infection model were carried out in female mice of CD1 strain as previously described (Håkansson et al., 2014). *In vivo* experiments were performed after prior approval from the local Ethics Committee for Animal Studies at the Administrative Court of Appeals in Gothenburg, Sweden (approval number: Dnr 26-2015).

The treatment-related systemic toxicity was assessed by observing general behavior and clinical signs including body posture, central excitation, mood, and motor activity. The local tolerability was assessed by observing incidence of erythema and oedema.

The doses of DPK-060 used in *ex vivo* and *in vivo* studies refer to the total concentrations of DPK-060 in the formulation and do not take into consideration the potential differences in the release pattern.

Statistical Analysis

Statistical significance between the groups was calculated using one-way ANOVA followed by LSD *post-hoc* test, with a value of $P < 0.05$ considered statistically significant. The statistical analyses were performed using SPSS Statistics (v24; IBM Corporation, Armonk, NY).

RESULTS

***In vitro* Antimicrobial Potency**

The MMC for DPK-060 in acetate buffer or when formulated with different nanocarriers in poloxamer gel was in the range of 1–5 μ g/ml (Table 2). No antibacterial activity was detected when these formulations were tested without any DPK-060 added (data not shown).

DPK-060 in acetate buffer or in poloxamer gel displayed bactericidal effect in time-kill assay at concentrations equal or higher than 2 μ g/ml with a significant reduction in the CFU numbers detected after 3 h of incubation (Figures 2A,B). DPK-060-loaded LNCs and ML-LNCs in poloxamer gel inhibited bacterial growth to the extent similar to that observed with DPK-060 in acetate buffer/poloxamer gel (Figures 2C,D). DPK-060-loaded cubosomes in poloxamer solution were less effective: the bactericidal effect was observed only at the highest concentration tested (8 μ g/ml; Figure 2E). Nanocarrier gels without DPK-060 did not display any significant inhibition in bacterial growth (Figures 2C–E).

***In vitro* Release**

The release of the peptide from DPK-060-loaded LNCs and cubosomes in poloxamer gel was first investigated by dialysis method. The results reveal that after 24 h of incubation, 100% of DPK-060 was released from the LNCs in poloxamer gel as well as from the poloxamer gel without any nanocarriers (Figure 3). In contrast, under these test conditions about 50–70% of DPK-060 remained encapsulated in the cubosomes (Figure 3). Of note, the release properties were similar comparing the formulations analyzed directly after preparation with those stored for 7 or 14 days before the dialysis experiment (Figure 3).

The peptide release efficiency from DPK-060-loaded LNCs in poloxamer gel was further investigated by Franz diffusion cell studies. The results reveal identical release profile for DPK-060 in poloxamer gel with or without encapsulation of the

TABLE 2 | MMC for DPK-060 in different formulations against *S. aureus*.

Test item	MMC (μ g/ml)
DPK-060 in acetate buffer	4.9
DPK-060 in poloxamer gel	1.2–2.4
DPK-060-loaded LNCs in poloxamer gel	2.4
DPK-060-loaded ML-LNCs in poloxamer gel	1.2
DPK-060-loaded cubosomes in poloxamer solution	4.9

The MMC test is a semiquantitative assay; the results are presented either as one concentration value or, in case there was a variation between the repetitions, as a concentration range.

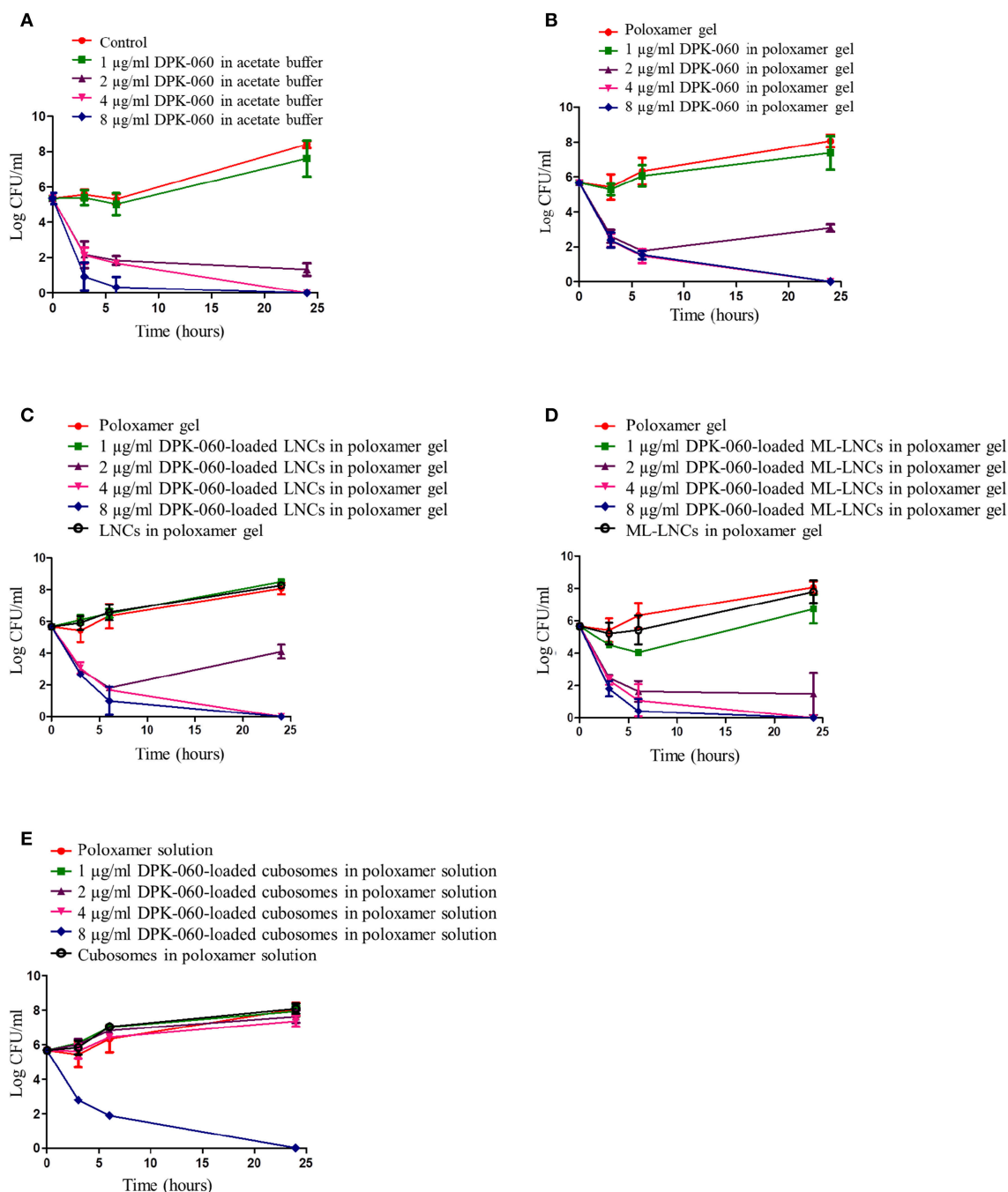


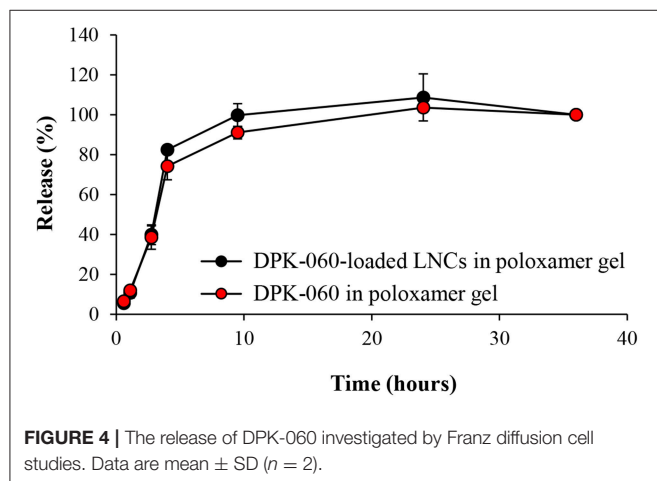
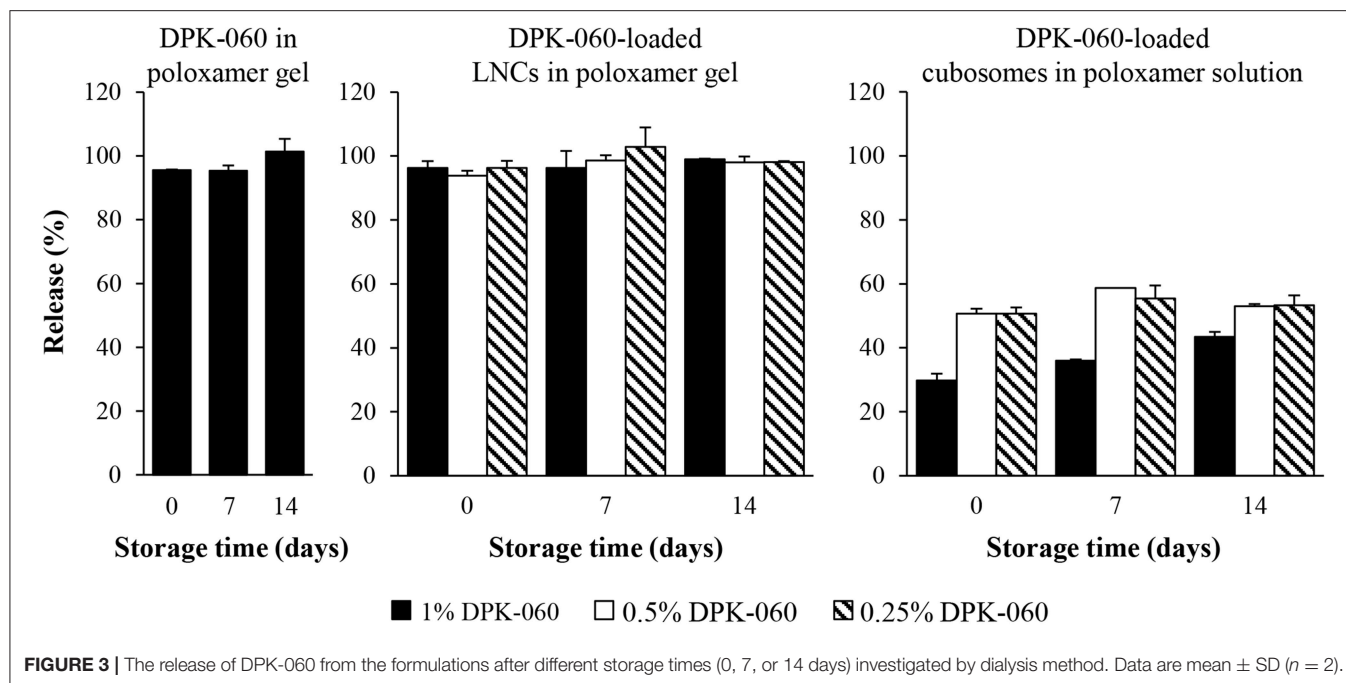
FIGURE 2 | Time-kill assay for DPK-060 formulated in acetate buffer (A), poloxamer gel (B), or in different nanocarriers in poloxamer gel (C–E) against *S. aureus*. Data are mean $\pm$ SD ($n = 3$). Samples were considered as bactericidal if there was at least 3 log reduction in the CFU number compared with the starting inoculum.

peptide into LNCs: approximately 40% of DPK-060 was released within 3 h of incubation and full release (100%) was observed at 10 h (Figure 4).

Antibacterial Effect in *ex vivo* and *in vivo* Wound Infection Models

The antibacterial activity of DPK-060-loaded nanocarriers (0.25, 0.5, and 1.0% of DPK-060) was investigated in an *ex vivo*

pig skin model. The formulations were administered 2 h post-infection with *S. aureus*, and the bacteria were harvested 4 h post-treatment (Figure 5A). DPK-060 in poloxamer gel, as well as DPK-060-loaded LNCs and ML-LNCs in poloxamer gel, significantly reduced the bacterial survival compared with sham (no treatment) as well as corresponding placebo (formulation only) and the concentration of 1% DPK-060 suppressed the bacterial survival with $\geq 99\%$ vs. the sham (Figure 5A).



The DPK-060-loaded cubosomes in poloxamer solution also diminished the microbial counts compared with sham/placebo; however, the anti-infectious potency was less pronounced compared with DPK-060-loaded LNCs/ML-LNCs (**Figure 5A**).

The *ex vivo* antimicrobial effect of selected formulations (1.0% DPK-060 in poloxamer gel, with or without nanocarriers) was further studied in the pig skin model after a longer follow-up period, i.e., the bacteria were harvested 24 h after the treatment (**Figure 5B**). In this setting, the bacterial survival was significantly reduced only in wounds treated with DPK-060 in poloxamer gel compared with corresponding placebo (**Figure 5B**).

The antimicrobial effect of DPK-060-loaded nanocarriers (0.25, 0.5, and 1.0% of DPK-060) was also studied in a murine *in vivo* model of surgical site infection. In this model, a silk suture contaminated with *S. aureus* was implanted into an incision

wound on the back of mice and assessment of the infection was performed 4 h post-treatment (**Figure 6**). The same model has been used previously to characterize the effect of systemic and topical antimicrobial agents and the results observed have closely correlated with efficacy in clinical trials with human subjects (Mcripley and Whitney, 1976; Gisby and Bryant, 2000; Rittenhouse et al., 2006). DPK-060 in poloxamer gel significantly suppressed the bacterial survival compared with sham as well as placebo (poloxamer gel) with a clear dose response relationship; the effect of 1% DPK-060 in poloxamer gel was comparable with the treatment with comparator 2% Bactroban (i.e., reduction in the bacterial survival with approximately 95% vs. the sham, **Figure 6**). Notably, DPK-060-loaded LNCs and cubosomes in poloxamer gel displayed minor/no antimicrobial effect in this model and no obvious dose response relationship was observed (**Figure 6**).

Safety and Local Tolerability

In connection to the local application of the formulations in the surgical site infection model in mice, with or without DPK-060, no systemic toxicity or local tolerability concerns were visually observed in any of the mice. According to *in vitro* EpiDerm Skin Irritation Test, the test item is considered to display an irritating potential in case the viability is reduced by $>50\%$ of the values measured for negative control sample. All the formulations tested, with or without DPK-060, displayed the cell viability of $\geq 90\%$ of the control sample.

DISCUSSION

The MMC results in this study corroborated the previous findings (Schmidtchen et al., 2009; Boge et al., 2016, 2017; Nordström et al., 2018) that human kininogen-derived peptide

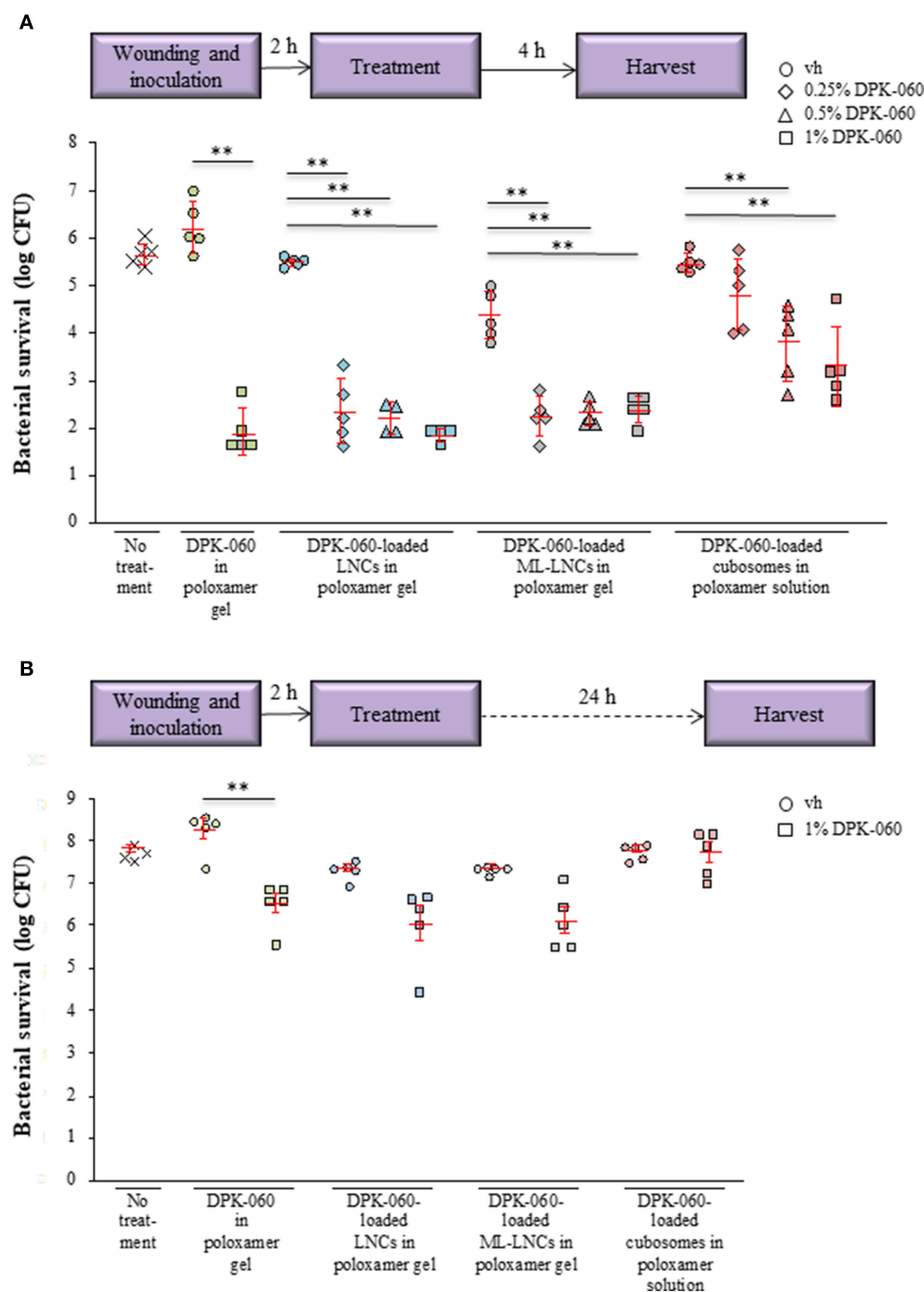
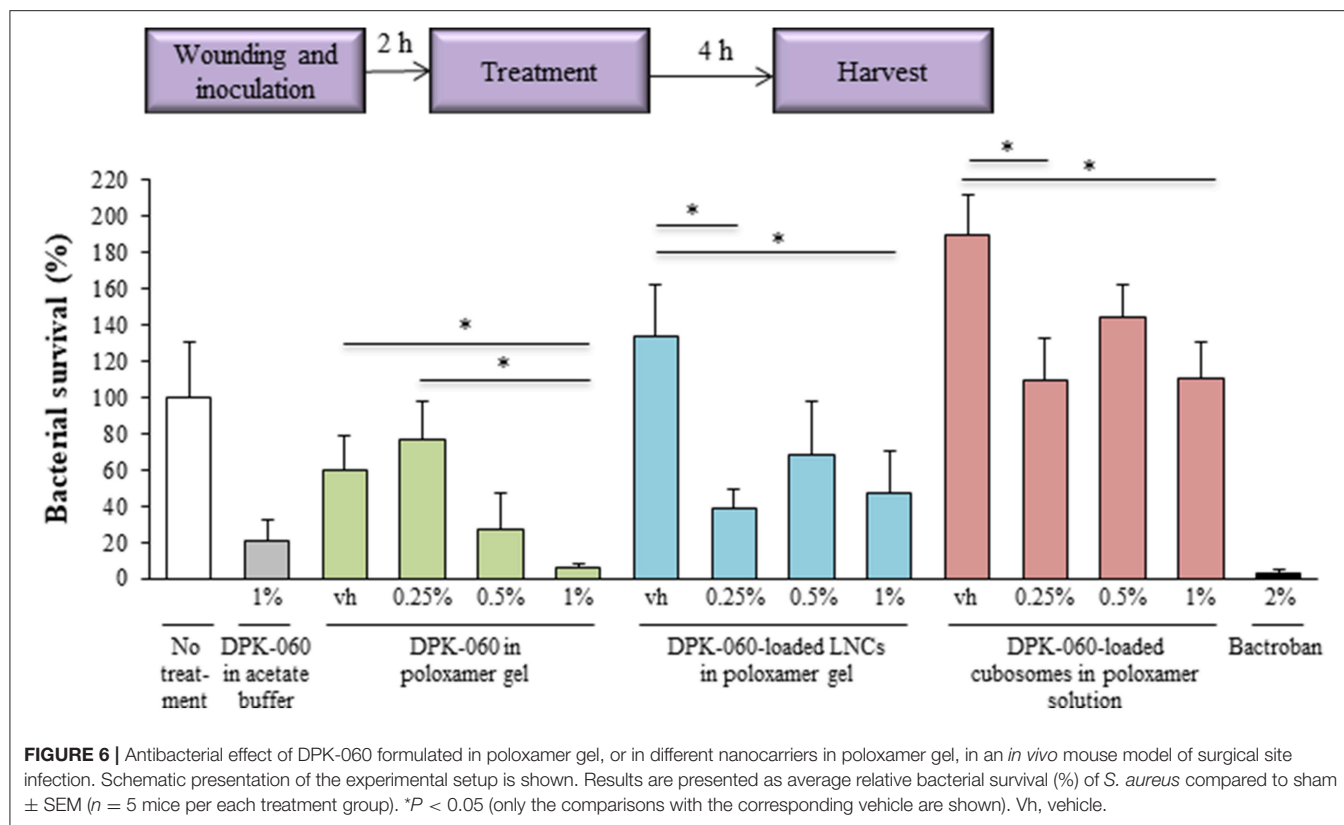


FIGURE 5 | Antibacterial effect of DPK-060 formulated in poloxamer gel, or in different nanocarriers in poloxamer gel, in an *ex vivo* wound infection model using pig skin. The bacterial survival was assessed 4 h (A) or 24 h (B) after the treatment. Schematic presentation of the experimental setup is shown. Data on bacterial survival of *S. aureus* are mean $\pm$ SEM ($n = 4$ –5 wounds per treatment group). ** $P < 0.01$ (only the comparisons with the corresponding vehicle are shown). Vh, vehicle.

DPK-060 in acetate buffer or in poloxamer gel displays an *in vitro* bactericidal action against *S. aureus* with activities in the micromolar range. *In vitro*, different AMPs including DPK-060, when adsorbed to nanoparticles, have shown enhanced antimicrobial activity both against planktonic bacteria and in reducing biofilm growth (Umerska et al., 2016a, 2017; Boge et al., 2017, 2018). In specific, antimicrobial surfactant monolaurin used in ML-LNCs has been suggested to act in synergetic

fashion with AMPs (Umerska et al., 2016a, 2017). In contrast, here we found that the MMC for DPK-060 formulated with different nanocarriers was in the similar range as the MMC for DPK-060 in acetate buffer or in poloxamer gel (1–5 $\mu\text{g/ml}$). Furthermore, in this study the DPK-060-loaded LNCs/ML-LNCs displayed similar microbicidal effect in time-kill assay compared with DPK-060 in acetate buffer/poloxamer gel (close-to-maximal effect at concentrations $\geq 2 \mu\text{g/ml}$), whereas DPK-060-loaded



cubosomes were less effective. In summary, while this study provides consistent evidence for the potent bactericidal action of DPK-060 *in vitro*, the encapsulation of this AMP into the nanocarriers failed to increase its antimicrobial efficacy in MMC or time-kill assay.

In addition to *in vitro* investigations, we also assessed the *ex vivo* and *in vivo* antimicrobial effect of DPK-060 in experimental wounds in pig skin and in mice, respectively, infected with *S. aureus*, which is one of the bacterial species most frequently causing human wound infections (Cardona and Wilson, 2015). We found that DPK-060 formulated in poloxamer gel caused a marked and statistically significant reduction in microbial counts with a clear dose-response relationship both *ex vivo* and *in vivo*. These data are consistent with the results of the previously completed clinical trial where total microbial count as well as count of Coagulase Negative Staphylococci (CoNS) and Gram-positive bacteria were significantly lower in eczematous lesions of patients with atopic dermatitis after 14 days of treatment with DPK-060 1% ointment (ClinicalTrials.gov Identifier: NCT01522391; EudraCT: 2007-007103-32).

Protection against proteolytic degradation by infection-affiliated enzymes has earlier been reported in *in vitro* studies when different AMPs were encapsulated into LNCs or cubosomes (Boge et al., 2017, 2019), which likely relates to the fact that the peptide is less accessible for proteolytic enzymes in nanoparticles compared with free peptide. Nanocarriers have also shown to enhance the skin penetration and delivery of several active substances (Lopes et al., 2006, 2007; Rattanapak

et al., 2012; Seo et al., 2013). On the basis of these previous findings, we hypothesized that the efficacy of DPK-060 in treating wound infections would be enhanced *in vivo* when adsorbed to nanoparticles. In contrary, we found that the antibacterial effect of DPK-060 was diminished in a mouse model of surgical site infection when encapsulated into LNCs or cubosomes compared with formulating the peptide in poloxamer gel only. Notably, the results of *in vitro* release experiments revealed that more than 50% of DPK-060 remained encapsulated in the cubosomes after 24 h of incubation; in case only the non-encapsulated “free” peptide is giving rise to bacterial killing, this likely contributed to the low efficacy of DPK-060-loaded cubosomes. However, we found that the release profile for DPK-060 was similar in poloxamer gel with or without encapsulation of the peptide into LNCs (about 40% of DPK-060 was released within 3 h) and the reason for low efficacy of DPK-060-loaded LNCs *in vivo* remains elusive.

We did not observe any significant reduction in cell viability with DPK-060 in any nanocarrier formulation tested when assessed by *in vitro* EpiDerm Skin Irritation Test. Furthermore, we found no visible signs of systemic toxicity or local irritation in murine surgical site infection model in connection to the administration of DPK-060-containing formulations. This is consistent with the results of the clinical trial, where no serious adverse events (SAE) were reported after dermal application of DPK-060 in atopic dermatitis patients, whereas adverse events (AEs) were observed at similar frequency in the DPK-060 and placebo groups (ClinicalTrials.gov Identifier: NCT01522391).

In conclusion, the present study confirms that DPK-060 has the potential to be an effective and safe drug candidate for the topical treatment of microbial infections; however, under the applied test conditions, the adsorption of the peptide to nanocarriers failed to show any additional benefits. Notably, in this study, a single administration of the test formulations, combined with a short follow-up time, was used both in *ex vivo* and *in vivo* assessments, which is considered a limitation of the experimental design. Repeated application over several days, and a longer follow-up period, would closer resemble the clinically relevant situation and may lead to different results in terms of both efficacy and safety.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

JH: experimental planning and was responsible for *in vivo* and *in vitro* studies, scientific input, performed the *in vivo* experiments. LR: planning of studies, scientific input, responsible for the

formulation studies. AU, JJ, TA, RR, and CB: performed *in vitro* studies and scientific input. LB and PT: performed formulation studies and scientific input. PKS: planning of *in vitro* and *in vivo* studies, scientific input. PS: planning of *in vitro* studies and scientific input. MM: overall responsible for the studies and corresponding author.

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Conflict of Interest Statement: At the time of the investigation, MM was employed at Promore Pharma AB and she continues to work for the company at consultancy basis. Promore Pharma is a biopharmaceutical company that develops peptide-based product candidates aimed for the bioactive wound care market.

The remaining 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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Semi-Mechanistic Modeling of Florfenicol Time-Kill Curves and *in silico* Dose Fractionation for Calf Respiratory Pathogens

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An important application of time-kill curve (TKC) assays is determination of the nature of the best PK/PD index ($fAUC/MIC$ or $fT\% > MIC$) and its target value for predicting clinical efficacy *in vivo*. VetCAST (the veterinary subcommittee of EUCAST) herein presents semi-mechanistic TKC modeling for florfenicol, a long acting (96 h) veterinary antimicrobial drug licensed against calf pneumonia organisms (*Pasteurella multocida* and *Mannheimia haemolytica*) to support justification of its PK/PD_{breakpoint} and clinical breakpoint. Individual TKC assays were performed with 6 field strains of each pathogen (initial inoculum 10^7 CFU/mL with sampling at times at 0, 1, 2, 4, 8, and 24 h). Semi-mechanistic modeling (Phoenix NLME) allowed precise estimation of bacteria growth system (K_{GROWTH} , natural growth rate; K_{DEATH} , death rate; B_{MAX} , maximum possible culture size) and florfenicol pharmacodynamic parameters (E_{MAX} , efficacy additive to K_{DEATH} ; EC_{50} , potency; Γ , sensitivity). PK/PD simulations (using the present TKC model and parameters of a florfenicol population pharmacokinetic model) predicted the time-course of bacterial counts under different exposures. Of two licensed dosage regimens, 40 mg/kg administered once was predicted to be superior to 20 mg/kg administered at 48 h intervals. Furthermore, we performed *in silico* dose fractionation with doses 0 – 80 mg/kg administered in 1, 2 or 4 administrations over 96 h and for MICs of 0.5, 1, 2, 4 mg/L with 2 inoculum sizes 10^5 and 10^7 CFU/mL. Regression analysis (I_{max} model) demonstrated that i) $fAUC/MIC$ outperformed $fT\% > MIC$ as PK/PD index and ii) maximum efficacy ($IC_{90\%}$) was obtained when the average free plasma concentration over 96 h was equal to 1.2 to 1.4 times the MIC of *Pasteurella multocida* and *Mannheimia haemolytica*, respectively.

Keywords: PK/PD, modeling and simulation, time-kill assay, antimicrobial susceptibility testing, *Pasteurella multocida*, *Mannheimia haemolytica*, VetCAST, bovine respiratory disease

INTRODUCTION

The aim of time-kill *in vitro* assays is to investigate the pharmacodynamics (PD) of antimicrobial drugs (AMD) by determining the rate of bacterial kill relative to drug concentration. Quantitative analysis of time-kill curve (TKC) data is more informative of the drug-bacteria relationship than snapshot indices, such as minimum inhibitory concentration (MIC). MIC

indicates only the net effect of a single AMD concentration on bacterial growth over a 24 h incubation period, while TKC establishes the rate of killing over a range of concentrations. Based on TKC data AMDs can be classified as time- or concentration-dependent in killing action (Toutain et al., 2017).

An important application of TKC data is determination of the best PK/PD index ($fAUC/MIC$ or $fT > MIC$) for predicting clinical efficacy *in vivo*, where $fAUC$ is area under plasma concentration-time curve and fT is the time the drug concentration exceeds MIC, for free drug concentrations. This has historically been established by correlating the reduction in bacterial count at 24 h from an initial inoculum count (Lees et al., 2015). Plots of log10 colony forming units (CFU)/mL at 24 h versus each of the two PK/PD indices allowed selection of the PK/PD index which best fits the sigmoidal E_{MAX} model (Lees et al., 2015). This approach was based on the net reduction of bacterial count with each concentration exposure, but did not utilize the time course (i.e., the shape) of individual kill curves. For human medicine, several advanced PK/PD models of TKC have incorporated the shape of the curve with time (Nielsen et al., 2007; Nielsen and Friberg, 2013, model D, **Figure 5**). This more advanced modeling enables estimation of the three pharmacodynamic (PD) parameters of AMD action, namely potency, efficacy and sensitivity. This approach therefore allows characterization the whole concentration-effect relationship.

In the present investigation, using historical TKC data (Sidhu et al., 2014), PD parameters of florfenicol against the calf pneumonia organisms, *Pasteurella multocida* (*P. multocida*) and *Mannheimia haemolytica* (*M. haemolytica*), have been established using the semi-mechanistic model proposed by Nielsen and Friberg (2013). The objective was to then conduct an *in silico* dose fractionation trial to determine the PK/PD index for florfenicol and these pathogens, best correlating with bacterial kill. Dose fractionation studies are generally conducted *in vivo* using rodent infection models, whereas in this study semi-mechanistic PD florfenicol models were used as a surrogate of rodent models to predict microbiological effects in response to a range of florfenicol dosage regimens. The ultimate goal was to compute a PK/PD breakpoint (PK/PD_{BP}) for the florfenicol clinical breakpoint (CBP) according to the procedures advocated by VetCAST (Toutain et al., 2017), where CBP is the MIC value used by microbiology laboratories to report the results of antimicrobial sensitivity testing (AST). PK/PD_{CO} is defined as the highest possible MIC for which a given percentage of animals in the target population (say 90%) achieve a pre-defined target value of the PK/PD index, the pharmacodynamic target (PDT) according to European Medicines Agency (EMA) terminology.

MATERIALS AND METHODS

Test Pathogens and MIC Determination

The test pathogens were *P. multocida* and *M. haemolytica*. MICs were determined in Mueller Hinton Broth (MHB) for six strains of each species, isolated from cases of calf pneumonia (Sidhu et al., 2014). Their origin and date of isolation are

summarized in the **Supplementary File S1**. Average florfenicol MICs were determined using 5 overlapping 2-fold-dilution series and were 0.4 and 0.5 mg/L for *P. multocida* and *M. haemolytica*, respectively.

Time-Kill Curves

Six individual TK assays were performed for each pathogen. Initial inoculum count was in the range 5×10^6 to 7×10^7 CFU/mL. Duration of incubation was 24 h with sampling at times of 0, 1, 2, 4, 8, and 24 h. Drug concentrations were expressed in the initial publication (Sidhu et al., 2014) as multiples of MIC (0 = growth control, 0.25, 0.5, 1, 2, and 4 times measured MIC). For data fitting, MICs were back calculated to mg/L. The lowest detectable count was 33 CFU/mL; lower counts were set as below the quantification limit (BQL). All TKC data sets analyzed for this study are included in the manuscript and the **supplementary File S2**.

Data Analysis

Pharmacodynamic data analyses were conducted using Phoenix® WinNonlin® 8.0 (Pharsight Corporation, St Louis, MO, United States). For each pathogen, the 6 TKC data sets were analyzed simultaneously using a non-linear mixed effect model (NLME). A semi-mechanistic structural model of bacterial growth, incorporating a compartment for growing drug-sensitive bacteria (S) (CFU/mL) and a compartment named persisters (P) (CFU/mL), corresponding to a pool of non-growing and insensitive-drug bacteria (phenotypic resistance) was adopted (**Figure 1**; Nielsen and Friberg, 2013).

Visual Inspection of TKC indicates an initial phase of slow growth. To capture the delay required to achieve a maximal steady-state growing rate, a mitigating function for K_{GROWTH} of the form was introduced (Equation 1):

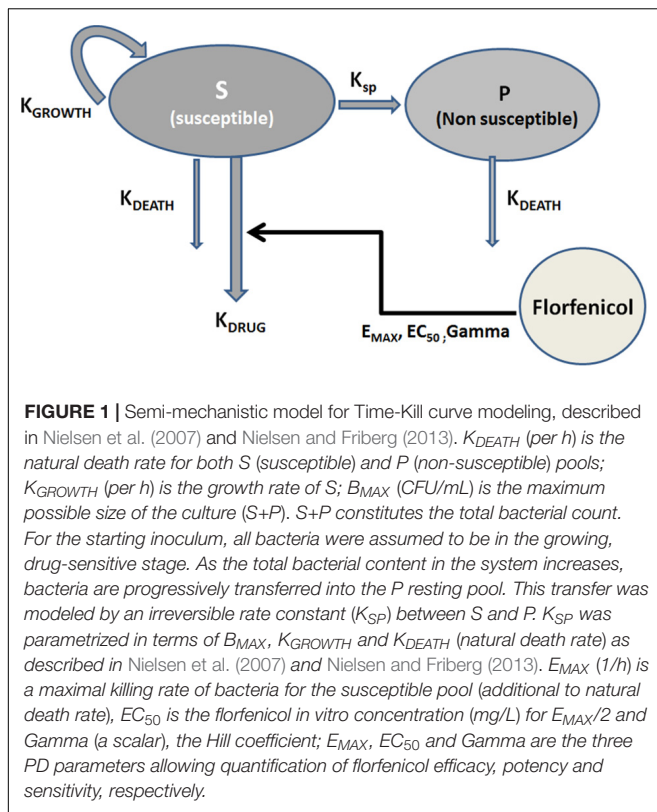
$$K_{GROWTH} = K_{GROWTHMAX} \times (1 - \text{EXP}(-\text{Alpha} \times \text{Time})) \quad (1)$$

where Alpha (per h) = rate constant to describe a progressive increase of K_{GROWTH} over time; at time 0, $K_{GROWTH} = 0$ then, K_{GROWTH} increases progressively to reach $K_{GROWTHMAX}$ with a mean time equal to $1/\text{Alpha}$. The lag phase corresponds to the physiological adaptation of the bacteria to the culture condition (induction of specific messenger RNA and protein synthesis and low cell density accounting for initial dilution of the exoenzymes that make nutrients readily available).

Florfenicol action was introduced in the model as a concentration-dependent killing rate $K_{DRUG(t)}$ (per h) acting in parallel with K_{DEATH} but for the S pool only. It was modeled according to the classical Hill equation (Eq. 2).

$$K_{DRUG(t)} = \frac{E_{MAX} \times C(t)^{Gamma}}{EC_{50}^{Gamma} + C(t)^{Gamma}} \quad (2)$$

where $C(t)$ is the florfenicol concentration (mg/L) at time t (the independent variable). $C(t)$ was the constant tested concentration when data were fitted to estimate the PD parameters but



$C(t)$ was obtained by solving the population PK model, when this equation was used for simulations (*vide infra*). E_{MAX} (1/h) is the maximal killing rate for the susceptible pool (additionally to natural death rate), EC_{50} is the florfenicol *in vitro* concentration (mg/L) for $E_{MAX}/2$ and Γ (a scalar), the Hill coefficient; E_{MAX} , EC_{50} and Γ are the three PD parameters providing quantitative indices of florfenicol efficacy, potency and sensitivity, respectively.

There were substantial differences in B_{MAX} across the six field strains of each pathogen. Therefore, a random component was introduced in the structural model to account for the inter-strain variability. Six individual B_{MAX} values were obtained, using an exponential model of the form (Eq. 3):

$$\theta_{1i} = \theta_1 \times \exp(\eta_{1i}) \quad (3)$$

where θ_1 is the typical population value of B_{MAX} , θ_{1i} the value of B_{MAX} for the i^{th} TKC assay, and η_{1i} (eta) the deviation associated with the i^{th} strain from the corresponding population value. This exponential model assumes a log-normal distribution of B_{MAX} . The between-strain variability of B_{MAX} was reported as coefficient of variation in the original scale, with an equation converting estimated variance terms to a coefficient of variation (CV%) (Eq. 4).

$$CV (\%) = 100 \times \sqrt{\exp(\omega^2) - 1} \quad (4)$$

The residual variability was modeled with an exponential error model of the form (Eq. 5):

$$Y_{ij} = \hat{Y}_{ij} \times \exp(\varepsilon_{ij}) \quad (5)$$

where $\hat{Y}_{ij}$ is the j^{th} response (CFU/mL) measured in the i^{th} curve in terms of CFU (no log-transformation of raw data), with ε_{ij} the common errors term having a mean of 0 and a variance σ^2_1 .

When there is only one exponential error model, the predictions and observations are automatically log-transformed by Phoenix and fitted in that space, so that the error model was actually a Log-additive error model.

Parameter estimates, with their associated SE and CV as a measure of precision, were based on minimizing an objective function value, using Laplace engine for the Maximum Likelihood Estimation.

For *P. multocida*, there were no values reported as below the quantification limit (BQL) due to some re-growth at 24 h. For *M. haemolytica*, data reported as BQL (7% of the data set) were retained in the analysis by using a likelihood-based approach according to the M3 method (Beal, 2001). Diagnostic plots determined whether the model was adequate: these included PRED (population (zero-eta) prediction) and IPRED (individual prediction) versus the dependent variable, Conditional weighted residuals (CWRES) and individual fitting. The overall adequacy of the model was established by plotting the Visual Predictive Check (VPC) i.e., a graphical comparison between the observed data and prediction intervals (20–80th percentiles) derived from the simulated data (data set simulated 500 times).

Secondary parameters computed were MIC and minimal bactericidal concentration (MBC). MIC and MBC indicate AMD PD parameters (efficacy, potency, sensitivity) but also test tube conditions [growth and death rates, duration of observation (often 18–24 h) and the initial inoculum load (usually 5×10^5 CFU/mL)]. According to Mouton and Vinks (2005), MIC is related to the aforementioned factors by eqs. 6A,B:

$$MIC = EC_{50} \times \left(\frac{K_{GROWTH} - 0.29}{E_{MAX} - (K_{GROWTH} - 0.29)} \right)^{\frac{1}{\Gamma}} \quad (6A)$$

where K_{GROWTH} , (actually $K_{GROWTH} - K_{DEATH}$) (for present data it is $K_{GROWTHMAX}$), EC_{50} , E_{MAX} , and Γ as defined above;

Time of measurement was fixed at 18 h and it is assumed that visible growth indicates an inoculum of 1×10^8 CFU/mL; hence, the constant 0.29 of eq. 6A is obtained from eq. 6B:

$$\frac{1}{\text{Time of measurement (18h)}} \times \ln \left(\frac{N(t)}{N(0)} \right) = 0.294 \quad (6B)$$

where $N(t)$ is the inoculum size at 18 h i.e., 10^8 CFU/mL and $N(0)$ is the initial inoculum i.e., 5×10^5 CFU/mL. When the initial load is not 5×10^5 CFU/mL as for the Sidhu et al. (2014), data eq. 6B should be edited to replace 0.29 by the *ad hoc* value; for example, using an initial count of 10^7 CFU/mL, the constant is no longer 0.29 but -0.127 .

Similarly, MBC is computed by replacing, in the previous equation, 10^8 by 5×10^2 CFU/mL; MBC corresponds to at least 99.9% kill from the initial inoculum (5×10^5 CFU/mL) (Mouton and Vinks, 2005); MBC is given by eq. 7:

$$MBC = EC_{50} \times \left(\frac{K_{GROWTH} + 0.383}{E_{MAX} - K_{GROWTH} + 0.383} \right)^{\frac{1}{\Gamma}} \quad (7)$$

The Phoenix model code is available on request and will be made available by the authors, without undue reservation, to any qualified researcher.

The estimated fixed parameters (E_{MAX} , EC_{50} , Γ , $K_{GROWTHMAX}$, K_{DEATH} , B_{MAX}) and α were reported as typical values with coefficient of variation.

Simulation of Two Possible Dosing Regimens and *in silico* Dose Fractionation to Select a PK/PD Index for Florfenicol

Selection of the best PK/PD index for florfenicol and its magnitude were calculated using the *in silico* PK/PD model, simulating several dosage regimens using eq. 2 with $C(t)$ being the predicted plasma florfenicol concentration obtainable *in vivo*. $C(t)$ was determined by solving the population PK model developed for florfenicol in calves by Toutain et al. (2017), which is a meta-analysis of PK studies in which calves were administered 40 mg/kg of florfenicol subcutaneously (300 mg/mL Solution for Injection). The design of the population pharmacokinetic study and the resulting estimated PK parameters used for these simulations are presented in **Supplementary File S3**. Using the PK/PD *in silico* model, the microbiological effect of two possible licensed dosing regimens

were simulated: single dose (40 mg/kg) versus 20 mg/kg twice at 48 h dosing interval. Using the same PK/PD model, dose fractionation was conducted for doses of 0, 2.5, 5, 10, 20, 30, 40 (licensed dose), 50, 60, and 80 mg/kg given, as a single administration, two administrations at 48 h interval or 4 administrations at 24 h intervals, yielding a total of 28 possible exposure patterns. Simulations were performed for two initial loads (10^5 and 10^7 CFU/mL) and for four MIC levels (0.5, 1, 2, and 4 mg/L). We assumed that differences in MIC were due solely to altered potency and not efficacy. For simulation at MICs of 0.5, 1, 2, and 4 mg/L, the EC_{50} fitted from TKCs was multiplied by a scaling factor converting measured MIC (0.4 mg/L for *P. multocida* and 0.5 mg/L for *M. haemolytica*) to the simulated MIC. For the bacteriological response, the cumulative Area Under the Curve of the total bacterial count over 96 h ($AUC_{bact96h}$) was used. Data were then log10 transformed for regression. When the bacterial count had decreased to 30 CFU/mL, it was considered that regrowth would not occur and curves were truncated for this cut-off value. PK/PD indices are conventionally determined using plasma protein unbound (free) concentration. The latest study at the time of writing reported that florfenicol protein binding was only 5% at the high concentration and was negligible at the low concentrations, representing a *fu* of essentially 1.0 (Foster et al., 2016). We therefore hypothesized that the binding of florfenicol to plasma protein could be ignored and that we simulated free plasma concentrations in the dose fractionation (*vide infra*). The area under the plasma concentration-time curve ($fAUC_{PK(0-96h)}$) and percentage time plasma concentration exceeded MIC within 96 h ($fT > MIC\%$) were computed using the statistical tool of Phoenix. The 28 pairs of $fT > MIC\%$ (independent variable) versus $AUC_{bact96h}$ (dependent variable) and $fAUC_{PK(0-96h)}/MIC$ (independent

TABLE 1 | Primary and secondary parameter estimates and precision (CV%).

		<i>Pasteurella multocida</i>		<i>Mannheimia haemolytica</i>	
Primary parameters ^a	Unit	Estimate	CV%	Estimate	CV%
Bacteria growth system parameters					
K _{GROWTHMAX}	1/h	0.97	5.1	1.58	10.7
K _{DEATH}	1/h	0.12	11.9	0.78	11.1
B _{MAX}	CFU/mL	5.2 × 10 ⁹	38.1	9.6 × 10 ⁸	34.6
alpha	1/h	0.22	8.9	0.93	12.8
Florfenicol pharmacodynamic parameters					
EC ₅₀	mg/L	0.46	7.5	0.70	7.2
gamma	scalar	2.74	8.9	2.63	11.9
E _{MAX} (maximal killing rate)	1/h	2.00	2.4	2.70	8.4
stdev0 (Ln domain)	scalar	1.11 (156 CV%)	9.9	1.05 (141 CV%)	5.5
Secondary parameters ^b					
MIC (10 ⁵ CFU/mL inoculum)	mg/L	0.35	5.9	0.43	8.1
MIC (10 ⁷ CFU/mL inoculum)	mg/L	0.40	6.4	0.49	7.1
stationary concentration	mg/L	0.41	6.5	0.50	7.0
MBC	mg/L	0.51	7.5	0.60	6.3

^a $K_{GROWTHMAX}$, maximal growth rate; K_{DEATH} , natural death rate; α , delay required to achieve maximal steady-state growth rate; B_{MAX} , maximum possible bacterial density; E_{MAX} , maximal increase in killing rate in addition to K_{DEATH} ; EC_{50} concentration required to achieve half of E_{MAX} ; γ , Hill coefficient. ^bMinimum Inhibitory (MIC) and Bactericidal (MBC) concentrations and stationary concentrations computed according to Mouton and Vinks (6).

variable) versus $AUC_{bact96h}$ (dependent variable) obtained for each MIC were fitted with an Inhibitory Effect Sigmoid I_{max} PD model (Model 108), Eq. 8:

$$Effect = E_0 - \left(\frac{I_{max} \times INDEX^{Slope}}{INDEX^{Slope} + INDEX_{50}^{Slope}} \right) \quad (8)$$

where E_0 is the maximum effect (obtained for the control curve for $C(t) = 0$), the maximum possible observed effect is $(E_0 - I_{max})$, I_{max} being the amplitude of maximal effect. $INDEX_{50}$ is the magnitude of the index ($fAUC_{PK(0-96h)}/MIC$ or $fT > MIC\%$) that achieves 50% of the I_{max} , and $Slope$ is the sigmoidicity factor, reflecting the steepness of the relationship. Curve fitting was performed with WinNonlin® using the non-linear least-squares algorithm. The coefficients of determination (R^2), the Akaike Information Criterion (AIC), and visual inspection of graphs were used to select the PK/PD index that best predicted the antibacterial effect. The $INDEX_{90\%}$ was computed as the breakpoint value of the predicting PK/PD index.

RESULTS

Time-Kill Curve Modeling

Parameter estimates from the TKC model (bacteria growth system and drug sub-models) are summarized in **Table 1**. The precision of the estimation of the parameter value was good in all cases (estimated CV% less than 38%).

Maximal growth rate ($K_{GROWTHMAX}$, per hour) was 0.97 for *P. multocida* (yielding a 0.71 h generation half-life) and 1.58 for *M. haemolytica* (yielding a 0.44 h generation half-life). The natural death rate (K_{DEATH} , per hour) was 0.12 for *P. multocida* (yielding a 5.9 h count-halving half-life) and 0.78 for *M. haemolytica* (yielding a 0.9 h count-halving half-life). The delay in achieving a maximal steady-state growing rate (α) was 0.22 h^{-1} for *P. multocida* and 0.93 h^{-1} for *M. haemolytica*, corresponding to half-lives to establish full growth capacity of 3 and 0.75 h, respectively. The maximum possible bacterial density of the cultures (B_{MAX}) was 5.2×10^9 CFU/mL for *P. multocida* and 9.6×10^8 CFU/mL for *M. haemolytica*. The CV% for inter-strain (assay) variability was 89% for *P. multocida* and 430% for *M. haemolytica*. Low values of eta-shrinkage (12% *P. multocida* and 4% for *M. haemolytica*) confirm the identifiability of the random effect on B_{max} .

The maximal drug-induced increase in bacterial killing rate (E_{max} , per hour) was 2.0 h^{-1} for *P. multocida* (yielding a 16.7-fold increase in overall death rate) and 2.7 h^{-1} for *M. haemolytica* (yielding a 3.5-fold increase in overall death rate). The *in vitro* concentration for achieving half the maximal effect (EC_{50}) was 0.46 mg/L for *P. multocida* and 0.70 mg/L for *M. haemolytica*, ranking favorably for average experimental MICs of 0.4 mg/L for *P. multocida* and 0.5 mg/L for *M. haemolytica*. The slope of the concentration-effect curve (γ , dimensionless scalar) was for 2.74 for *P. multocida* and 2.63 for *M. haemolytica*.

The plot of the observed natural logarithm of bacterial counts (CFU/mL, the dependent variable DV) versus individual predicted count values (IPRED) for *P. multocida* and

M. haemolytica is presented in **Figure 2**. Visual predictive check (VPC) for *P. multocida* and *M. haemolytica* are shown in **Figure 3**.

Comparison of Microbiological Response for Two Possible Modalities of Florfenicol Administration

The *in silico* predicted microbiological efficacy of the two approved dosage regimens for florfenicol were similar for two inoculum sizes (low 10^5 and high 10^7 CFU/mL) for *P. multocida* and *M. haemolytica* at MICs of 0.5, 1, 2, and 4 mg/L (**Figure 4**). For an MIC of 2 mg/L, the single administration of 40 mg/kg was clearly superior to the two administrations of 20 mg/kg at 48 h interval for both *P. multocida* and *M. haemolytica* and with both inoculum counts. For an MIC of 4 mg/L, none of the dosage regimens were predicted to be efficacious by the *in silico* PK/PD model.

Dose Fractionation *in silico*

Figure 5 illustrates the fitting comparison for prediction of $\log_{10}AUC_{bact(0-96h)}$ (I_{max} sigmoid model) using $fAUC_{PK(0-96h)}/MIC$ or $fT > MIC\%$ as the predictive variable for MICs 0.5, 1, 2, and 4 mg/L and for inoculum strengths of 10^5 and 10^7 CFU/mL for both *P. multocida* and *M. haemolytica*. The fitting for MIC 4 mg/L was excluded due to the limited efficacy of even the highest dosage regimen. In all cases, $fAUC/MIC$ was a better PK/PD index than $fT > MIC$ over 96 h. For *P. multocida*, the goodness of fit values, averaged for inoculum sizes of 10^5 and 10^7 CFU/mL and for all MICs, were better for $fAUC_{PK(0-96h)}/MIC$ (AIC = 76.9, $R^2 = 0.939$) than for $fT > MIC$ (AIC = 81.3, $R^2 = 0.934$). For *M. haemolytica*, the goodness of fit values, averaged for inoculum sizes of 10^5 and 10^7 CFU/mL and for all MICs, were also better for $fAUC_{PK(0-96h)}/MIC$ (AIC = 84, $R^2 = 0.924$) than for $fT > MIC$ (AIC = 86.3, $R^2 = 0.924$).

The critical value for 90% of the maximal *in silico* possible anti-bacterial action for $fAUC_{PK(0-96h)}/MIC$ was solved using Eq. 8 for the two inoculum strengths actually tested (10^5 and 10^7 CFU/mL) and for MICs of 0.5, 1, and 2 mg/L (**Table 2**).

Data in **Table 2** indicate that the critical value of the PK/PD index ($fAUC_{PK(0-96h)}/MIC$) to achieve 90% of maximal effect) was dependent of the tested MIC but relatively similar for both bacterial species. For a MIC of 1 mg/L, the critical value for $fAUC_{PK(0-96h)}/MIC$ for *P. multocida* was 115 and 134 h for inocula of 10^5 and 10^7 CFU/mL, respectively. The corresponding values were 127 and 133 h for *M. haemolytica*. These values indicate that, to achieve 90% of maximal efficacy for a pathogen having a MIC of 1 mg/L, the average free plasma florfenicol concentration over 96 h should be equal to 1.19- and 1.32-fold the MIC for *P. multocida* and 1.40 and 1.39 fold the MIC for *M. haemolytica*.

DISCUSSION

This study is the first to quantify, for veterinary pathogens, the three basic PD parameters of an AMD from TKC analysis,

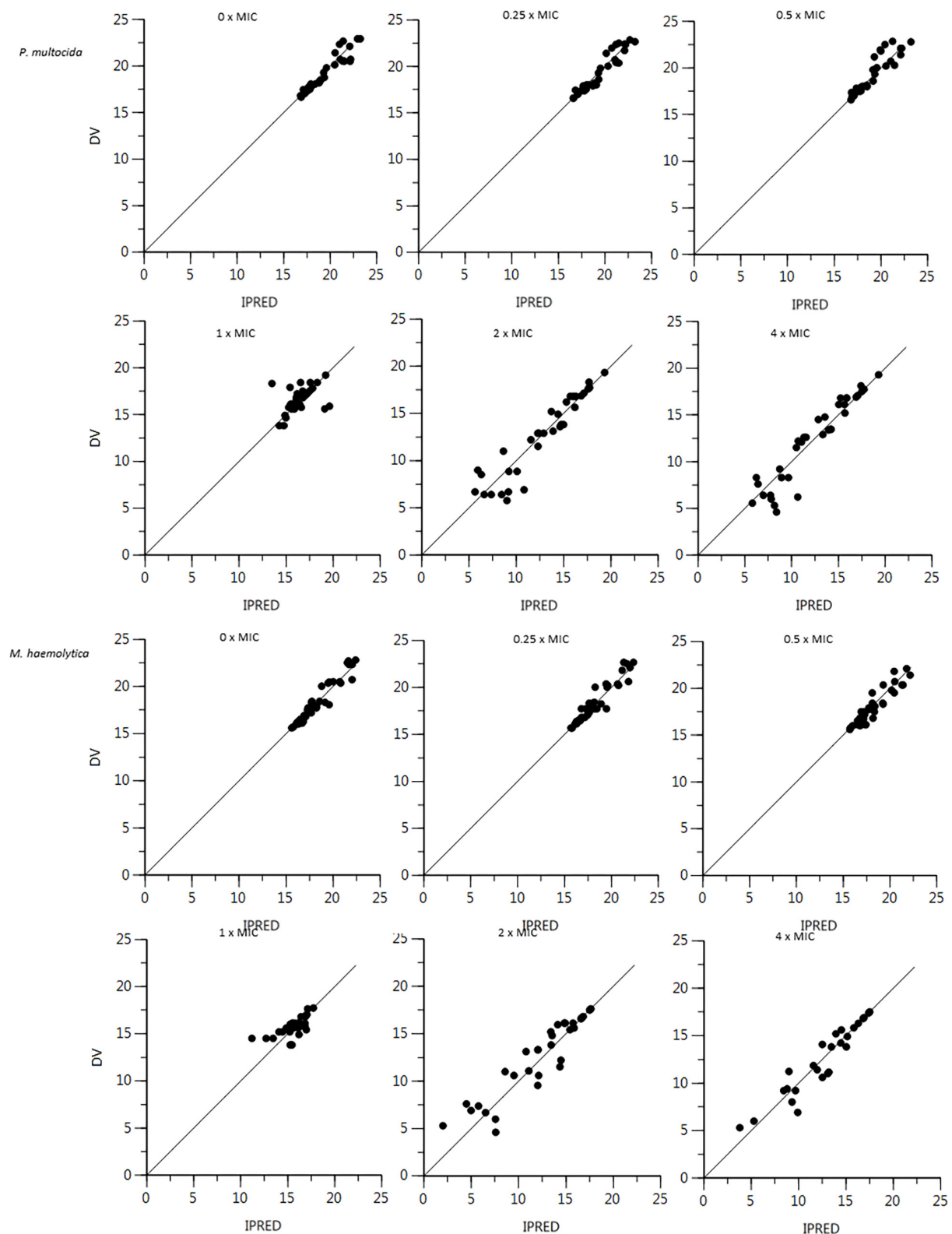
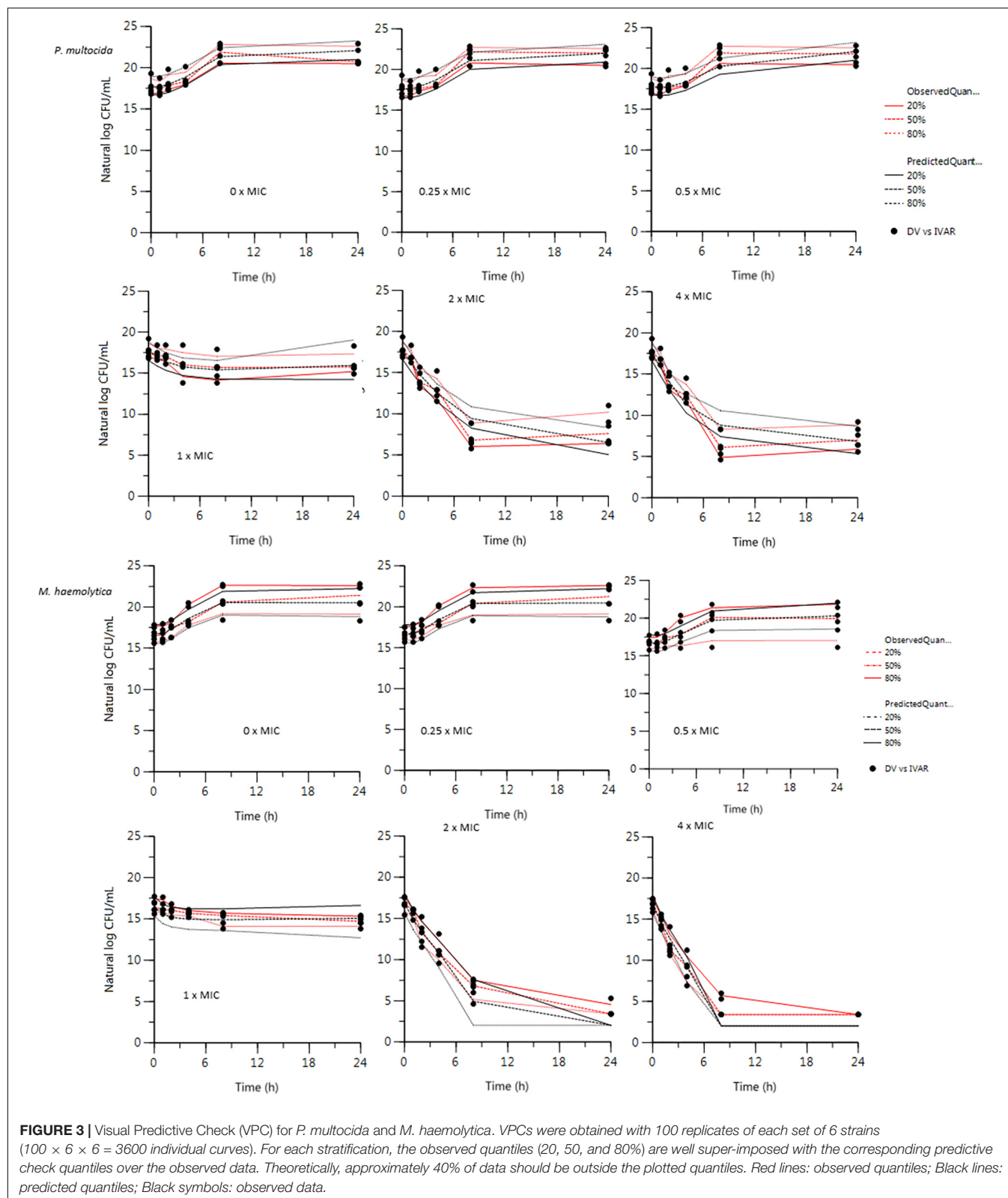
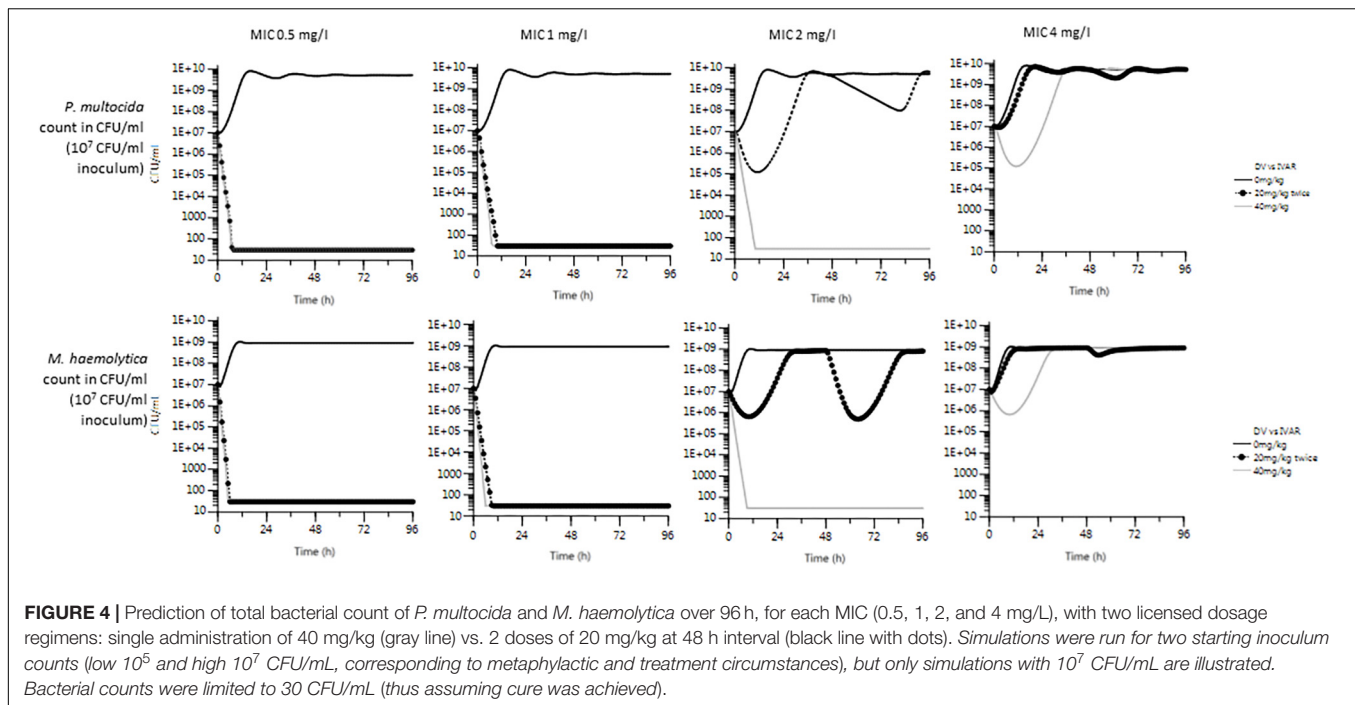


FIGURE 2 | Plot of the dependent variable (DV) i.e., observed natural logarithm of bacterial counts (CFU/mL) versus individual predicted count values (IPRED) for *P. multocida* and *M. haemolytica*. Individual strain predictions are obtained by setting random effects to the “post hoc” or empirical Bayesian estimate of the random effects for the individual from which the DV observation was made. Thus, the plot shows observed versus fitted values of the model function. Ideally, they should fall close to the line of unity $y = x$.



namely efficacy (E_{MAX} maximum killing rate), potency (EC_{50}) and sensitivity (slope of the concentration-effect relationship). These data have been obtained for florfenicol and two major

calf pathogens, *P. multocida* and *M. haemolytica*. The classical index describing quantitatively AMD action is MIC. However, MIC is not a genuine PD parameter; it is a reproducible hybrid



variable measured under standard conditions. Actually, MIC is dependent not only on the three PD parameters but also on *in vitro* conditions (growth and death rates of the tested pathogen, duration of observation and the initial inoculum load). The numerical value of each MIC therefore depends on seven separate factors, as explicitly indicated in eqs. 6A,B.

The advantage of dissecting MIC into these dependency components is to identify the test tube conditions that can be regarded as confounding factors from the actual PD properties that are of primary interest, not only for AMDs but for drugs of all classes. The three parameters have been dissected out and quantified by modeling TKC data. In contrast with MIC, as a crude index of AMD action, TKCs describe time course as well as magnitude of antibacterial action over the 18–24 h duration of exposure. This enables capture of the pattern of bacterial killing with semi-mechanistic models of the type used in the present paper (Figure 1). This model has recently been evaluated against similar PK/PD models proposed by others and using Monte Carlo Simulations. It was concluded that, under constant drug concentrations, as in this study, the median PD parameter estimates were within 10% of the true value and the precision was < 20% (Jacobs et al., 2016).

The calculations indicate that potency and efficacy of florfenicol were of the same order of magnitude for the two pathogens investigated. The strains belonged to the distribution of the wild population for the two pathogens as EUCAST epidemiological cut-off values (ECOFFs) values are 1 and 2 mg/L for *P. multocida* and *M. haemolytica*, respectively. In future studies, it would be valuable to subject to the same modeling process strains belonging to resistant sub-populations; this would reveal how resistance is phenotypically expressed

(for example, as either an increase in EC_{50} and/or a reduction in $EMAX$). Such data would enable interpretation of mechanisms of emergence of resistance, using the same conceptual framework for drugs of other pharmacological classes, when analyzing drug-receptor interaction. Such analysis is a major tool in the quest for developing new drugs (Kenakin, 1997).

The analysis presented in this paper adds a new dimension to bactericidal killing curves by converting them into proxies of an infection model. This required linking *in vivo* PK data to a PD TKC model able to predict the temporal dynamics of bactericidal activity. The PK data are generated by solving a model readily obtained through either classical or population investigations. The PD model predicts a microbiological response for a given drug exposure at two inoculum levels of 10^5 and 10^7 CFU/mL (corresponding to metaphylactic and treatment circumstances, respectively). Currently, rodent models are widely used but they raise questions of cost and ethical use of animals in research. As an alternative to animal studies, the hollow fiber model has been developed as a dynamic infection model (Michael et al., 2014) but its use in veterinary medicine has not yet been reported. Hollow fiber technology is costly and resource demanding; few alternatives like chemostats can be explored but have their own limitations. The present adaptation of TKC results offers the advantages of using historical data and its availability for many veterinary pathogens. Hence, data meta-analysis, as presented in this article, provides, at low cost and with benefits for animal ethics, a new approach to selection of a PK/PD index to predict clinical efficacy of AMDs used in veterinary medicine.

The selected PD model simulated the time course of bactericidal activity of florfenicol, with pathogen exposure

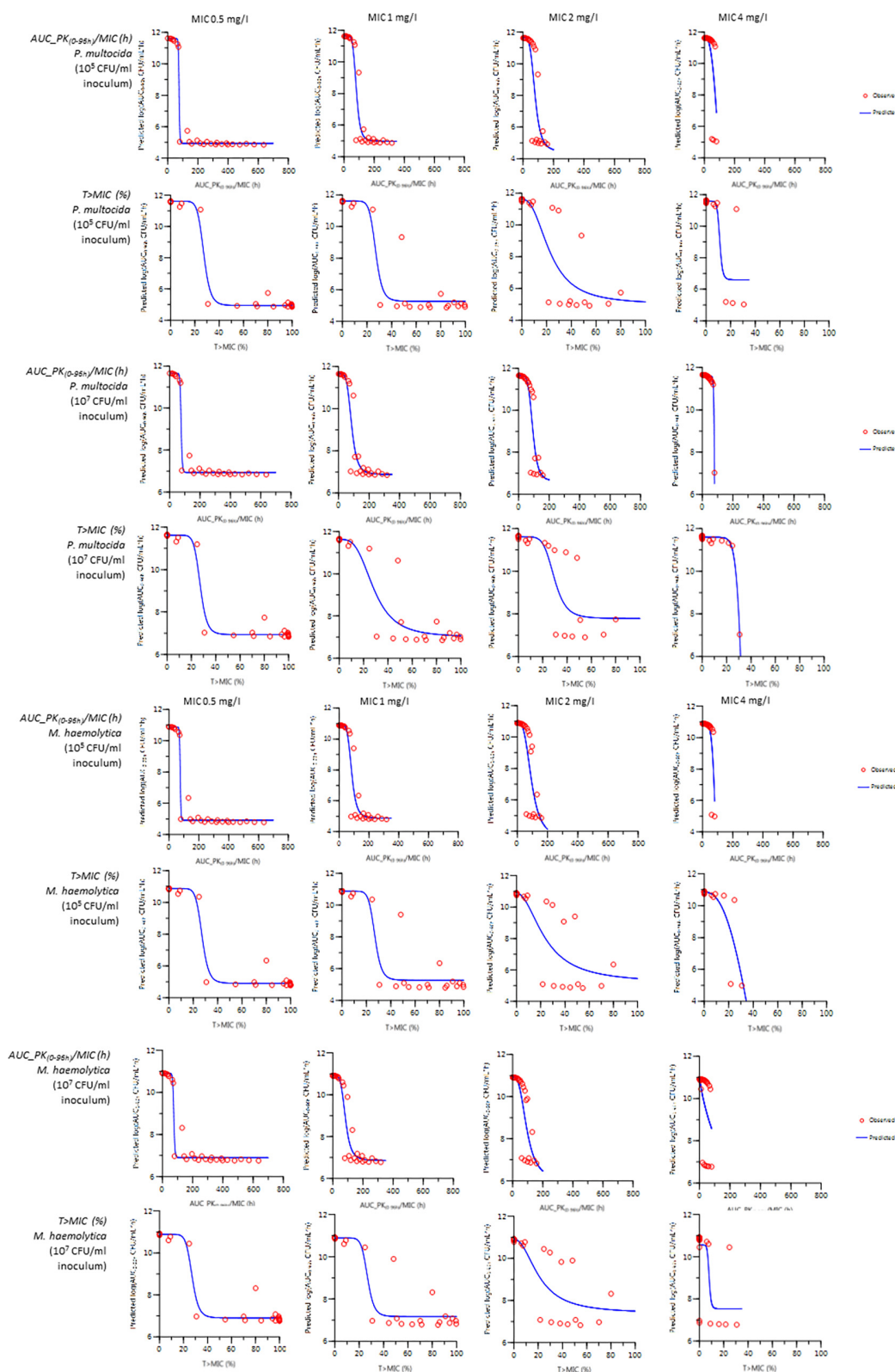


FIGURE 5 | Comparison of fitting for prediction of $\log_{10}$ AUCbact(0-96h) (I_{max} sigmoid model) with $fAUC_{PK(0-96h)}/MIC$ or $T > MIC\%$ as a predictive variable for MIC of 0.5 to 4 mg/L (from left to right) for inoculum sizes of 10^5 and 10^7 CFU/mL for both *P. multocida* and *M. haemolytica*. A sigmoid I_{max} model predicted $\log_{10}$ AUCbact(0-96h) from the value of the PK/PD indices: either $fAUC_{PK(0-96h)}/MIC$ (top row) or $T > MIC\%$ (bottom row) were used as a predictive variables for MICs of 0.5 to 4 mg/L (from left to right) for inoculum sizes of 10^5 and 10^7 CFU/mL for both *P. multocida* and *M. haemolytica*.

TABLE 2 | Critical value of the PK/PD index ($fAUC_{PK(0-96h)}/MIC$, unit h) to achieve 50% or 90% of the maximal possible *in silico* bacteriological effect.

Efficacy (% Emax)		<i>P. multocida</i>		<i>M. haemolytica</i>		Average free plasma concentration required over 96 h (expressed in MIC-fold, unitless) to achieve 90% of the maximal efficacy ^b	
		50%	90%	50%	90%		
MIC (mg/L)		$fAUC_{PK(0-96h)}/MIC$ (h) ^a				<i>P. multocida</i>	<i>M. haemolytica</i>
10 ⁵ CFU/mL inoculum	0.5	75	81	75	81	0.84	0.84
	1	81	115	83	127	1.19	1.32
	2	79	133	94	186	1.38	1.93
10 ⁷ CFU/mL inoculum	0.5	75	84	75	83	0.87	0.86
	1	85	134	84	133	1.40	1.39
	2	92	137	94	195	1.43	2.03

^aFigures for $fAUC_{PK(0-96h)}/MIC$ (h) were computed from equation 8 for two inoculum strengths (10⁵ and 10⁷ CFU/mL) and for MICs of 0.5, 1, and 2 mg/L. ^bThe corresponding average plasma concentration to achieve over 96 h (expressed in multiples of MIC) to ensure 90% efficacy was computed by dividing these critical values by 96 h. Bold fonts highlight values for MIC of 1 mg/L.

actually obtained *in vivo* after administration to calves of the reference florfenicol formulation (Nuflor®). To achieve this, the PD component of the model with its estimated parameters was solved using plasma florfenicol concentrations as predicted by a florfenicol population model (Supplementary File S3, also see Toutain et al., 2019).

Thus, several florfenicol exposure scenarios were simulated to generate corresponding killing curves. This leads to the conclusion that a single florfenicol dose of 40 mg/kg should be more efficacious in bactericidal effect than an alternative dosing regimen of two 20 mg/kg dose at a 48 h interval. According to a meta-analysis from DeDonder and Apley (2015), both dosage regimen were equally efficacious (absolute risk reduction of morbidity) versus negative control.

In order to propose a PK/PD breakpoint for florfenicol based on the VetCAST approach, the first step is to select an appropriate PK/PD index predicting efficacy. A PK/PD approach is superior to using a target CFU at 24 h as it allows the description of the onset, rate and extent of killing and a data-based determination as to whether an AMD is time or concentration-dependent. Florfenicol is used solely in veterinary medicine, so that historically no dose fractionation rodent studies are available to determine the most appropriate PK/PD index predicting efficacy. For determination of the best PK/PD index, *in silico* simulation approaches are scientifically attractive, ethically acceptable and low cost alternatives to *in vivo* dose fractionation studies. This *in silico* approach has been validated for human medicine for the main AMD classes (Nielsen and Friberg, 2013). To select $fAUC/MIC$ or $fT > MIC$ as the PK/PD index of choice, it is necessary to establish the influence of both level (concentration) and shape of exposure to florfenicol on the efficacy of its bactericidal effect, as predicted by the PD model. In this study, from simulated killing curves obtained with 10 florfenicol dose levels ranging from 0 to 80 mg/kg and divided into one, two or four administrations at differing dosing intervals, 28 killing curve profiles were generated. These were then modeled using the classical E_{max} model, with the PK/PD index as independent variable and $fAUC_{(0-96h)}$ under the killing curves as dependent variable. For MICs of 0.5, 1, and 2 mg/L, $fAUC/MIC$ was systematically superior to $fT > MIC$

in predicting bacterial killing, although for the lowest MIC (0.5 mg/L) both indices were acceptable. For an MIC of 2 mg/L, the relationship degraded for $fT > MIC$ but remained acceptable for $fAUC/MIC$. For an MIC of 4 mg/L, both indices failed to predict adequately the florfenicol response or lack thereof.

The selection of $fAUC/MIC$ as the best PK/PD index for florfenicol is consistent with a previous report that, regardless of AMD class, $fAUC/MIC$ is the most appropriate index when terminal half-life is long (Nielsen and Friberg, 2013).

In this study, results of simulations are presented using free plasma concentration of florfenicol, as free plasma concentration is the best proxy for concentration in the biophase. In non-lactating dairy cattle, plasma protein binding ranged from 19 to 23% (Bretzlaff et al., 1987). However, a recent investigation reported that the degree of florfenicol binding in 6-month old steers was either very low (5%) or negligible (Foster et al., 2016). Such low binding differs from another recent study (Mzyk et al., 2018). Investigating the influence of age (1 to 168 days) on degree of florfenicol plasma protein binding, these authors reported binding ranging from 12 to 42% in one-day old, and from 11 to 32% in 168-day old animals, at a concentration of 1 mg/L. In light of these inter-study differences, and as the selected PK/PD index is $fAUC/MIC$, it would be a simple matter to apply a correction for unbound fraction during the computation of the PK/PD cut-off for florfenicol by Monte Carlo simulation. On this basis, it is concluded that for both *P. multocida* and *M. haemolytica* maximum efficacy (actually 90%) over 96 h is obtained when the average free plasma concentration is equal to the 1.2 to 1.4 times the respective MIC.

DATA AVAILABILITY

All datasets analyzed for this study are included in the manuscript and the Supplementary Files S1–S3.

AUTHOR CONTRIBUTIONS

PL and PS generated raw data. LP retrieved and validated raw data. P-LT and LP performed the modeling analysis and

drafted the manuscript. All authors critically reviewed several drafts of the 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/fmicb.2019.01237/full#supplementary-material>

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Conflict of Interest Statement: 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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A Newly Isolated *Bacillus subtilis* Strain Named WS-1 Inhibited Diarrhea and Death Caused by Pathogenic *Escherichia coli* in Newborn Piglets

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Bacillus subtilis is recognized as a safe and reliable human and animal probiotic and is associated with bioactivities such as production of vitamin and immune stimulation. Additionally, it has great potential to be used as an alternative to antimicrobial drugs, which is significant in the context of antibiotic abuse in food animal production. In this study, we isolated one strain of *B. subtilis*, named WS-1, from apparently healthy pigs growing with sick cohorts on one *Escherichia coli* endemic commercial pig farm in Guangdong, China. WS-1 can strongly inhibit the growth of pathogenic *E. coli* *in vitro*. The *B. subtilis* strain WS-1 showed typical *Bacillus* characteristics by endospore staining, biochemical test, enzyme activity analysis, and 16S rRNA sequence analysis. Genomic analysis showed that the *B. subtilis* strain WS-1 shares 100% genomic synteny with *B. subtilis* with a size of 4,088,167 bp. Importantly, inoculation of newborn piglets with 1.5×10^{10} CFU of *B. subtilis* strain WS-1 by oral feeding was able to clearly inhibit diarrhea ($p < 0.05$) and death ($p < 0.05$) caused by pathogenic *E. coli* in piglets. Furthermore, histopathological results showed that the WS-1 strain could protect small intestine from lesions caused by *E. coli* infection. Collectively, these findings suggest that the probiotic *B. subtilis* strain WS-1 acts as a potential biocontrol agent protecting pigs from pathogenic *E. coli* infection.

Importance: In this work, one *B. subtilis* strain (WS-1) was successfully isolated from apparently healthy pigs growing with sick cohorts on one *E. coli* endemic commercial pig farm in Guangdong, China. The *B. subtilis* strain WS-1 was identified to inhibit the growth of pathogenic *E. coli* both *in vitro* and *in vivo*, indicating its potential application in protecting newborn piglets from diarrhea caused by *E. coli* infections. The isolation and characterization will help better understand this bacterium, and the strain WS-1 can be further explored as an alternative to antimicrobial drugs to protect human and animal health.

Keywords: *Bacillus subtilis*, genomic analysis, biocontrol agent, newborn piglets, *Escherichia coli*

INTRODUCTION

Bacillus was first reported by Christian Gottfried Ehrenberg in 1835 (Sella et al., 2015). Since then, *Bacillus* has been found in a wide variety of organisms, such as pigs, and environments, such as ponds and soil (Borsodi et al., 2011; Gu et al., 2015; Wei et al., 2016). *Bacillus* is a common bacterium and is Gram-positive, rod-shaped with a size range of 0.3–22 $\mu\text{m} \times 1.2\text{--}7.0 \mu\text{m}$, spore-forming, and aerobic-to-facultative (Cowan, 1974). A circular colony with rough, opaque, fuzzy white or slightly yellow, and jagged edges was observed by culturing *Bacillus* on nutrient agar (Lu et al., 2018). The genus *Bacillus* was initially proposed by Cohn in 1872 (Harwood, 1989), since then the genus has been expanded with many novel strains. In general, *Bacillus* can be classified into three categories based on the morphology of the spore: (1) seven species including *Bacillus subtilis* in the first genus with ovular- or pillar-shaped spores and with sporocyst that is not significantly expanded; (2) nine species including *Bacillus circulans* in the second genus with oval spore and enlarged sporocyst; and (3) *Bacillus sphaericus* in the third genus with a circular spore and enlarged sporocyst (Ruth et al., 1973).

Bacillus is widely used in industry, agriculture, and the medical field since a variety of functional *Bacillus* strains are extensively used in the production of industrial enzymes, bioinsecticides, antibiotics, and other products (Schallmey et al., 2004). *B. subtilis* is one of the most commonly functional strains. Currently, several studies have confirmed that *B. subtilis* has great application value in animal husbandry. It was reported that pig feed with *B. subtilis* natto could significantly improve meat quality and flavor (Sheng et al., 2016). Liu et al. (2017) also demonstrated that dietary corn bran fermented by *B. subtilis* MA139 could decrease gut cellulolytic bacteria and microbiota diversity in finishing pigs. In addition, *B. subtilis* as delivery vectors is also used to develop vaccines against TGEV in pigs (Mou et al., 2016). Additionally, more functions such as the antimicrobial activity of *B. subtilis* have been identified with the discovery and isolation of new strains (Sato et al., 2001; Phelan et al., 2013; Piewngam et al., 2018; Bartolini et al., 2019; Swartzendruber et al., 2019). For example, it has been found by Bartolini et al. (2019) that the stress-responsive alternative sigma factor (SigB) of *B. subtilis* enhanced antifungal proficiency by increasing the synthesis of lipopeptide surfactin. In addition, Piewngam proved that fengycin-producing *Bacillus* could inhibit *Staphylococcus aureus* colonization in mice (Piewngam et al., 2018).

The accurate identification of a new bacterium is an essential part of studying its function. The traditional techniques to identify a new bacterium are mainly based on the use of selective media by observing bacterial colony characteristics and morphology and by biochemical tests (Lu et al., 2018). Although these methods can preliminarily identify the type of the new strain, the detailed information such as genus, genomic composition, and functional proteins remains unclear. After the conserved bacterial genomic regions are amplified and sequenced, the genus of the bacteria will be accurately identified by homology analysis with the sequences from

GenBank (Wellinghausen et al., 2009). These methods contribute to rapid and accurate identification of new bacterial strains.

Despite various applications of *B. subtilis* in many fields, detailed information underlying resistance against intestinal infections in the pig remains unclear. In this study, we isolated one strain of *B. subtilis* WS-1 from apparently healthy pigs raised with sick cohorts on one *Escherichia coli* endemic commercial pig farm in Guangdong, China and analyzed its genome. In addition, *B. subtilis* WS-1 was identified to inhibit the growth of pathogenic *E. coli* *in vitro* and *in vivo*, which implied that the isolate of *B. subtilis* WS-1 could have potential usage in the future.

MATERIALS AND METHODS

Bacterial Strain and Experimental Newborn Piglets

E. coli strain 4–1, belonging to serogroup O149:K88 and containing LT and ST genes, was isolated from the same commercial pig farms as *B. subtilis* WS-1 and was confirmed to be highly pathogenic to the newborn piglets. Four-day-old crossbred (Duroc $\times$ Landrace $\times$ Big White) healthy conventional female newborn piglets without diarrheic symptoms were procured from Wen's Foodstuffs Group Co, Ltd. (Guangdong, China). All piglets were fed a mixture of skim milk powder (Inner Mongolia Yili Industrial Group Co., Ltd., China) with warm water. The animal study was approved by the Institutional Animal Care and Use Committee of the Sun Yat-sen University (Guangdong, China), and animals were treated in accordance with the regulations and guidelines of this committee.

Reagents and Culture Medium

The Tryptic Soy Broth (TSB) medium and Tryptic Soy Agar (TSA) medium were purchased from Becton, Dickinson and Company (USA). The Oxford cup and Gram Stain Kit were purchased from Guangzhou Heyue Biotechnology Co., Ltd. (China). Endospore Stain Kit was purchased from Solarbio Company (China). TIANamp Bacteria DNA Kit was purchased from TIANGEN Biotech Co., Ltd. (Beijing, China). The Premix Taq™ (LA Taq™ Version 2.0 plus dye) and pMD19-T were purchased from Takara (Dalian, China).

Bacteria Isolation and Inoculum Preparation

Fresh pig feces (10 g) were collected from apparently healthy pigs growing with diarrheic cohorts on one *E. coli* endemic commercial pig farm in Guangdong, China. Prior to isolation, pig feces were mixed with 90 μl sterile 1 $\times$ phosphate buffer saline (PBS; pH 7.4), then incubated in an orbital shaker incubator (Shanghai Bluepard Instruments Co., Ltd., China) at room temperature with a shaking speed of 180 rpm for 30 min, then serially diluted up to 10^{-7} with a sterile distilled water. Isolation of bacteria from this mixture was done with a serial dilution technique in TSA medium (BD, USA). Bacteria

were purified by repeated streaking and single colony culture at 37°C for 17–24 h. A total of 35 unknown bacterial isolates were recovered in TSB medium (BD, USA). Exponential phase growing cultures were washed twice with sterile 1 × PBS and maintained at 4°C until use.

Screening of the Antimicrobial Isolates Using the Oxford Cup Method

The Oxford cup method was performed as previously described with some modifications (Bian et al., 2016). Briefly, the culture fluid of *E. coli* strain 4–1 was mixed with the TSA medium at 50°C and poured into a bacterial culture dish. The Oxford cups containing 80 µl (7.2×10^8 CFU/ml) culture fluid of 35 unknown bacteria strains were affixed to the uniform coating on the medium, placed at 37°C for 18–24 h, and the inhibitory rings were observed and measured with a Vernier caliper (Guangzhou Heyue Biotechnology Co., Ltd., China). The Oxford cups containing culture fluid without bacteria were used as control.

Identification of the No. 1 Unknown Bacterial Strain

(1) The No. 1 unknown bacterial strain was examined for being a member of the family *Bacillus* by means of endospore staining according to the manufacturer's instruction (Solarbio Company, China), Gram staining (Lu et al., 2018), and biochemical trait test (Cowan, 1974) and further by enzyme activity (Cowan, 1974). (2) Molecular identification of the *Bacillus* isolate by PCR and total DNA of the No. 1 unknown bacterial strain were prepared according to the manufacturer's instruction (TIANGEN Company, China). The universal bacterial primers for the 16S rRNA gene of *Bacillus* (sense: 5'-AGAGTTGATCCTGGCTAAG-3'; antisense: 5'-GGTTACCTTGTTACGACTT-3') were designed with reference to a previous publication (Lu et al., 2018) and were synthesized by Sangon Company (Shanghai, China). The PCR was performed in a volume of 50 µl containing 1 µl of DNA, 25 µl Premix Taq™ (LA Taq™ Version 2.0 plus dye), upstream and downstream primer (50 µmol/L) each at 1 µl and 22 µl ddH₂O. The thermal cycling parameters were as follows: 94°C for 5 min; 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 15 min. The positive PCR products were cloned into the pMD19-T (TaKaRa, Dalian) and sequenced by Sangon Company (Shanghai, China). Sequence alignments of 16S rRNA of different *Bacillus* species were performed using the DNASTar Lasergene 7.0. A genome homology analysis and phylogenetic trees were constructed by using the maximum likelihood method with MEGA 5 software¹ based on the 16S rRNA nucleotide sequences of 14 *Bacillus* strains from different countries.

Genome Sequencing and Bioinformatics Analysis

After we successfully identified the No. 1 unknown strain as a member of *B. subtilis*, which was thereafter named as

B. subtilis WS-1, the complete genome was sequenced by the PacBio platform (Single Molecule, Real-Time technology) (Magigene, Guangdong) as previously described with some modifications (Cameron et al., 2015; Tanizawa et al., 2015; Carrión et al., 2018). Briefly, the bacterial genomic DNA was extracted as described above. DNA integrity and purity were monitored on 1% agarose gels, and the concentration and purity of DNA were measured using Qubit 2.0 (Thermo Fisher Scientific Waltham, USA) and Nanodrop one (Thermo Fisher Scientific Waltham, USA) at the same time. Then, the qualified genomic DNA was fragmented with G-tubes (Covaris) and end-repaired to prepare SMRTbell DNA template libraries (with a fragment size of >10 kb selected by bluepippin system) according to the manufacturer's specification (PacBio, Menlo Park, USA). Library quality was detected by Qubit 3.0 Fluorometer (Life Technologies, Grand Island, NY, USA), and average fragment size was estimated on an Agilent 4,200 (Agilent, Santa Clara, CA, USA). SMRT sequencing was performed on the Pacific Biosciences RSII sequencer (PacBio, Menlo Park, USA) according to standard protocols. The low-quality reads were filtered by the SMRT 2.3.0 and assembled to generate one contig without gaps after sequencing. For the genome component prediction, the whole genome sequence was performed by Gene Marks for coding gene prediction (Besemer et al., 2001), diamond and BLAST for gene annotation, and PHAST for pre-phage prediction (Zhou et al., 2011). Related functional proteins were analyzed by BLAST. The genome overview was created by Circos (Krzywinski et al., 2009) to show the annotation information. Genomic synteny was analyzed by MUMmer software (Delcher et al., 2002) based on the alignment results with *B. subtilis* (GenBank no: AL009126.3).

Measurement of Bacterial Growth

To determine the growth rate of *B. subtilis* WS-1 (3.4×10^4), the strain was grown in TSB medium at 37°C for 48 h with agitation, and the CFU was determined at 0, 6, 12, 18, 24, 30, 36, 42, and 48 h.

Experimental Infection With the *E. coli* 4–1 Strain After *B. subtilis* Strain WS-1 Treatment in Conventional Newborn Piglets

Twelve newborn piglets were randomly divided into two groups (6 piglets/group) and were housed in two separate rooms. Prior to inoculation, newborn piglets were confirmed negative for the major porcine enteric viruses (PDCoV, PEDV, TGEV, and PRoV) by testing the rectal swabs collected from the newborn piglets on day 1 as previously described (Xu et al., 2018). On day 0, newborn piglets in group 1 were orally inoculated with 5 ml/day of TSB medium for 3 days. Newborn piglets in group 2 were orally inoculated with 5 ml/day of TSB medium containing a total of 5×10^9 CFU of the *B. subtilis* strain WS-1 (1 ml of medium contained

¹<http://www.megasoftware.net/>

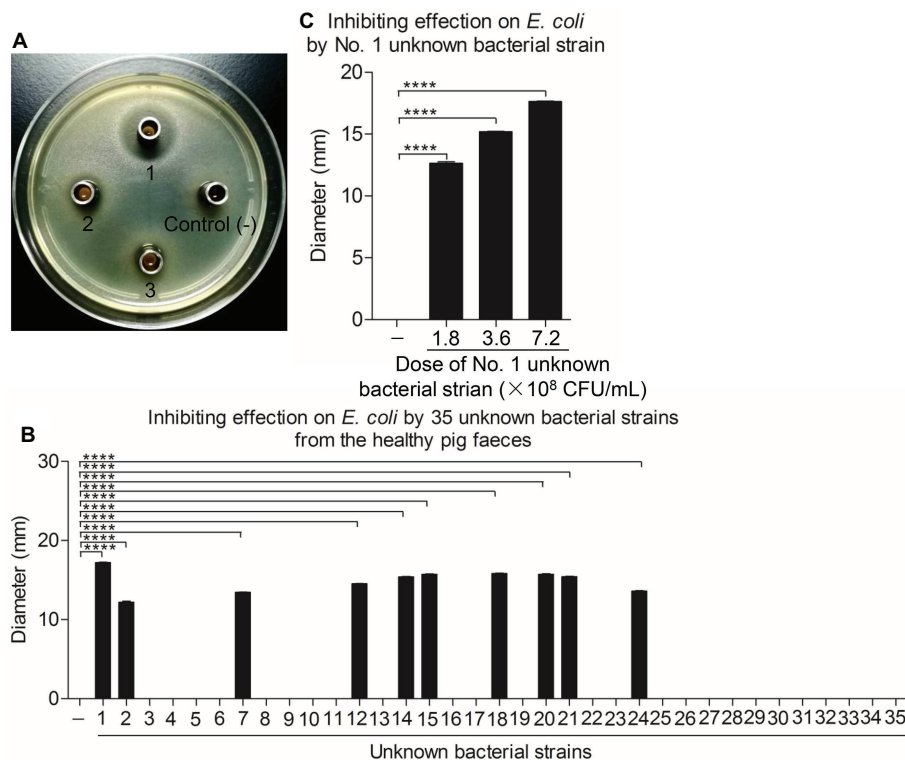


FIGURE 1 | Ten unknown bacterial strains from healthy pig feces significantly inhibited the growth of *E. coli* *in vitro*. **(A,B)** Inhibiting effect on *E. coli* of total 35 unknown bacterial strains isolated from the healthy pig feces. *E. coli* strain 4–1 was maintained on bouillon agar medium (10^8 CFU/ml); the Oxford cup with 80 μ l single bacterial culture fluid was placed in the middle. After 24 h, the inhibitory ring was observed and measured with ruler. **(C)** The No. 1 unknown bacterial strain significantly inhibited the growth of *E. coli*. *E. coli* strain 4–1 was maintained on bouillon agar medium (10^8 CFU/ml); the Oxford cups with 80 μ l of No. 1 unknown bacterial culture fluid containing different amounts of 1.8×10^8 , 3.6×10^8 , and 7.2×10^8 CFU/ml were placed in the middle. The inhibitory rings were observed and measured with a ruler. Data are representative of three independent experiments. Data are represented as mean $\pm$ SD, $n = 3$. **** $p < 0.0001$.

1×10^9 CFU of *B. subtilis* strain WS-1) for 3 days. Afterward, all piglets were orally challenged with 5 ml TSB medium containing 1×10^{10} CFU of the *E. coli* 4–1 strain. The piglets were observed daily for clinical signs of diarrhea and lethargy. One piglet from each group was necropsied at 3 days post challenge (d.p.c). At necropsy, the fresh duodenum, jejunum, and ileum were collected and fixed by 10% formalin for histopathology analysis. In addition, the mortality rate of newborn piglets in different treatment groups was recorded daily from day 1 to day 6 after challenge for protection rate analysis.

Histological Staining

Histological staining was performed as previously described (Xu et al., 2018). Briefly, tissue samples of the duodenum, jejunum, and ileum of the piglets from the *B. subtilis* strain WS-1 treatment and control groups were routinely fixed in 10% formalin for 36 h at room temperature and then dehydrated in graded ethanol, embedded in paraffin, cut in 5- μ m sections, and mounted onto glass slides. After the sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E), the slides were examined and analyzed with conventional light microscopy (Nikon, Japan).

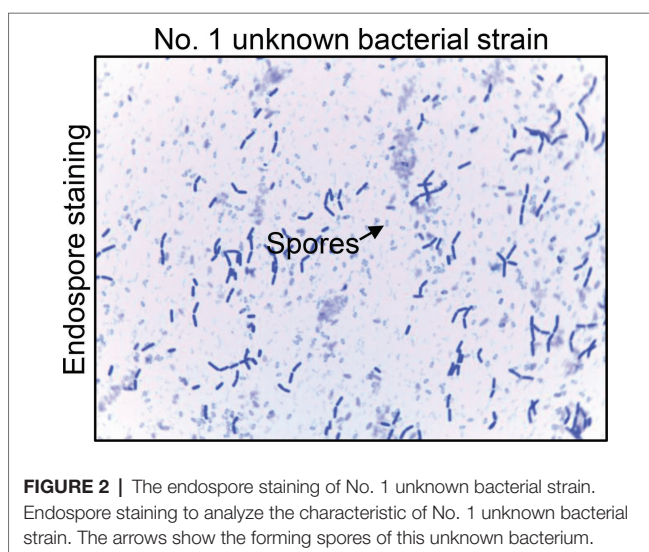


FIGURE 2 | The endospore staining of No. 1 unknown bacterial strain. Endospore staining to analyze the characteristic of No. 1 unknown bacterial strain. The arrows show the forming spores of this unknown bacterium.

Statistical Analysis

Statistical comparisons were performed using GraphPad Prism software. The significance of the differences between the treatment group and control in the inhibitory rings, diarrhea rate, and survival rate was determined by ANOVA and Mann-Whitney accordingly.

TABLE 1 | Biochemical traits of the *Bacillus* isolate (*B. subtilis* strain WS-1).

Test	Result	Test	Result	Test	Result
Glycerin	+	Inositol	+	Inulin	+
Erythritol	–	Mannitol	+	Melezitose	–
D-arabinose	–	Sorbitol	+	Raffinose	+
L-arabinose	+	α -methyl-D mannose	–	Starch	+
Ribose	+	α -methyl-D glucoside	+	Glycogen	+
D-xylose	+	N-acetyl-glucosamine	–	Xylitol	–
L-xylose	–	Amygdalin	+	Gentian disaccharide	–
Amoxicil	–	Arbutin	+	D-Turanose	+
β -methyl-D-xyloside	–	Esculin	+	D-lyxose	–
Galactose	–	2-Hydroxybenzyl alcohol	+	D-Tagatose	–
Glucose	+	Cellobiose	+	D-arabinitol	–
fructose	+	Maltose	+	L-arabinitol	–
Mannose	+	Lactose	–	Gluconate	–
Sorbose	–	Melibiose	+	2-keto-gluconate	–
L-Rhamnose	–	Sucrose	+	5-keto-gluconate	–
Dulcitol	–	Trehalose	+		

RESULTS

Ten Unknown Bacterial Strains Isolated From the Feces of Apparently Healthy Pigs Inhibit the Growth of Pathogenic *E. coli* *in vitro*

Some apparently healthy piglets without any symptoms of diarrhea were found on one *E. coli* endemic commercial pig farm in Guangdong, China. To determine the protective agent against the diarrhea outbreak in this pig farm, fresh feces from one healthy pig were collected and used to isolate the bacteria. Of the 10 g of feces examined, 35 unknown bacterial strains were isolated. Furthermore, we found that 10 unknown bacterial strains could markedly inhibit the growth of *E. coli* by the Oxford cup method (Figures 1A,B). Of them, the No. 1 unknown bacterial strain had the best antibacterial effect in a dose-dependent manner (Figures 1B,C).

A Strain of *B. subtilis* Was Identified

The No. 1 unknown bacterial strain was identified as *Bacillus* by morphological and biochemical examinations. The isolate displayed the morphology of *Bacillus* by visual and microscopic observations and was shaped as Gram-positive (data not shown) *Bacillus*, which could form spores (Figure 2). A biochemical test was further employed to analyze the characteristic of this bacterium. The results clearly showed that the strain was up to 99% in consistency with the standard of *Bacillus* (Table 1). These results indicated that the No. 1 unknown bacterial strain was a strain of *Bacillus*. To determine the species of *Bacillus* of this strain, we further analyzed the 16S rRNA by sequencing the PCR-amplified product. As shown in Figure 3,² the No. 1 unknown bacterial strain shared 99% identity with *B. subtilis* based on the sequence of 16S rRNA. Phylogenetic analysis showed that the No. 1 unknown bacterial strain was clustered into a clade with *B. subtilis* B4 from Hubei, China.

Taken together, the No. 1 unknown bacterial strain belongs to *B. subtilis* and was named WS-1.

Complete Genome Sequencing and Analysis of *B. subtilis* Strain WS-1

The complete genome of *B. subtilis* strain WS-1 was acquired and uploaded onto GenBank (No. CP024921). The genome of *B. subtilis* strain WS-1 has a size of 4,088,167 bp (Figure 4A), with G + C content being 43.8%. Some genes encoded by this strain of *Bacillus* have protease, lipase, and amylase activity (Table 2), indicating that the WS-1 strain might have antimicrobial activity. In addition, approximately 89% of nucleotides were predicted as protein-coding regions, and 86.8% (3,704) of the open reading frames were annotated with known proteins. Some putative proteins were predicted to be associated with antimicrobial or probiotic activity by the Swiss-Prot database (Table 3). We also confirmed that the WS-1 strain contained sporulation genes like *CgeA*, *CotB*, and *CotZ* (data not shown). These data confirmed that the WS-1 strain shares 100% genomic synteny with *B. subtilis* (Figure 4B).

The Growth Rate of *B. subtilis* Strain WS-1 *in vitro*

To determine the growth rate *in vitro*, we inoculated *B. subtilis* strain WS-1 in TSB medium and detected the living bacterial count at corresponding time points. As shown in Figure 5, the *B. subtilis* strain WS-1 grew exponentially post inoculation and reached a plateau 12 h post inoculation, which lasted for at least 36 h, indicating that *B. subtilis* strain WS-1 could adapt to the TSB medium *in vitro*.

B. subtilis Strain WS-1 Inhibits Diarrhea and Death Caused by *E. coli* in Newborn Piglets

Since *B. subtilis* strain WS-1 was confirmed to significantly inhibit the growth of *E. coli* *in vitro*, we attempted to determine whether WS-1 has the same effect *in vivo*. We experimentally infected

²<http://www.megasoftware.net>

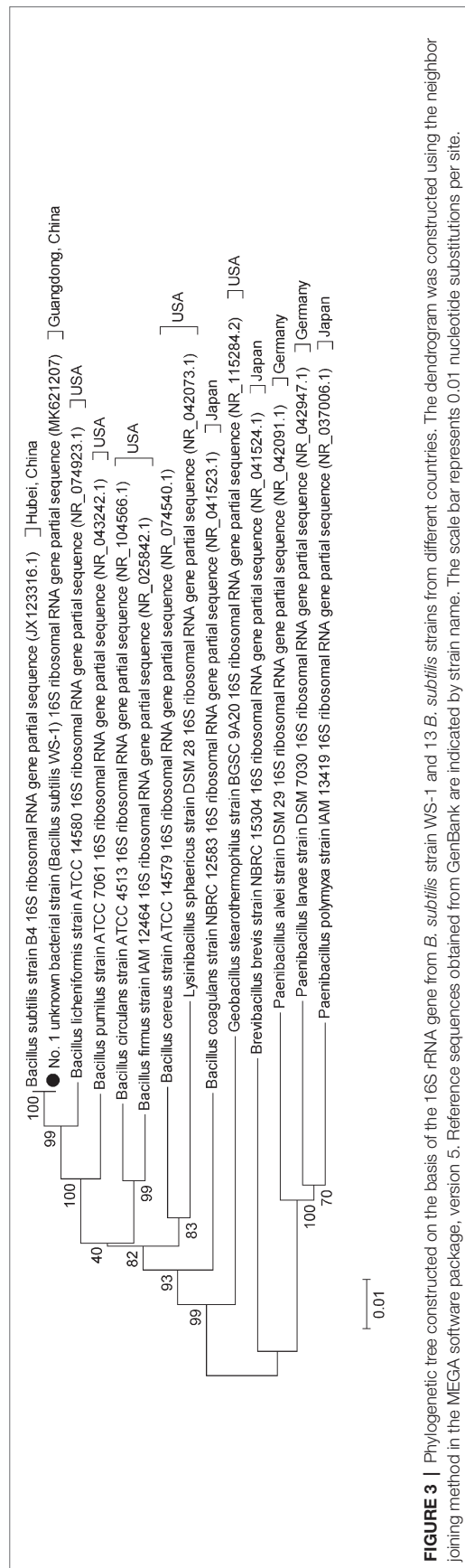


FIGURE 3 | Phylogenetic tree constructed on the basis of the 16S rRNA gene from *B. subtilis* strain WS-1 and 13 *B. subtilis* strains from different countries. The dendrogram was constructed using the neighbor joining method in the MEGA software package, version 5. Reference sequences obtained from GenBank are indicated by strain name. The scale bar represents 0.01 nucleotide substitutions per site.

newborn piglets with *E. coli* strain 4–1 after *B. subtilis* strain WS-1 treatment. As expected, the newborn piglets pre-inoculated with TSB medium containing *B. subtilis* strain WS-1 *via* oral feeding showed a lower diarrhea rate (16.7%) at the first day. By contrast, all newborn piglets (100%) pre-inoculated with TSB medium without *B. subtilis* strain WS-1 *via* oral feeding showed acute and severe watery diarrhea in the first 2 days (**Figure 6A**), indicating that *B. subtilis* strain WS-1 might serve as a probiotic in newborn piglets. Importantly, two piglets died that pre-inoculated with TSB medium without *B. subtilis* strain WS-1 *via* oral feeding group at 2 d.p.c., and no piglets died in the *B. subtilis* strain WS-1 treatment group during the study (**Figure 6B**). Taken together, these results demonstrated that *B. subtilis* strain WS-1 works as a probiotic to inhibit *E. coli* infection *in vivo*.

Histopathological Results of Newborn Piglets Infected With *E. coli* After *B. subtilis* Strain WS-1 Treatment

To determine the histological changes in the intestine of newborn piglets infected with *E. coli* after *B. subtilis* strain WS-1 treatment, piglets were necropsied at 3 d.p.c. As shown in **Figure 7**, blunt intestinal villus was observed in the duodenum, jejunum, and ileum in the TSB medium only group, while the villus in the *B. subtilis* strain WS-1 treatment group remained intact (**Figures 7A–F**).

DISCUSSION

Since the first report of *B. subtilis* by Christian Gottfried Ehrenberg in 1835 (Sella et al., 2015), this ancient bacteria had been widely detected and isolated in many organisms and environments (Borsodi et al., 2011; Gu et al., 2015; Wei et al., 2016). Many subsequent studies demonstrated that *B. subtilis* had a variety of functions. Although *B. subtilis* was widely used in the field of fermentation and livestock and poultry breeding (Nguyen et al., 2015; Liu et al., 2017; Amorim et al., 2019), currently limited information is available regarding the protection of *B. subtilis* against intestinal diseases in newborn piglets. In the present study, we reported an isolate of *B. subtilis* from apparently healthy pigs in a pathogenic *E. coli* endemic commercial farm with severe diarrhea symptoms in piglets, which could inhibit the growth of *E. coli in vitro* and protect piglets from diarrhea and death caused by pathogenic *E. coli* in newborn piglets.

Diarrhea in piglets is mainly caused by viruses and bacteria such as Porcine epidemic diarrhea virus (PEDV) and *E. coli* (Vlasova et al., 2014; Garcia-Menino et al., 2018), which results in significant economic losses to the pig industry. Occasionally, some apparently healthy pigs without symptoms of diarrhea were found commingling with *E. coli* diarrheic piglets. To determine the protective agent in these pigs, 35 unknown bacterial strains from fresh feces of one healthy pig were isolated and 10 unknown bacterial strains were determined to markedly inhibit the growth of *E. coli* and *Salmonella typhi* (data not shown) by the Oxford cup method (**Figure 1**), indicating that these unknown strains might

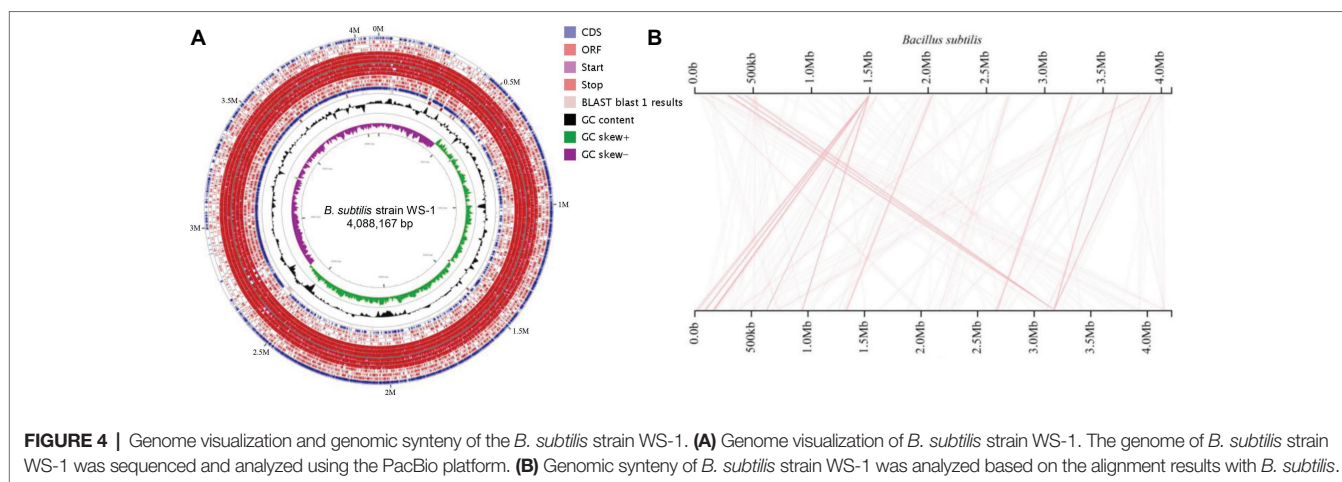


TABLE 2 | Enzyme activity analysis of *B. subtilis* strain WS-1.

Variety	Nucleotide position*	Enzyme activity (U/ml)
Protease	(-) 210183-211151, 684509-686197, 725622-727559, 758838-761258, 1122374-1122967, 2696411-2697742, 2813385-2814671, 2814668-2815948, 2829137-2830327, 2847680-2848948, 2882897-2884300, 2884317-2884862, 3384242-3385801, 3415417-3418101, 3467502-3468782, 3906905-3907594; (+) 516616-517818, 1045409-1046851, 1283746-1285122, 1364459-1365088, 1450962-1451639, 1783051-1784313, 1784465-1786123, 1786304-1788628, 1804131-1804667, 1812254-1813120, 1895220-1895846, 2095165-2095638, 2134016-2135539, 2187051-2188331, 2300552-2301154, 2306573-2307229, 2439721-2441121, 2710268-2711596, 3031354-3032919, 3135226-3137355, 3185998-3186957, 3466390-3467463, 3498168-3498779, 3899889-3900581	40.1
Lipase	(-) 163484-164155, 3661219-3661836, 4066571-4067212; (+) 2415760-2416569	28.6
Amylase	(-) 126705-128684	158.2

*Nucleotide position is numbered based on *B. subtilis* WS-1 strain (CP024921).

(+): Positive-sense strand; (-): negative-sense strand.

be the key to protect from diarrhea in these pigs. Of them, the No. 1 unknown bacterial strain had the best antibacterial effect. After analysis through conventional bacterial identification methods, the No. 1 unknown bacterial strain was identified as Gram-positive bacteria of *Bacillus*, having protease, lipase, and amylase activity, which might be utilized to generate antimicrobial substances such as bioactive peptides (Daroit et al., 2012) and chloramphenicol esters (Dong et al., 2017) to inhibit bacterial biofilm formation (Vaikundamoorthy et al., 2018). Bosshard et al. (2003) demonstrated that 95–99% similarity for 16S rRNA gene sequencing between two bacteria hints toward a similar species, while >99% indicates the same bacterium. In the study, the No. 1 unknown bacterial strain was identified as

a member of *B. subtilis* by the analysis of 16S rRNA gene sequencing and therefore named as *B. subtilis* WS-1. To further characterize the isolate, the complete genome of *B. subtilis* strain WS-1 was sequenced and analyzed. The WS-1 strain shares 100% genomic synteny with *B. subtilis* and encoded multiple functional proteins like lipopeptides, which might be associated with the antibacterial activity of the WS-1 strain.

Bacterial resistance is becoming more common with the abuse of antibiotics over the past years, and it urgently demands an alternative to antimicrobial drugs. Of note, several groups have confirmed the protective role of *Bacillus* strains as probiotics *in vivo* (Wu et al., 2014; Ramesh et al., 2015). Collectively, these previous results confirmed that *Bacillus* strains could antagonize *Vibrio parahaemolyticus* and *Aeromonas hydrophila*. Swine enteric colibacillosis affects all sectors of the pig industry and all cycles of production. Compared with other age groups, newborn piglets are more vulnerable to swine enteric colibacillosis. We hypothesized that *B. subtilis* strain WS-1 was also capable of antagonizing colibacillosis in newborn piglets based on its probiotic effect *in vitro*. Therefore, we experimentally infected newborn piglets with the pathogenic *E. coli* after *B. subtilis* strain WS-1 treatment. As expected, *B. subtilis* strain WS-1 was able to work against diarrhea and death caused by *E. coli* in newborn piglets (Figure 6), indicating that the isolated *B. subtilis* strain WS-1 also had a probiotic effect *in vivo* and might be used as an alternative of antimicrobial drugs. In addition, we also found that none of the WS-1 strain-inoculated newborn piglets showed any clinical signs (data not shown) before *E. coli* infection, proving that *B. subtilis* strain WS-1 is safe for pigs. Furthermore, no evident gross lesions were observed in the intestinal tract of the *B. subtilis* strain WS-1 treatment piglets at necropsy at 3 d.p.c. (data not shown). Similarly, no microscopic lesions were observed in the small intestine in the *B. subtilis* strain WS-1 treatment group after *E. coli* infection (Figure 7), which furthered our understandings of the role of the *B. subtilis* as a probiotic. It was reported that several lipopeptides such surfactin, secreted by *B. subtilis*, can confer strong antipathogenic effects and thus benefit the

TABLE 3 | Functional proteins prediction of *B. subtilis* strain WS-1.

Name	Nucleotide position*	Number
Polyketides	(-) 2714457-2722088, 2722103-2738647, 2738637-2751425, 2751441-2754173, 2754134-2764267, 2764255-2765067, 2765212-2765610, 2765607-2780180, 2780225-2780974, 2781014-2781793, 2781781-2783043, 2783044-2784291, 2784579-2786513, 2786501-2786881, 278678-2787852, 2788356-2789222, 2790462-2791079;	18
Lipopeptides	(+) 3033214-3034185 (-) 20315-21268, 90550-91134, 132322-133203, 147031-147990, 164284-165411, 600839-601852, 962123-963040, 1243143-1243943, 1249990-1250721, 1428128-1429255, 1570007-1571287, 1865158-1865979, 2955757-2956074, 2956062-2956208, 3207178-3208827, 3350410-3352047, 3463183-3464229, 3586153-3586992, 3608704-3609702, 3784560-3785786, 3824696-3825895, 4012394-4013515, 4063703-4065709 (+) 44837-45643, 87329-88180, 267938-269866, 272995-273948, 608703-609497, 650251-651399, 683398-684237, 740201-741358, 1074976-1075785, 1113395-1114648, 1160684-1161949, 1207777-1208697, 1264758-1265702, 1306601-1307425, 1318172-1318681, 1318687-1319430, 1523167-1524108, 1581821-1583335, 1660893-1661702, 1661692-1662528, 1726112-1727431, 1965356-1966111, 2058797-2059528, 2125691-2126728, 2214635-2215402, 2415760-2416569, 2437636-2438457, 2457696-2458565, 2676968-2677603, 3500643-3501521, 3651495-3652505, 3745050-3746072	55
Flagella	(-) 1138642-1139100, 2861243-2862133, 2862130-2863230, 2863230-2865263, 2865296-2866378, 2866378-2867157, 2867165-2867410, 2867449-2868114, 2868107-2868766, 2869169-2870305, 2870295-2871293, 2871327-2871749, 2871746-2871961, 2872001-2872762, 2872817-2873239, 2873236-2874699, 2875337-2875780, 2877096-2877857, 2877841-2878857, 2878870-2880480, 2880526-2880852, 2880857-2881312, 2881309-2881704, 2892718-2892999; (+) 921972-922808, 922854-923651, 1029455-1030963, 1030974-1031870, 1032513-1032944, 1034454-1034783, 1034801-1034947, 1034977-1036296, 1036318-1036719, 1625025-1625846, 1625836-1626564, 3137648-3138550, 3138522-3139307	37
Exopolysaccharide biosynthesis	(+) 556979-558043, 1143948-1144784, 1149168-1150202, 1152318-1152968, 1153006-1154139, 1154136-1155086	6
Enolase	(-) 3144704-3145948; (+) 3395921-3397036	2

*Nucleotide position is numbered based on *B. subtilis* WS-1 strain (CP024921).
(+): Positive-sense strand; (-): negative-sense strand.

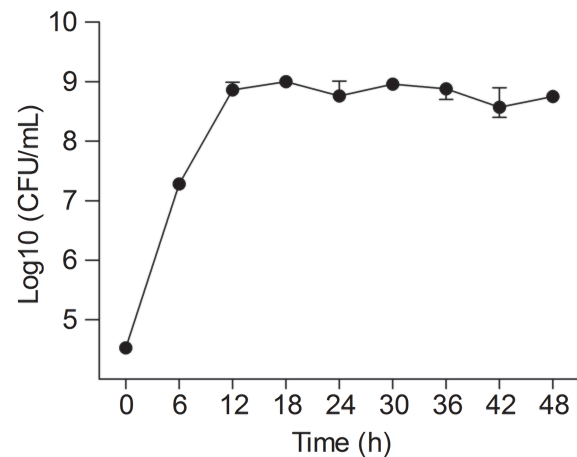
Growth of *B. subtilis* strain WS-1 in vitro culture

FIGURE 5 | Measurement of bacterial growth. The growth curves of *B. subtilis* strain WS-1. Bacteria were grown in bouillon fluid medium at 37°C for 48 h with agitation, and the Colony Forming Unit (CFU) was determined at 0, 6, 12, 18, 24, 30, 36, 42, and 48 h. The data are representative of three independent experiments. Data are represented as mean $\pm$ SD, $n = 3$.

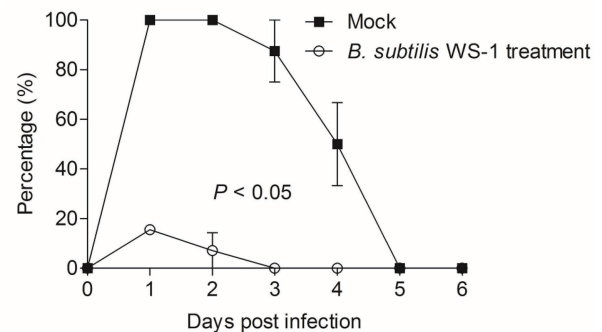
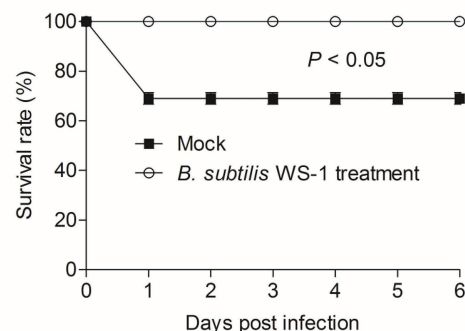
A The diarrhea rate of *E. coli*-inoculated newborn piglets**B** The survival rate of newborn piglets post infection with *E. coli*

FIGURE 6 | *B. subtilis* strain WS-1 inhibited diarrhea and death caused by *E. coli* in newborn piglets. Newborn piglets were first orally fed with TSB medium or TSB medium containing *B. subtilis* strain WS-1. At 3 days, all piglets were orally challenged with *E. coli* strain 4-1. The diarrhea rate (**A**) and survival rate (**B**) of newborn piglets post-challenge with *E. coli* between the control group and the *B. subtilis* strain WS-1 treatment group were recorded daily from the first day to the sixth day after challenge. The data are representative of two independent experiments. Data are represented as mean $\pm$ SD, $n = 6$ or $n = 7$.

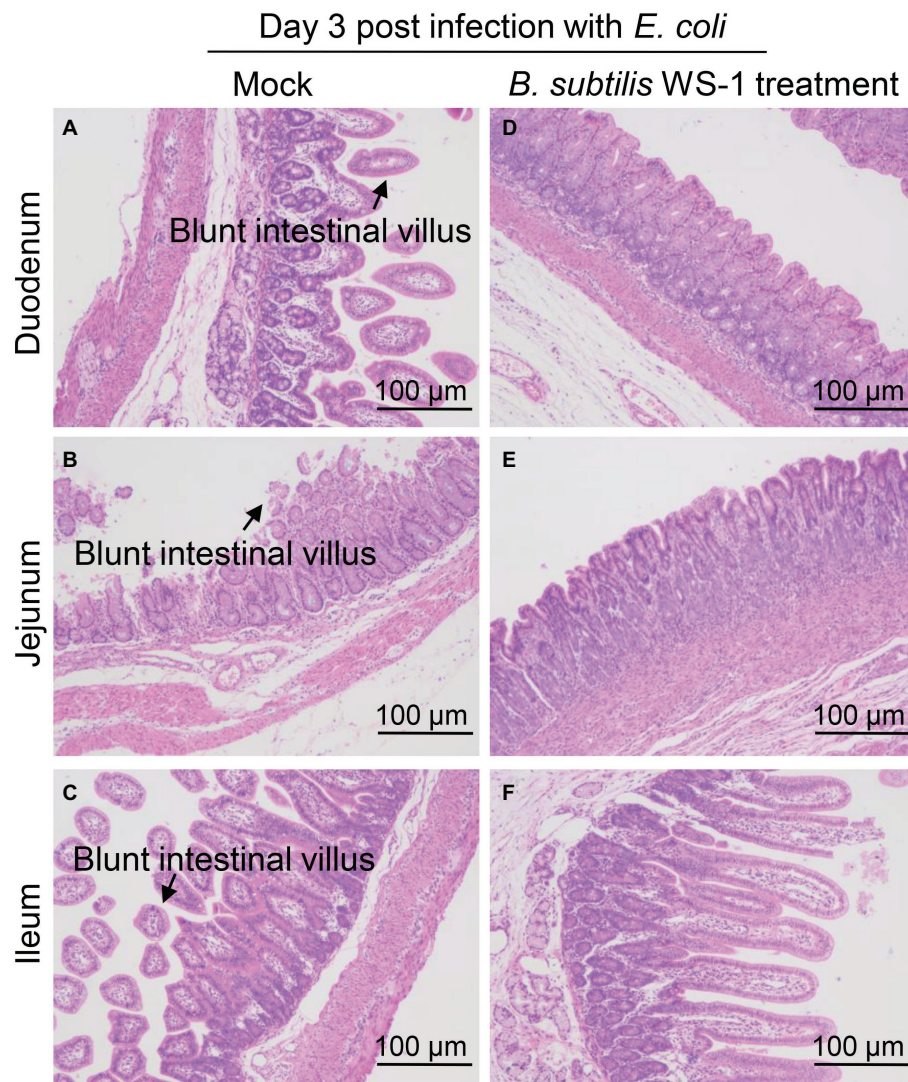


FIGURE 7 | Intestinal changes in newborn piglets post-challenge with *E. coli* of the control group and the *B. subtilis* strain WS-1 treatment group. **(A–C)** Hematoxylin and eosin (H&E)-stained intestinal tissue section (blunt intestinal villus was indicated by arrows) of a control piglet at 3 d.p.c. **(D–F)** H&E-stained intestinal tissue section of a *B. subtilis* strain WS-1 treatment piglet at 3 d.p.c. The data are representative of two independent experiments. Data are represented as mean $\pm$ SD, $n = 1$ or $n = 2$.

host by balancing the intestinal microbiome (Zhou et al., 2018). Polyketide from the seaweed-associated heterotrophic bacterium *B. subtilis* MTCC 10403 has potential antibacterial activities against clinically important pathogens (Chakraborty et al., 2017). In addition, some studies concluded that some proteins involved in aggregation, such as flagella, might be associated with probiotic effects (Kleta et al., 2014). We determined that some genes in the WS-1 genome encode these proteins (Table 3). Whether the interference effect of WS-1 on *E. coli* infection in newborn piglets was associated with these putative proteins needs to be further explored. Nevertheless, there are still several important questions that need to be addressed. For example, what is the exact underlying mechanism of *B. subtilis* strain WS-1 inhibiting enteric diseases caused by *E. coli* in newborn piglets? Can *B. subtilis* strain

WS-1 resist enteric diseases caused by viruses like PEDV in pigs? Does *B. subtilis* strain WS-1 have other applications apart from protecting the intestinal tract? Elucidation of these questions will elevate our understandings of the function of *B. subtilis* strain WS-1 and may help extend its application in many fields.

In summary, we isolated a field strain of *B. subtilis* from apparently healthy pigs growing with sick cohorts on one *E. coli* endemic commercial pig farm in Guangdong, China. Remarkably, inoculation of newborn piglets with 1.5×10^{10} CFU of WS-1 by oral feeding could prevent diarrhea and death caused by pathogenic *E. coli* in piglets. Collectively, these findings suggest that *B. subtilis* strain WS-1 has great potential to be explored as biocontrol agent protecting piglets from enteric diseases in pigs.

ETHICS STATEMENT

The animal study was supervised by the Institutional Animal Care and Use Committee of Sun Yat-sen University (IACUC-2018-000178), and animals were used in accordance with regulations and guidelines of this committee.

AUTHOR CONTRIBUTIONS

YC and YD conceived and designed the experiments. YD, ZX, GY, WL, and DY performed the experiments. YD and ZX

analyzed the data. YC, QZ, JL, LC, YZ, and CX contributed the reagents, materials, and analysis tools. ZX wrote the manuscript. YC checked and finalized the manuscript. All authors read and approved the final manuscript.

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Anti-outer Membrane Vesicle Antibodies Increase Antibiotic Sensitivity of Pan-Drug-Resistant *Acinetobacter baumannii*

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Acinetobacter baumannii often causes serious nosocomial infections. Because of its serious drug resistance problems, complex drug resistance mechanism, and rapid adaptation to antibiotics, it often shows pan-drug resistance and high fatality rates, which represent great challenges for clinical treatment. Therefore, identifying new ways to overcome antibiotic resistance is particularly important. In this study, mice immunized with *A. baumannii* outer membrane vesicles (AbOMVs) produced high IgG levels for a long time, and this antiserum significantly increased the small molecule intracellular aggregation rate and concentrations. *In vitro* experiments demonstrated that the combined use of anti-AbOMV serum and quinolone antibiotics significantly increased the sensitivity of the bacteria to these antibiotics. Mouse sepsis model experiments demonstrated that delivery of these antibodies using both active and passive immunization strategies significantly improved the susceptibility to quinolone antibiotics, improved the survival rate of mice infected with *A. baumannii*, and reduced the bacterial load in the organs. In a pneumonia model, the combination of serum anti-AbOMVs and levofloxacin improved levofloxacin sensitivity, which significantly reduced the bacterial loads in the lung and spleen compared with those of the antibiotic or antibody alone. This combination also significantly reduced lung inflammatory cell infiltration and inflammatory cytokine aggregation. In this study, the main protein targets that bound to these antibodies were identified. Structural modeling showed that seven of the proteins were porins. Therefore, we speculated that the anti-AbOMV antibodies mainly improved the intracellular aggregation of antibiotics by affecting porins, thus improving susceptibility to quinolone antibiotics. This study provides a method to improve susceptibility to existing antibiotics and a novel idea for the prevention and treatment of pan-drug-resistant *A. baumannii*.

Keywords: antibiotic resistance, *Acinetobacter baumannii*, antibodies, outer membrane vesicles, outer membrane proteins

INTRODUCTION

Acinetobacter baumannii is widely found in nature and is prone to causing infections in the skin, respiratory tract, and urinary system. It is also an important conditional pathogen in hospitals (An and Su, 2018). At present, the *A. baumannii* infection rate continues to rise with the widespread use of antibacterial drugs and has increased in various invasive procedures, and this bacteria has become the main pathogen responsible for nosocomial infections. Of concern, the degree of antibiotic resistance of *A. baumannii* is extremely severe, and the numbers of multidrug-resistant (MDR) and pan-drug-resistant (PDR) strains in intensive care units in particular are increasing, which not only pose great difficulties for clinical treatment but also represent great challenges for nosocomial infection control (Ben-Chetrit et al., 2018).

The resistance mechanisms of *A. baumannii* include inhibition of membrane permeability, efflux pumps, drug-inactivating enzymes, and drug target changes. When multiple resistance mechanisms work together, *A. baumannii* shows severe drug resistance. Bacteria reduce penetration of antibiotics into the cell by altering the structures or modulating the expression levels of outer membrane proteins (OMPs) to affect their permeability. Additionally, bacteria can initiate efflux systems and prevent antibacterial drugs from reaching their effective therapeutic concentrations in the bacteria, which then can escape the bactericidal effects of the antibiotics (Smani et al., 2014; Krishnamoorthy et al., 2017).

Specific antibodies can activate complement, neutralize toxins and viruses, promote phagocytosis, and function by activating and antagonizing targets (Casadevall and Pirofski, 2004). Currently, antibody drugs have been widely used for infectious and autoimmune diseases and tumor immunotherapy (Pagan et al., 2018; Tang et al., 2018). In immuno compromised patients, lack of antibiotic efficacy is very common, which indicates that clearance of bacterial infections results from a combination of the host immune defense and antibiotic sterilization in the patients. A combination of two fully humanized monoclonal antibodies directed against CDA1 and CDB1 with metronidazole or vancomycin significantly reduced the recurrence of *Clostridioides difficile* infection (Lowy et al., 2010). In addition, antibodies targeting *Pseudomonas aeruginosa* PcrV and Psl effectively increased antibiotic sensitivity (DiGiandomenico et al., 2014). The method, which involves linking antibodies and antibiotics with linker molecules to target intracellular pathogens, is more effective than treatment with antibiotics alone (Mariathasan and Tan, 2017). In addition, the combined use of anti-efflux pump protein SerA antibodies and antibiotics improved susceptibility to antibiotics against *Stenotrophomonas maltophilia* (Al-Hamad et al., 2011). These findings suggest that antibody-antibiotic combination drugs have broad application potential.

The OMPs of *A. baumannii* present an important correlation with bacterial drug resistance. Most OMPs are exposed on the cell surface and thus can easily be bound by antibodies. Therefore, a method that can identify effective antibody-binding OMP targets related to drug resistance and antibodies to reverse bacterial resistance will have great significance. However,

studies of regulation of drug resistance *A. baumannii* using antibodies are lacking.

The outer membrane vesicles of *A. baumannii* (AbOMVs), which range in size from 10 to 300 nm, are released and secreted extracellularly from the outer membrane by bacteria during growth. Their natural components are mainly phospholipids, OMPs, lipopolysaccharides (LPSs), and soluble periplasmic proteins (Kulp and Kuehn, 2010). Our previous study showed that immunization with AbOMVs produced high levels of antibodies against *A. baumannii*, which protected mice from infection by a drug-resistant strain (Huang et al., 2014). These anti-AbOMV antibodies work against the major OMPs of *A. baumannii* and activate phagocytes to opsonize and kill the bacteria, but the effects of these antibodies on the function of target proteins have not been reported. In this study, we used AbOMVs to immunize mice and obtained polyclonal antibodies that could increase the aggregation of small molecules in bacterial cells *in vitro* and allow antibiotics to rapidly reach high intracellular concentrations. The results showed that the combined use of the antibodies and quinolone antibiotic could effectively improve antibiotic susceptibility both *in vitro* and *in vivo*. Ten major OMPs that reacted with these antibodies were obtained by mass spectrometry analysis and structure prediction. This study provides a basis for screening of antibody-antibiotic combination drugs that are more effective, target multiple OMPs, and can prevent and treat PDR *A. baumannii* infections.

MATERIALS AND METHODS

Ethics Statement

The animal experimental procedures were approved by the Ethics Committee of Animal Care and Welfare, Institute of Medical Biology, CAMS (Permit Number: SYXK (dian) 2010-0007) in accordance with the animal ethics guidelines of the Chinese National Health and Medical Research Council (NHMRC) and the Office of Laboratory Animal Management of Yunnan Province, China. All efforts were made to minimize animal suffering.

All human participants submitted a signed informed consent form to participate in the study. The protocol complied with the Helsinki Declaration and was approved by the Institutional Review Boards of the Institute of Medical Biology, Chinese Academy of Medical Sciences and Peking Union Medical College.

Bacterial Strains and Mice

All *A. baumannii* strains were isolated from patients hospitalized at the Second Affiliated Hospitals of Kunming Medical University (Kunming, China). All strains are drug-resistant. The *A. baumannii* ATCC 19606 strain was obtained from the American Type Culture Collection (ATCC). Female C57BL/6N mice (6–8 weeks of age) were maintained under specific pathogen-free (SPF) conditions.

OMV Preparation

OMV preparation was based on a previously described protocol (Huang et al., 2014). A single Ab112 colony was inoculated

and cultured overnight in LB without (named AbOMVs or Ab112-OMVs) or with a sub-minimum inhibitor concentration (sub-MIC) of ceftriaxone (32 $\mu\text{g/mL}$), amikacin (256 $\mu\text{g/mL}$), azithromycin (256 $\mu\text{g/mL}$), ampicillin (256 $\mu\text{g/mL}$), or levofloxacin (2 $\mu\text{g/mL}$) under different temperatures (25, 30, and 45°C) or in Mueller-Hinton (MH) medium without antibiotics (named MH-AbOMVs). *A. baumannii* ACTT19606 was cultured in LB medium without antibiotics (named 19606-OMVs). The bacterial culture was centrifuged at $14,000 \times g$ for 30 min, and the supernatant was filtered through a 0.45- μm membrane (Millipore, Merck). The filtered fraction was concentrated by ultrafiltration with a 500,000 nominal molecular weight cut off (500,000 NMWC) column (GE Healthcare). The concentrate was ultracentrifuged at $200,000 \times g$ for 4 h at 4°C. The vesicle pellets were resuspended in phosphate-buffered saline (PBS; 0.02 mol/L phosphate buffer with 0.15 mol/L NaCl, pH 7.4) and then filtered through a 0.45- μm membrane. The absence of viable bacteria in the OMV preparations was determined by spreading aliquots on agar plates to test for bacterial growth. The OMVs were quantified using the Bradford reagent according to the manufacturer's instructions.

Preparation of Outer Membrane Protein Complexes (OMPCs)

Outer Membrane Protein Complex preparation was based on a previously described protocol (McConnell et al., 2011). *A. baumannii* Ab112 was grown in 500 mL of LB medium to an optical density at 600 nm (OD₆₀₀) of 0.8, and the pelleted bacteria were resuspended in PBS and lysed by sonication. Unlysed cells were removed by centrifugation at $4000 \times g$ for 5 min, and the supernatant was centrifuged at $20,000 \times g$ for 1 h to pellet the cell envelopes. Inner membranes were selectively solubilized with 5 mL of 2% N-laurylsarcosinate by incubation at 37°C for 30 min. The insoluble fraction was pelleted by centrifugation at $20,000 \times g$ for 1 h and then washed with PBS.

Electron Microscopy

Electron microscopy was based on a previously described protocol (Huang et al., 2014). The OMV sample was fixed with 2.5% cold glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4) for 2 h at 4°C and post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at 4°C; then, the sample was observed and imaged using a transmission electron microscope (Hitachi) at 80 kV.

Dynamic Light Scattering (DLS)

Dynamic light scattering was based on a previously described protocol (Huang et al., 2016a). AbOMVs were measured by DLS using the Zetasizer Nano ZS (Malvern Instruments) to detect the size distribution, which was reflected by the polydispersity index (PDI) with a range between 0.0 (monodispersed) and 1.0 (entirely heterodispersed).

Mouse Immunizations

The mouse immunizations were based on a previously described protocol (Huang et al., 2014). The vaccine was prepared by

mixing AbOMVs (isolated from Ab112) with an equal volume of 2 mg/mL of Alum adjuvant (Thermo Scientific). Then, each mouse was immunized subcutaneously three times with 100 μL of the vaccine containing 2 μg or 0.2 μg of AbOMVs per mouse at weeks 0, 2, and 4. An additional group of mice was injected with a mixture of PBS and adjuvant to serve as the control.

Antibody Measurements Using an Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA based on a previously described protocol (Huang et al., 2016a). AbOMV-specific IgG responses were measured using an ELISA. Briefly, 96-well plates were coated overnight with 25 $\mu\text{g}/100 \mu\text{L}$ of AbOMVs per well. The plates were incubated with the collected serum samples (diluted 1:1000) incubated with anti-mouse IgG secondary antibodies (diluted 1:10,000, Santa Cruz) and developed with an alkaline phosphatase substrate.

Assessment of Intracellular Small Molecule Accumulation

This experiment refers to a previously reported research method (Coldham et al., 2010). All strains were cultured overnight at 37°C and used to inoculate fresh medium, which was incubated for an additional 5 h at 37°C. In addition, 10^7 CFU/100 μL of the strains and 80 μL of anti-AbOMVs or control serum (diluted 1:320) were added to a white 96-well plate and co-incubated for 1 h at 37°C. Hoechst 33258 (HT) (25 μM) was added (20 μL) to each well to a final concentration of 2.5 μM . Fluorescence was read from the top of the wells using excitation and emission filters of 352 and 461 nm, respectively, every 5 min for 15 min on the Synergy4 microplate reader (Biotek) and imaged with a fluorescence microscope (Nikon) at 15 min.

MICs

The MICs were determined using an agar doubling dilution method similar to that recommended by the Clinical Laboratory Standards Institute (CLSI) (Randall et al., 2001), with the main exception being that Luria-Bertani agar was used instead of Mueller Hinton agar. Bacteria grown overnight at 37°C in LB broth were diluted 1:10 in normal saline and inoculated with a multipoint inoculator onto agar containing suitable dilutions of an antibiotic (ceftriaxone, ciprofloxacin, levofloxacin, amikacin, gentamicin, ampicillin, or imipenem). A preliminary study was performed using bacteria incubated with different serum dilutions (1:1 to 1:1024), and the results suggested that the 1:320 dilution was the best anti-serum dilution. Control or anti-AbOMV serum and bacteria (final concentration of 5×10^5 CFU/mL) was incubated at 37°C for 1 h and then added to the plates. The plates were incubated overnight at 37°C, and the MICs were recorded as the lowest concentration that inhibited growth.

Kinetic Growth Curve Analyses

Levofloxacin was tested at 1/16 of the MIC (1 µg/mL), 1/4 of the MIC (4 µg/mL) or the MIC (16 µg/mL). Ciprofloxacin was tested at 1/4 of the MIC (8 µg/mL) and combined with anti-AbOMVs or control serum (diluted 1:320). Ab112 at a concentration of 5×10^5 CFU/mL in LB was used for all growth curve assays. Samples were collected at 0, 2, 4, 8, 16, and 24 h, diluted in LB and plated in 10-fold dilutions on LB plates. The plates were incubated at 37°C overnight, and the CFUs were counted.

Mouse Infection and Antibiotic Therapy

For the active immunization studies using the sepsis model, female C57BL/6N mice were immunized with 2 µg of AbOMVs or the Alum control at weeks 0, 2, and 4. At week 40, $\sim 1 \times 10^7$ CFU/200 µL [$\sim 10 \times$ of the median lethal dose (LD50)] of *A. baumannii* Ab112 cells with 10% porcine mucin (w/v; Sigma-Aldrich) were administered intraperitoneally to each mouse. For the passive immunization studies in the sepsis and pneumonia models, the mice were administered the Ab112 strain by intraperitoneal (i.p) or intranasal (i.n) challenge. The bacterial dose of the abdominal cavity was the same as that of the active immunization experiment. The intranasal challenge dose was 50 µL of live Ab112 dissolved in PBS at a concentration of 10^9 CFU/mL. A total of 50 µL of 30 mg/kg of levofloxacin and 50 µL of anti-AbOMV serum (collected at week 40 from the vaccinated mice) were administered in the tail vein 1 h after bacterial challenge, and the mice were treated every 12 h for 3 days.

Survival Rates and Bacterial Burdens

This experiment refers to the previous research method (Huang et al., 2016a). For the sepsis and pneumonia model, the mice were monitored continuously for 7 days to determine the survival rate after challenge with Ab112. The bacterial burdens in the lung and spleen were measured 12 h after challenge with *A. baumannii* by plating 10-fold dilutions on LB plates. The plates were incubated at 37°C overnight, and the CFUs were counted. The results are expressed as CFU/g.

Analysis of Lung Inflammation

The lung inflammation analysis was based on a previously described protocol (Huang et al., 2014). For the pneumonia model, the mice were anesthetized 12 h after infection, and lung was collected. After homogenization, the supernatants were kept for cytokine measurements. The concentrations of cytokines (IL-1β and IL-6) were measured using ELISA (eBioscience) according to the supplier's instructions. Lung tissues were embedded in paraffin and serially sectioned (5 mm) sagittally. The specimens were stained with hematoxylin and eosin to examine peribronchial and alveolar inflammatory cell accumulation. Lung inflammation was scored according to the following definitions (Noto et al., 2017): 0, no pathology; 1, minimal infiltrates of neutrophils in alveolar spaces; 2, low numbers of neutrophils in alveoli; 3, moderate numbers of neutrophils and hemorrhage in alveoli with occasional lobar involvement and focal necrosis of alveolar-wall neutrophils in bronchioles; 4, marked numbers of neutrophils, consolidation, and widespread alveolar necrosis.

Immunoblotting and Tandem Mass Spectrometry Analyses

This experiment refers to the previous research method (Huang et al., 2016a). *A. baumannii* Ab112 whole cells (WCs) and OMVs were separated by SDS-PAGE. WCs were transferred to a PVDF membrane. *A. baumannii* Ab112 OMV (AbOMV) antiserum or control serum was used as the primary antibody (diluted 1:1000), and horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Invitrogen) was used as the secondary antibody at a 1:10,000 dilution. The blots were developed with an electrochemiluminescence (ECL) substrate (Thermo Scientific).

Tandem matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF)-TOF mass spectrometry analysis was performed by Sangon Biotech (Shanghai) Co., Ltd., to identify the protein bands of interest by SDS-PAGE. The samples were removed directly from the gel.

Homology Modeling and Sequence Identity Analysis

The homology modeling was based on a previously described protocol (Lin et al., 2013). All proteins were modeled by homology *in silico* using the SWISS-MODEL automated protein structure homology modeling server¹. The sequence homology of these 10 proteins to approximately 2,832 reported *A. baumannii* strains, and amino acid homology with human proteins or other non-homologous strains that excluded *A. baumannii* was analyzed using NCBI BLAST. The homology was divided into seven intervals (100–96%, 95–91%, 90–86%, 85–81%, 80–76%, 75–71%, and 70–0%); the percentages of sequence homology of the 10 proteins from the entire database are fully displayed in a heat map.

Statistical Analyses

All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, Inc.). The survival rates were compared using the non-parametric log-rank test. One-way ANOVA with Tukey's multiple comparison test was used to analyze the IgG levels, cytokine concentrations, histology score, fluorescence units, bacterial growth, and bacterial burden. Differences were considered significant if the *P* value was <0.05. All graphed values represent the mean, and the error bars represent the standard error.

RESULTS

Mice Immunized With AbOMVs Were Able to Induce a Prolonged IgG Antibody Response

The vesicles secreted by PDR *A. baumannii* Ab112 were collected, and the morphology of the AbOMVs were observed under a transmission electron microscope in a random field of view (Figure 1A). Their diameter was determined by a particle size analyzer to be 248.7 ± 5.6 nm, with a polydispersity

¹<http://swissmodel.expasy.org>

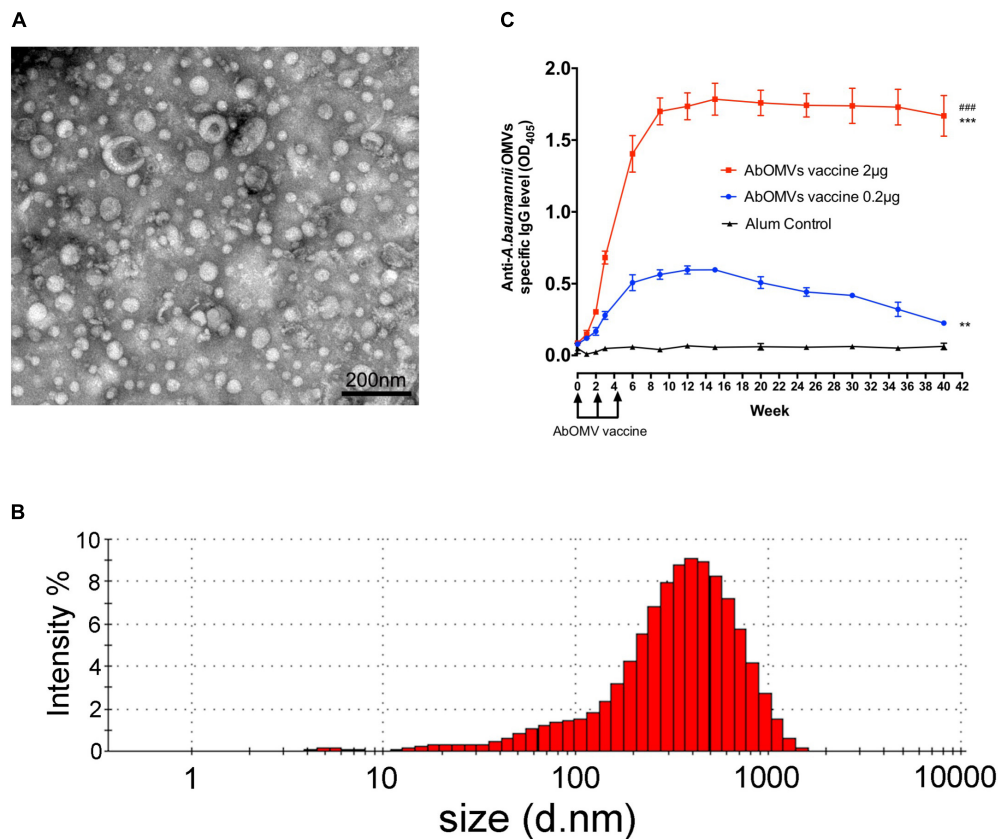


FIGURE 1 | Generation of AbOMVs and mouse immunization. **(A)** OMVs secreted by the Ab112 strain were observed under a transmission electron microscope in a random field of view, bar: 200 nm. **(B)** The OMV diameters were analyzed using a particle size analyzer. **(C)** ELISA was performed to measure the specific anti-AbOMV IgG antibody levels after mice were immunized with the AbOMVs ($n = 5$). #Represents the comparison between the 2 and 0.2 μg dose groups; *represents the comparison between each group and the Alum control group; ***/### $P < 0.001$; ** $P < 0.01$.

index ranging from $\text{DLS} 0.45 \pm 0.018$ (Figure 1B). C57BL/6N mice were immunized with AbOMVs as a vaccine. After three immunizations, both the 2 and 0.2 μg dose groups produced IgG specific to the AbOMVs compared with the adjuvant control group. The IgG level in the 2 μg dose group was significantly higher than that in the 0.2 μg dose group; the 2 μg dose group sustained a high IgG antibody level for up to 40 weeks, whereas the IgG level in the 0.2 μg dose group had begun to decrease by 15 weeks (Figure 1C). These results showed that the AbOMV vaccine could rapidly induce a high and prolonged IgG response.

Anti-AbOMV Serum Significantly Increased the Intracellular Aggregation of Small Molecules

The uptake rate of the small molecule fluorescent dye HT by drug-resistant *A. baumannii* Ab112 was slow, and HT was enriched intracellularly at a low concentration. The mean fluorescence unit value at 15 min was only 1089. After addition of control serum from normal mice, the mean fluorescence unit value at 15 min was 2475. After addition of the anti-AbOMV serum, the ability of the bacteria to take up HT was rapidly improved, and the mean fluorescence unit value at 15 min

was 4922. This experiment demonstrated that addition of anti-AbOMV serum significantly increased HT uptake, suggesting that anti-AbOMV serum could increase the aggregation rate and small molecule concentrations in PDR *A. baumannii* cells (Figure 2A). The intracellular HT aggregation level was observed using fluorescence microscopy. The fluorescence appeared specifically in the bacterial cells, and the number of bacteria that were fluorescently stained after the addition of anti-AbOMV serum was significantly increased compared with that of the serum control and PBS groups (Figure 2B). We used nine clinically isolated PDR *A. baumannii* strains to examine whether this anti-AbOMV serum had universality. The results showed that the anti-AbOMV serum significantly increased the intracellular aggregation of HT in all strains, but the HT uptake efficiency varied among the different strains (Figure 2C).

Anti-AbOMV Serum Increased the Sensitivity of PDR *A. baumannii* to Antibiotics *in vitro*

The effect of the anti-AbOMV serum on susceptibility of the Ab112 strain to seven common antibiotics was examined. We found that administration of ceftriaxone together with the

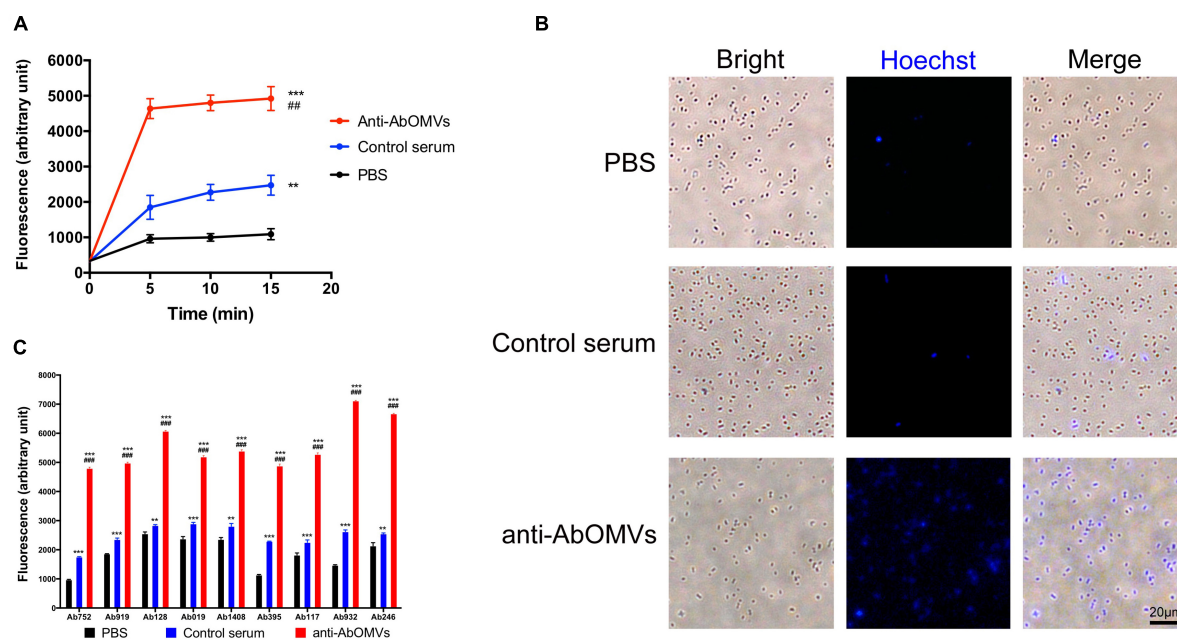


FIGURE 2 | Anti-AbOMV serum significantly increased the aggregation of small molecules in cells. **(A)** The antibodies were incubated with the Ab112 strain for 30 min; then, HT was quickly added, and the dynamic uptake efficiency of HT by the strain was tested every 5 min. The experiment was repeated four times. *Indicates comparison with the PBS group; #indicates comparison with the control serum group. **(B)** After the Ab112 strain was incubated with HT for 15 min, intracellular staining of the bacteria was observed by fluorescence microscopy; the size of each image is the same, bar: 20 μ m. **(C)** Fluorescence uptake data were measured 15 min after 9 PDR *A. baumannii* strains were mixed with HT. The experiment was repeated four times. ***/### $P < 0.001$; **/## $P < 0.01$.

anti-AbOMV serum reduced the MIC value of the antibiotic from 256 to 128 μ g/mL. Administration of the quinolone antibiotics ciprofloxacin and levofloxacin together with the anti-AbOMV antibodies reduced the MICs of these antibiotics from 32 to 8 μ g/mL and from 16 to 4 μ g/mL, respectively, which were four times lower than the MICs of the control serum group. The MIC value decreased to 512 μ g/mL after the amino acid antibiotic amikacin was combined with the anti-AbOMV antibodies, whereas the MIC of gentamicin still exceeded the highest detection value of 1024 μ g/mL. Similarly, the MICs of ampicillin and imipenem combined with the anti-AbOMV serum still exceeded the maximum detection value (Table 1). Combining the anti-AbOMV serum with 1/16 of the levofloxacin MIC could not reverse the antibiotic resistance of

the bacteria *in vitro* (Figure 3A). When the MIC concentration of levofloxacin was reached, both the anti-AbOMV and control serum combined with levofloxacin had a bactericidal effect (Figure 3B). When the amount of levofloxacin used was 1/4 of the MIC, combination with the anti-AbOMV serum significantly inhibited bacterial growth; even combination with the control antiserum showed some inhibitory effect, although only a small number of bacteria were killed (Figure 3C). When ciprofloxacin at a concentration of 1/4 of the MIC was used in combination with the anti-AbOMV serum, significant inhibition of bacterial growth was also observed (Figure 3D). These results showed that administration of the anti-AbOMV serum together with quinolone antibiotics could reduce the antibiotic resistance of *A. baumannii*. The reversal of levofloxacin resistance by the anti-AbOMV serum was evaluated in nine different PDR *A. baumannii* strains, and the results showed reduction of drug resistance for 77.78% (7/9) of the strains. Among them, the MIC values were reduced fourfold for five strains and twofold for two strains and did not change for two strains (Table 2). These results showed that the anti-AbOMV antibodies was able to significantly reduce the drug resistance of *A. baumannii in vitro*, especially to quinolone antibiotics.

TABLE 1 | Determination of MIC values for the Ab112 strain when anti-AbOMV serum was used together with different types of antibiotics.

Antibiotic	MIC (μ g/ml)		
	PBS	+Control serum	+Anti-AbOMVs
Ceftriaxone	256	256	128
Ciprofloxacin	32	32	8
Levofloxacin	16	16	4
Amikacin	> 1024	> 1024	512
Gentamicin	> 1024	> 1024	> 1024
Ampicillin	> 1024	> 1024	> 1024
Imipenem	> 1024	> 1024	> 1024

Anti-AbOMVs Increased Susceptibility of Drug-Resistant *A. baumannii* to Levofloxacin in the Mouse Sepsis Model

To generate the sepsis model, mice were immunized subcutaneously with AbOMVs three times and challenged

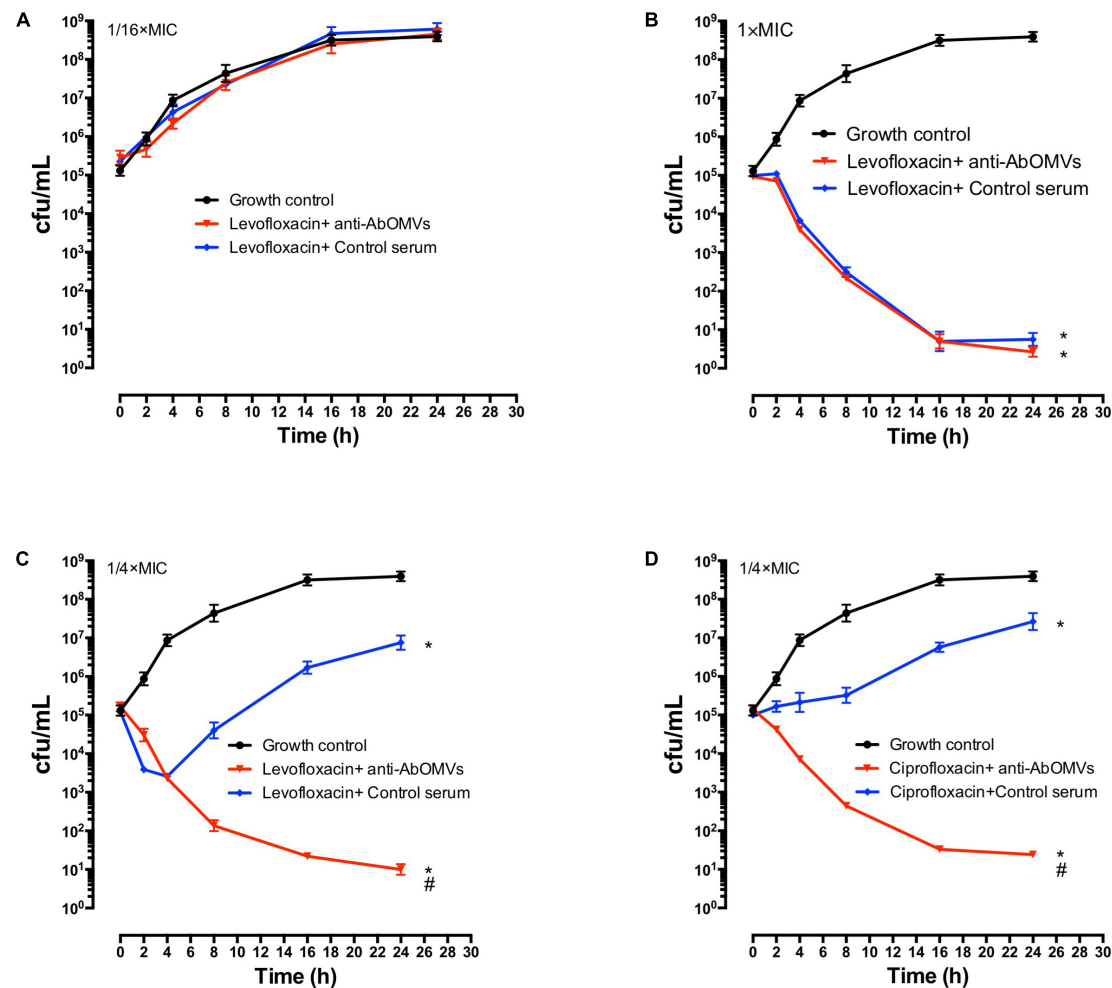


FIGURE 3 | Anti-AbOMV serum improved the sensitivity of PDR *A. baumannii* to antibiotics *in vitro*. Levofloxacin at concentrations of (A) 1/16 of the MIC, (B) 1x the MIC, and (C) 1/4 of the MIC was combined with anti-AbOMV or control serum for 24 h and then viable bacteria (CFU/mL) were counted. (D) Ciprofloxacin at a concentration of 1/4 of the MIC was combined with anti-AbOMV or control serum for 24 h and bacteria were counted (CFU/mL). *Indicates anti-AbOMV antiserum or control serum compared with the PBS control; #indicates anti-AbOMVs compared with the control serum group. The experiment was repeated three times. */# $P < 0.05$.

intraperitoneally with 10^8 CFU/mouse ($10 \times \text{LD}_{50}$) of strain Ab112 at 40 weeks, followed by administration of levofloxacin at a dose of 30 mg/kg 1 h after challenge. The drug was administered once every 12 h, and the treatment was continued for 3 days. The survival of the mice was observed continuously for 7 days (Figure 4A). The survival rate of the mice infected after immunization with the AbOMVs was significantly increased when they were treated with levofloxacin and remained at 83.33% until day 7 (Figure 4B). The lungs and spleens of the mice were harvested 12 h after bacterial challenge to examine the bacterial loads in their organs. The results showed that mice vaccinated with the AbOMV vaccine had a reduced bacterial load in their lungs and spleen; when levofloxacin was used together with the treatment, the bacterial loads in the lungs (Figure 4C) and spleen (Figure 4D) were significantly reduced compared with those of the vaccination alone group. Antisera were collected from actively immunized mice, and passive immunization combined

with antibiotics experiments was performed in the mice. The results showed that the combination of anti-AbOMV antiserum with levofloxacin significantly increased the survival rate of the infected mice compared with those of the groups treated with antibiotics and the anti-AbOMV antiserum alone (Figure 4E). The above experiments showed that immunization with both the AbOMV vaccine and anti-AbOMV serum combined with levofloxacin increased the susceptibility of drug-resistant *A. baumannii* to antibiotics.

Anti-AbOMV Antibodies Combined With Levofloxacin Significantly Reduced Lung Inflammation in the Mouse Pneumonia Model

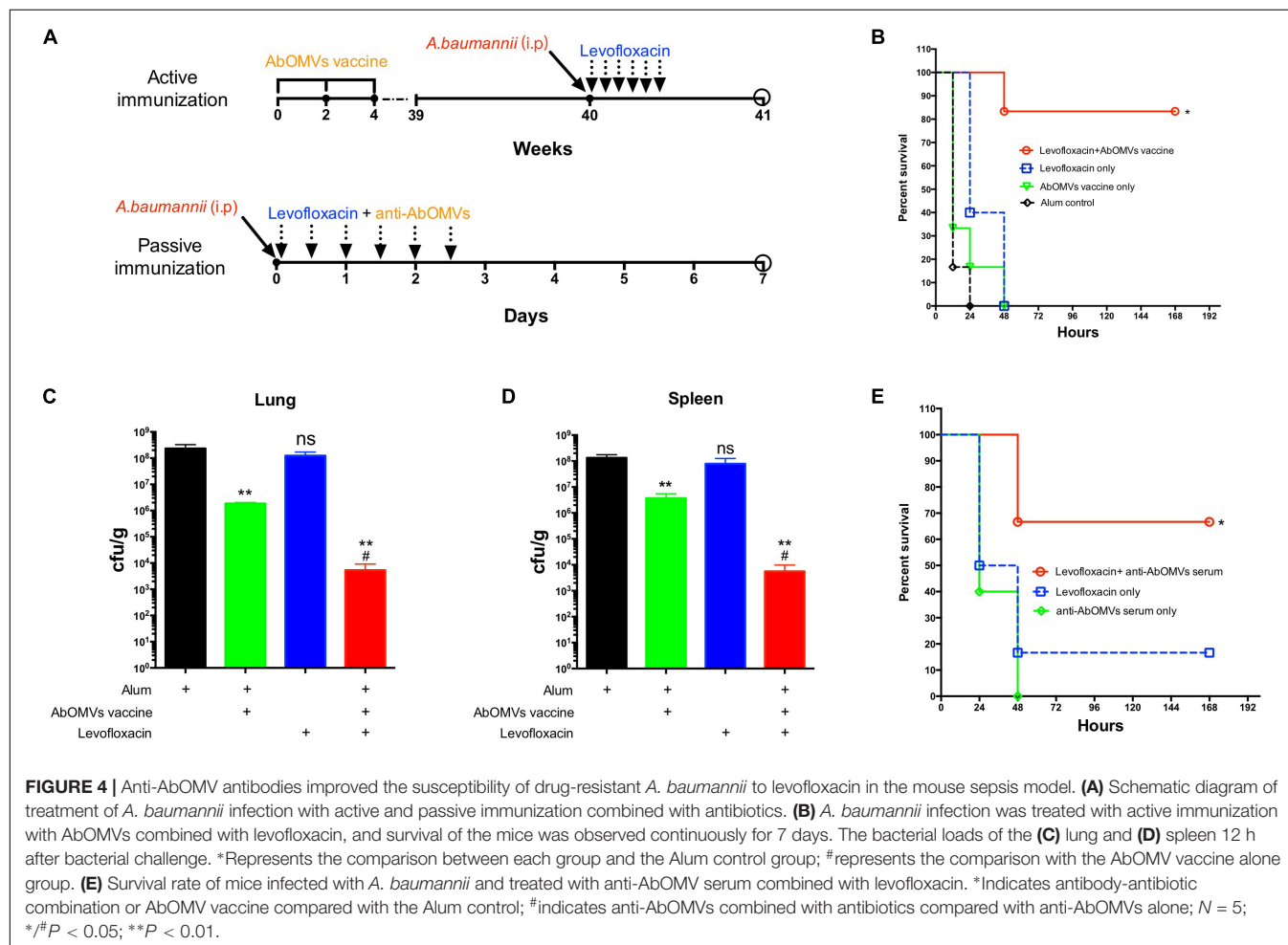
Since *A. baumannii* often causes lung infections in patients, the pneumonia model has greater significance for evaluation

TABLE 2 | Determination of MIC values of different strains when anti-AbOMV serum was used together with levofloxacin.

Strains	MIC ($\mu\text{g/ml}$)		
	+PBS	+Control serum	+Anti-AbOMVs serum
Ab752	32	32	16
Ab919	16	16	4
Ab128	4	4	1
Ab019	16	16	16
Ab1408	16	16	4
Ab395	32	32	16
Ab117	16	16	16
Ab932	32	32	8
Ab246	16	16	4
	% of bacteria to increase antibiotic sensitivity	0%	77.78%

of infection. Therefore, after completing the intraperitoneal infection model, we continued to evaluate the ability of the anti-AbOMV antiserum to increase antibiotic sensitivity in

the mouse pneumonia model. After intranasal infection with *A. baumannii*, the mice were given anti-AbOMV serum and levofloxacin using a passive immunization approach every 12 h (Figure 5A). The combination of anti-AbOMV antiserum with levofloxacin significantly increased the survival rate of the infected mice compared with those of the groups treated with antibiotics and the anti-AbOMV antiserum alone (Figure 5B). At 12 h post-infection, the anti-AbOMV antiserum combined with levofloxacin significantly reduced the bacterial loads in the mouse spleen (Figure 5C) and lung (Figure 5D) compared to those of the mice administered only antibiotics or the antibodies. The inflammatory cytokines IL-1 β (Figure 5E) and IL-6 (Figure 5F) were also significantly lower in the lung homogenate supernatant after anti-serum and antibiotic combination treatment. In addition, lung showed limited tissue damage with mild inflammatory cell infiltration in alveoli after anti-AbOMV serum and antibiotic combination treatment. In contrast, lung of levofloxacin and anti-AbOMV antibodies treatment alone showed more severe neutrophil and lymphocyte aggregation in the peri- and endo-bronchial spaces, and severe hemorrhage and inflammatory cell infiltration in alveoli (Figure 5G). The histology score suggested the tissue destruction and inflammation in the lungs. The results showed



that the combination of anti-AbOMV antiserum and levofloxacin significantly reduced the pathology of the lung compared with those obtained in the mice administered antibiotics or antibody treatment alone (**Figure 5H**). In summary, since the anti-AbOMV antibodies reverse the resistance of *A. baumannii* to levofloxacin, the antibody and levofloxacin combination shows a more effective bactericidal effect and thus effectively reduces inflammation in the lungs.

The Anti-AbOMV Antibodies Reversed Bacterial Resistance Mainly by Binding to Outer Membrane Porins

Western blotting analysis was performed to detect the main bacterial proteins that were immunoreactive with the anti-AbOMV antibodies. The results showed no immune reaction between normal mouse antiserum and *A. baumannii* cells. The anti-AbOMV antiserum had stronger immune reactions with 10 *A. baumannii* proteins. The corresponding AbOMV protein bands were analyzed by mass spectrometry, and these 10 proteins were found to generate a strong immune response (**Figure 6A**, left). Since these 10 proteins were the major immunogenic proteins, we investigated whether OMPCs or OMVs obtained using different preparation methods would have the same function. To this end, we carefully analyzed the protein compositions of OMVs derived from the standard ATCC19606 strain (19606-OMVs), OMPCs from Ab112 (Ab112-OMPCs), and OMVs derived from the clinical strain Ab112 (Ab112-OMVs) produced under different culture conditions by SDS-PAGE to explore the possibility of using other methods to prepare immunogens and improve antibiotic sensitivity. The results showed that the Ab112-OMPCs contained all of the major antigens, but the composition was more complex. Most of the major proteins on the 19606-OMVs were identical to those on the Ab112-OMVs. The Ab112-OMVs still contained most of the 10 immunogenic proteins after incubation at different temperatures and with different antibiotics and media, but the expression levels of these proteins changed (**Figure 6A**, right). These results suggested that the bacterial culture conditions had an effect on the protein compositions of OMVs. Therefore, the OMV preparation process must be stable.

Seven proteins had a porin OMP structure based on protein structure homology-modeling (**Figure 5B**), suggesting that these antibodies enhanced intracellular aggregation of antibiotics mainly by affecting porins. BLAST analysis of the amino acid sequence homology of these 10 proteins demonstrated that they had high sequence similarity in *A. baumannii*. After sequence homology analysis with approximately 2832 *A. baumannii* strains, we found homology with all strains with the following exceptions: 29.61% of the strains for Caro, 24.42% of the strains for the putative long-chain fatty acid transport protein, 13.25% of the strains for Omp25, and 3.79% of the strains for Omp22. The sequence conservation for these proteins in these strains was 81, 76, 70, and 70%, respectively. In most of the other strains, the homology for all 10 proteins was greater than 81%. In fact, most proteins had more than 96% homology among most strains (**Figure 6C**). Comparison to human proteins showed that OmpA

and OmpW had the highest similarity at 34.04 and 32.26%, respectively. No other OMPs had similar proteins.

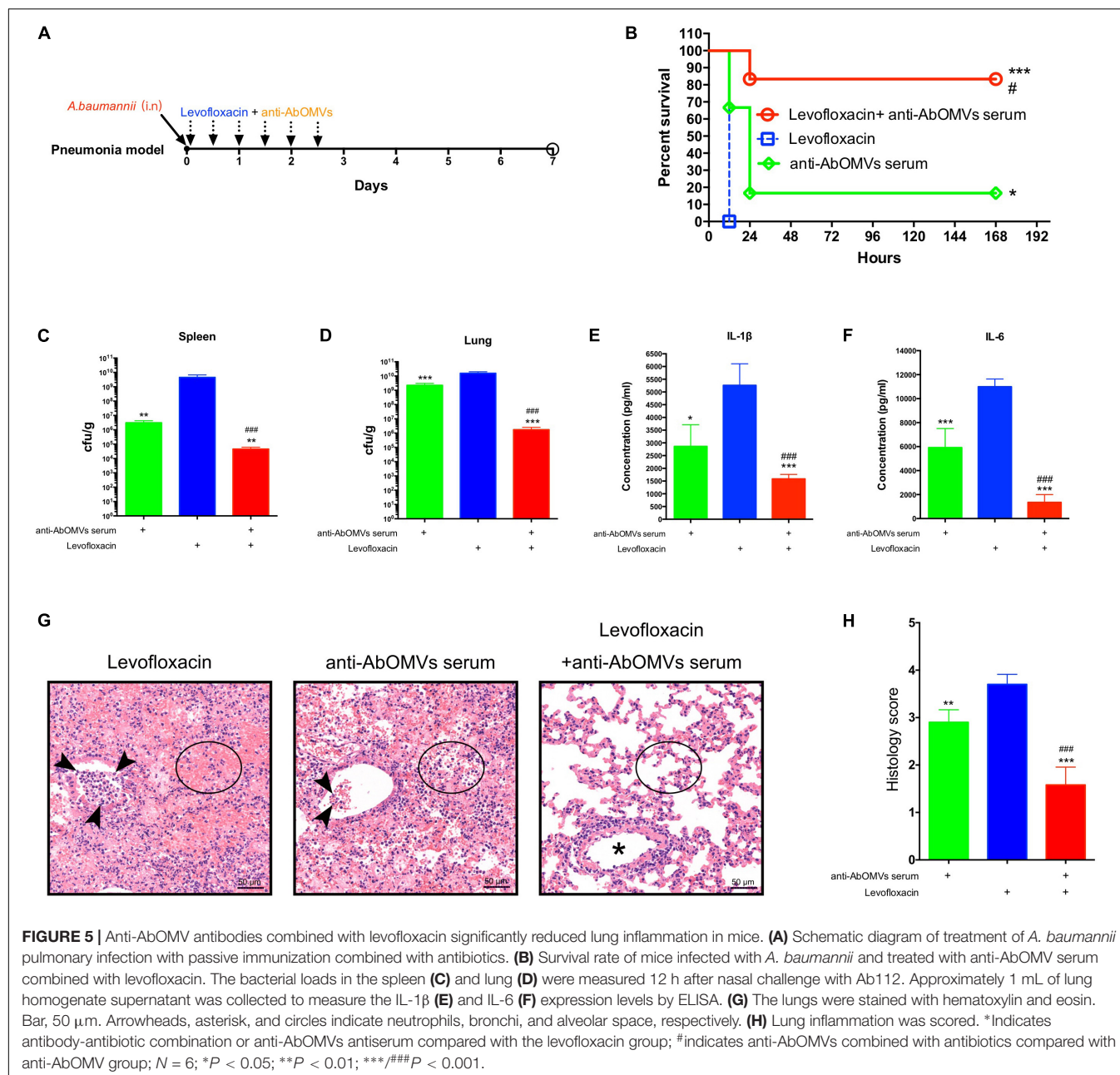
In summary, this result indicates that the anti-AbOMV antibodies are well conserved among all *A. baumannii* strains and has weak cross-reactivity with human proteins, which is conducive to follow-up studies of antibody drugs.

DISCUSSION

At present, increasing numbers of multi-drug-resistant bacteria are appearing in the clinic. These bacteria seriously threaten the lives of patients, but development of corresponding antibacterial drugs has progressed slowly. *Enterococcus faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *E. coli* (ESKAPE) are the most resistant bacteria. To combat these bacterial infections, therapy combining antibodies with antibiotics has been carried out in clinical trials (DiGiandomenico and Sellman, 2015). Therefore, the method of using antibodies to promote antibacterial effects warrants further study (Domenech et al., 2018).

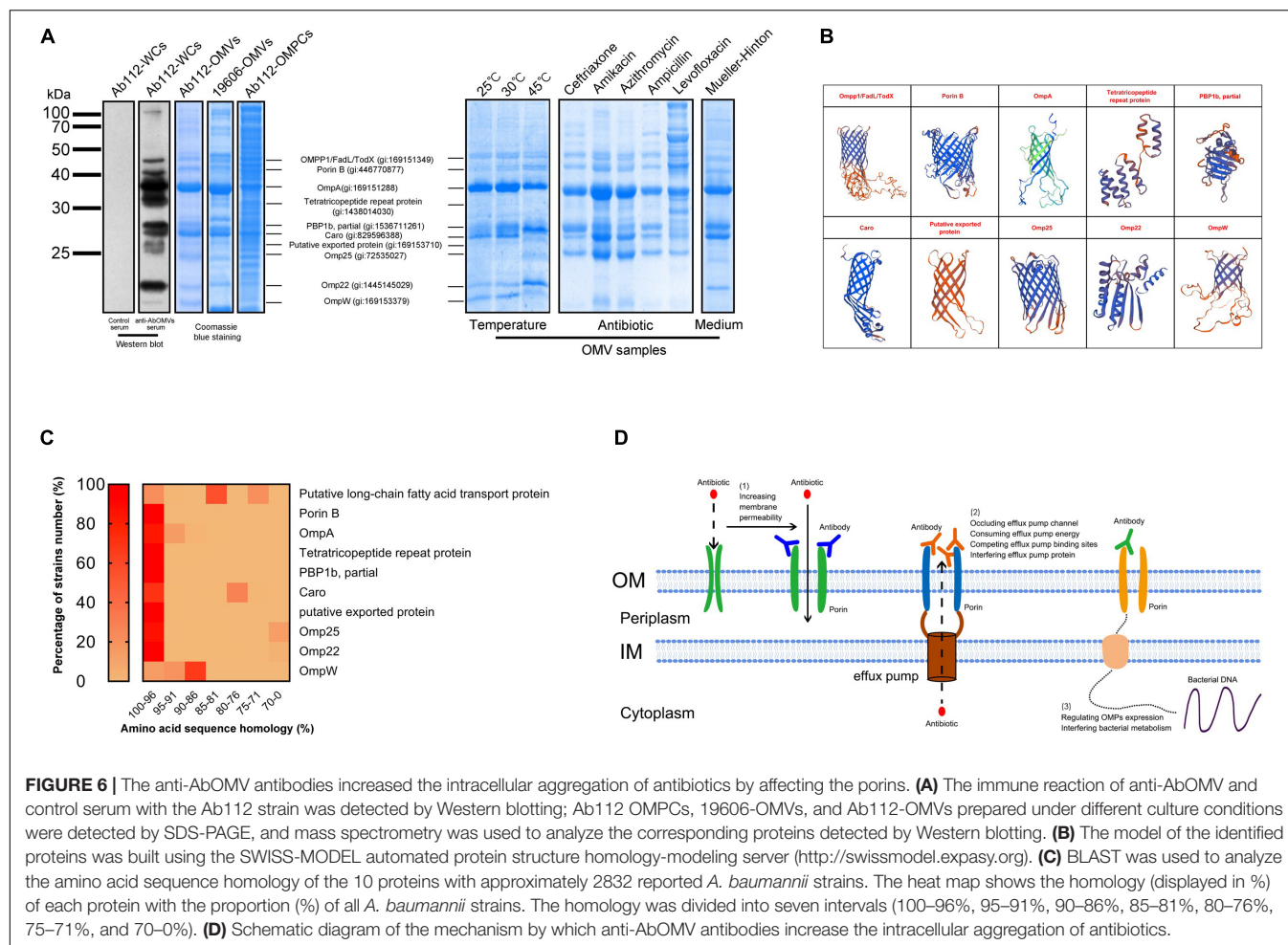
A. baumannii outer membrane vesicles can stimulate the body to generate an immune response and long-term immunological memory (Vidakovics et al., 2010; Romeu et al., 2014); therefore, AbOMVs can induce higher IgG levels and a prolonged response as a vaccine (**Figure 1C**). This study showed that the antibodies could bind to *A. baumannii* OMPs to reduce drug resistance, thereby demonstrating the extensive value of the AbOMV vaccine in preventing infection and reversing drug resistance. We used active immunization experiments with the AbOMV vaccine to demonstrate its ability to induce antibody production and interfere with *A. baumannii* drug resistance (**Figure 4B**). We also demonstrated that exogenous anti-AbOMV antibodies could increase susceptibility to levofloxacin *in vivo* using passive immunization experiments (**Figures 4D, 5**). In addition, the AbOMV antibodies reduced the MICs of quinolone antibiotics by fourfold *in vitro* (**Figures 3C,D**). These results provide a basis for the combined use of antibodies-antibiotics to prevent and treat PDR *A. baumannii* infections. Since our previous study showed that the anti-AbOMV antibodies delivered via both active and passive immunization could inhibit *A. baumannii* infection at the LD50 challenge dose (Huang et al., 2014), the mice in this study were challenged at a dose of 10× the LD50 to highlight the bactericidal effect of the combined use of antibiotics and antibodies *in vivo*. Challenging mice with large doses of *A. baumannii* will kill a large number of immune cells, thereby affecting the bactericidal effect of the AbOMV vaccine when administered alone. However, combined use of antibodies with antibiotics can rapidly kill bacteria, thereby increasing the survival rate of the mice.

Drug-inactivating enzyme-mediated bacterial resistance is very serious and difficult to reverse. For example, oxacillinase, cephalosporinase (AmpC), extended-spectrum β -lactamase (ESBL), metallo- β -lactamase (MBL), glycoside-modifying enzymes, and 16S ribosomal RNA transferase ArmA expression allows *A. baumannii* to acquire resistance to different types of antibiotics (Potron et al., 2015). Bacterial strains with coexistence



of these enzymes and other resistance mechanisms exhibit a higher level of resistance. Differences in antibiotic resistance also largely depend on penetration of antibiotics into the cell, and bacteria control the concentrations of intracellular antibiotics through a synergistic relationship between active efflux and outer membrane penetration (Krishnamoorthy et al., 2017). When the external factors affect the OMPs, the membrane permeability is increased or the efflux effect is blocked. Then, the external antibiotic molecules can enter the cell in large quantities, and the antibiotic absorption rate is elevated, which to a certain extent offsets the impact of the efflux effect and the action of drug-inactivating enzymes on antibiotics. Simultaneously, the bacterial intracellular enzymes will leak

after the membrane permeability is changed, thereby reducing the concentrations of intracellular drug-inactivating enzymes and enzyme-mediated drug resistance. Similarly, the use of efflux pump inhibitors can effectively reduce drug resistance to quinolone antibiotics (Bhattacharyya et al., 2017). **Table 1** shows the effect of the anti-AbOMV antiserum on different types of antibiotics. Our goal was to find the best antibiotic type for combination with the anti-AbOMV antiserum by screening. In this study, the combination of the anti-AbOMV antibodies with quinolone antibiotics (ciprofloxacin and levofloxacin) showed the best combined efficacy. This results may have occurred because resistance of *A. baumannii* to quinolone antibiotics is mainly caused by changes in membrane



permeability and effects on active drug efflux. Therefore, a large increase in the intracellular accumulation of antibiotics will have a significant effect on reversing the resistance to these antibiotics. Ampicillin, ceftriaxone, and imipenem are examples of penicillins, cephalosporins and carbapenems, respectively, which are β -lactam antibiotics. *A. baumannii* resistance to β -lactam antibiotics is mainly mediated by β -lactamase and includes a reduction in outer membrane permeability, efflux pump activation and changes in penicillin-binding protein expression. The severe resistance of *A. baumannii* to ampicillin and imipenem may be the result of MDR mechanisms; thus, anti-AbOMV antiserum can increase intracellular accumulation of the drug but does not effectively reverse the resistance of *A. baumannii*. Additionally, the resistance mechanisms of bacteria for aminoglycoside antibiotics (amikacin and gentamicin) include production of aminoglycoside-modifying enzymes (AMEs) and 16S rRNA methylase and decreased outer membrane permeability and drug efflux. Among these mechanisms, AMEs and the 16S rRNA methylase are most important. The AMEs modify the amino and hydroxyl groups on the aminoglycoside antibiotic side chain, and the 16S rRNA methylase methylates the target of the aminoglycoside antibiotic. Both mechanisms can effectively block binding of

the aminoglycoside antibiotics to the target, resulting in a loss of antibacterial activity of the antibiotics. The combination of antibiotics and the anti-AbOMV antibodies can increase the intracellular antibiotic concentration, which must counteract the effect of the AMEs and the 16S rRNA methylase (especially ArmA) to prevent antibiotic binding (Liou et al., 2006). If the drug target is highly modified, it may not be able to exert effective bactericidal activity even if a large amount of antibiotics are taken up by bacteria, thus limiting the ability of the anti-AbOMV antiserum to reverse aminoglycoside antibiotic resistance (Doi et al., 2007; Yu et al., 2007). The emergence of strong drug resistance is not the result of a single drug resistance mechanism but often is the result of a combination of MDR mechanisms. These drug resistance mechanisms synergistically cause strong drug resistance in bacteria. Therefore, for strains with strong drug resistance, overcoming only one drug resistance mechanism (i.e., increasing intracellular drug aggregation) will not be sufficient to reverse the drug resistance, which is why the effect of the anti-AbOMV antiserum is limited. In the follow-up study, we will continue to optimize the “antibody-antibiotic” combination strategy and carefully explore the relationship between anti-AbOMV antibodies and antibiotic resistance mechanisms to design more effective “antibody-antibiotic”

drugs. Consequently, when designing antimicrobial drugs, consideration of other bacterial resistance mechanisms is purposefully included. At present, prevention and treatment methods for PDR *A. baumannii* include nanoparticle-mediated antibiotic delivery, combined use of antibiotics and antibiotics, combined use of efflux pump inhibitors and antibiotics, and combined use of antimicrobial peptides and antibiotics. However, the combined use of antibodies and antibiotics for the prevention and treatment of *A. baumannii* has not been reported. Therefore, identifying antigen targets that can reverse drug resistance is an important goal.

The bacterial culture temperature, media, and addition of antibiotics can affect the OMV yield and protein composition (Kulp and Kuehn, 2010; McConnell et al., 2011; Yun et al., 2018). The Ab112-OMV preparation method used in this study cultivated Ab112 in LB medium without antibiotics at 37°C. However, whether OMVs collected under other culture conditions would have the same function was not clear. We prepared AbOMVs by changing the bacterial culture conditions (temperature, antibiotics, and media) and examined the differences between OMV protein components before and after these changes. The results showed that some OMPs expression levels on the OMVs were changed in the different preparation (Figure 6A, right). Therefore, we recommend using a standard procedure for OMV preparation. Antibiotic stimulation can increase the OMV yield, which will help improve the preparation efficiency. Therefore, to explore the optimal AbOMV production scheme and more broadly discuss this function of AbOMVs, we cultured Ab112 with five different antibiotics at sub-MIC doses. The harvested OMVs showed some changes in protein bands compared to those of AbOMVs prepared without antibiotics. Although the protein content changed, most proteins were still present on the OMVs. Therefore, we believe that separately analyzing the “antibiotic-OMVs” of interest is necessary to determine whether OMVs prepared under different drug stimulation also have the ability to improve antibiotic susceptibility *in vitro* and *in vivo*.

Differences in OMP expression levels exist between susceptible and resistant *A. baumannii* strains. The membrane proteins associated with antibiotic efflux are often highly expressed in drug-resistant bacteria, but the pore protein associated with antibiotic uptake is often expressed at a low level. Therefore, using AbOMVs secreted by drug-resistant bacteria to prepare antibodies could induce the production of more specific antibodies according to the resistance mechanism of the drug-resistant bacteria. In this study, we used OMVs secreted by PDR bacteria to induce antibodies and reverse bacterial resistance. To explore this function of bacterial OMVs more widely, we also wanted to know whether OMVs from the standard strains had this function. To address this question, we prepared OMVs from the standard strain ATCC19606 (19606-OMVs) and compared the protein components with AbOMVs from clinical strain Ab112. The results showed that the 19606-OMV protein composition was identical to most of the proteins on the AbOMVs (Figure 6A). Therefore, we believe that the 19606-OMVs can perform the same function as the Ab112-OMVs. However, clearly determining whether OMVs from the

standard strains have the ability to reverse bacterial resistance also requires more experiments. Furthermore, we were interested in whether OMPCs had the same function as the AbOMVs. Therefore, we extracted OMPCs from the Ab112 strain and compared protein bands with those of the Ab112-OMVs using SDS-PAGE. The results showed that the protein components of the OMPCs were very complex, but the main protein bands in the Ab112-OMVs were all present in the OMPCs (Figure 6A). Therefore, we believe that the OMPCs can induce the body to produce more antibody components, including these 10 major antibodies. However, because the advantages of these 10 proteins may be attenuated by a large number of heteroproteins in the OMPCs, we are not sure of their ability to induce these major antibodies. Regardless, we believe that the protein components of OMVs are simpler than those of OMPCs and that the induced antibodies are more targeted.

In this study, a preliminary analysis of the main protein targets involved in the interaction between the antibodies and bacteria was performed starting with the antibodies targeting the drug-resistant AbOMVs. The 10 main antigen targets we identified were very likely to be the key targets affecting the level of outer membrane permeability or efflux pump activity. The rough structural simulation showed that seven proteins were porins and three were non-porins (Figure 6B). A sequence conservation comparison of these OMPs with known databases demonstrated that these 10 proteins were highly conserved in *A. baumannii* (Figure 6C). Therefore, the anti-AbOMV antibodies generated by this method had extensive cross-reactivity in *A. baumannii*. However, because these proteins exhibit low similarity to human proteins, autoantibody-associated immune disease will not occur during combined use of antibodies and antibiotics. In addition, although our current study was only carried out with *A. baumannii*, we believe that the strategy by which antibodies affect bacterial resistance can be extended to other drug-resistant bacteria, such as drug-resistant *P. aeruginosa*, *K. pneumoniae*, *E. coli* and other ESKAPE pathogens.

The OMPs identified and obtained in this study had a direct association with antibiotic resistance. For *A. baumannii*, the pore-forming activity of the major pore protein OmpA is very low (Iyer et al., 2018). OmpA has been confirmed to be a target of the vaccine against *A. baumannii* (Zhang et al., 2016) and is one of the most important virulence factors of this bacterium (Lee et al., 2010). Furthermore, deletion of the OmpA protein in *A. baumannii* increases susceptibility to antibiotics (Kwon et al., 2017). When the OmpA gene is damaged, the antibiotic susceptibility to chloramphenicol, nalidixic acid, and aztreonam is increased, indicating that OmpA is also involved in drug efflux (Smani et al., 2014). OmpA is both osmotically competent and involved in antibiotic efflux, and therefore the function of OmpA is not singular. These results indicate that OmpA is an important target for the development of new antimicrobial agents and vaccines, but whether anti-OmpA antibodies can reverse bacterial resistance has not been reported. Caro is directly associated with uptake of carbapenem antibiotics and often shows low expression in drug-resistant bacteria (Mussi et al., 2005), but its other functions are not clear. Porin B is an OprB-like protein.

The efflux pump BpEAB-OprB system of *Burkholderia* is not associated with resistance to aminoglycosides but can mediate drug resistance to fluoroquinolones, lincosamides, macrolides, and tetracyclines (Mima and Schweizer, 2010). Omp25 is a major porin, and its protein expression level correlates with drug resistance to antibiotics (Rumbo et al., 2013). OmpW is an effective antigen target against *A. baumannii* (Huang et al., 2015). One study reported under-expression of the protein in carbapenem-resistant strains (Tiwari et al., 2012); however, other studies showed that the OmpW expression level in carbapenem-resistant *A. baumannii* was higher than that of the standard strain, indicating that OmpW might also be involved in drug efflux. Therefore, the function of OmpW in antibiotic resistance in *A. baumannii* is not clear. Omp22 may also be an effective vaccine target (Huang et al., 2016b). The 22.5-kDa protein is associated with resistance to antibiotics (Fernandez-Cuenca et al., 2003), but the other functions of this protein are not clear. Taken together, the OMPs identified in this study are associated with drug resistance in *A. baumannii*, and their binding to anti-AbOMV antibodies has the potential to affect their functions and reverse drug resistance.

Efflux pump antibodies in combination with antibiotics improve the sensitivity of drug-resistant bacteria to antibiotics (Al-Hamad et al., 2011). However, when an important OMP is affected by antibodies, bacteria will regulate the expression of other proteins on the outer membrane to form a drug resistance-associated OMP interaction network and enable survival (Wu et al., 2016). Therefore, these proteins work in concert to influence bacterial membrane permeability and efflux, and achieving a good result with a single antibody acting on a single target may be difficult. Anti-AbOMV antibodies are complex antibody components against multiple major porins and have more advantages than a single antibody. However, these anti-AbOMV antibodies also have the possibility of inducing useless antibodies or counteracting antibody components. One of the proteins identified in this study was penicillin-binding protein 1b (PBP1b), which has dual *trans*-transferase and transpeptidase activities and is involved in bacterial peptidoglycan synthesis (Bertsche et al., 2005). PBP1b plays an important role in maintaining cell morphology. Loss of PBP1b impairs bacterial biofilm formation and motility (Kumar et al., 2012; King et al., 2017). Penicillin and ampicillin have high affinity for PBP1b, and binding of these antibiotics interferes with bacterial peptidoglycan synthesis, resulting in bacterial death (Reinert, 2009). The resistance of *A. baumannii* to carbapenem antibiotics is largely due to changes in the PBP protein. When PBP changes, antibacterial drugs do not recognize the bacteria, and the bacteria can successfully escape antibiotic-induced killing (Vila and Marco, 2002; Fernandez-Cuenca et al., 2003; Cayo et al., 2011). In this study, we found that PBP1b was included on the OMVs is a truncated form. This truncated PBP1b was discovered by a genome-wide association study (GWAS) and RNA-seq analysis in December 2018 (gi: 15371121261), but the article has not been published. This protein may represent a cryptic mechanism for drug-resistant *A. baumannii*. We first discovered this truncated PBP1b (aa400-667) on OMVs of drug-resistant *A. baumannii*;

the sequence represented the region from amino acids 400 to 667 of the full-length sequence, for a total of 268 amino acids. The protein molecular weight is 28 kDa, which is exactly the same as our SDS-PAGE result. This truncated form of PBP1b is not present in the ATCC 19606 standard strain (**Figure 6A**), which demonstrates that the truncated protein is unique to drug-resistant strains and may be the product of full-length PBP1b. More interestingly, (1) as the culture temperature increases, the expression level of this truncated PBP1b on OMVs significantly increases. (2) β -Lactam antibiotics, such as ceftriaxone and ampicillin, result in high expression of this truncated PBP1b on OMVs, whereas other types of antibiotics do not affect its expression. This finding may represent an undiscovered, novel and important drug resistance mechanism by which *A. baumannii* escapes β -lactam antibiotics through actively truncating PBP1b under antibiotic pressure. (3) The expression of truncated PBP1b also increases in MH culture medium, which may be related to the ionic strength of different media (Jira et al., 2018). At present, very few studies have investigated the function of PBP1b in *A. baumannii* strains. We cannot clearly explain the function and significance of this truncated PBP1b in bacterial survival, but we believe that this truncated PBP1b is related to bacterial resistance, which warrants in-depth research. In summary, identification of the individual components in anti-AbOMV antibodies has great importance and will aid in the discovery of new bacterial drug resistance mechanisms and the identification of antibody combinations that can effectively reverse bacterial resistance, which is the goal of our future analyses.

Acinetobacter baumannii develops drug resistance by reducing the intracellular accumulation of antibiotics, and thus the resistant strain Ab112 shows less HT fluorescence. The control serum was shown to promote HT aggregation, because serum may contain efflux pump inhibitors and complement the effect of bacterial membrane permeability and the efflux pump levels (Ramos-Sevillano et al., 2012; Blanchard et al., 2014). In contrast, the anti-AbOMV serum interacts with porins associated with drug resistance and has been shown to significantly increase intracellular HT aggregation. Although we do not have a good explanation for the bactericidal effect of the combined use of AbOMV antibodies and antibiotics, we believe that they have many properties that can directly or indirectly affect the microorganism, and we speculate that the mechanisms by which anti-AbOMV antibodies promote the intracellular aggregation of antibiotics are as follows (**Figure 6D**). (1) Anti-AbOMV antibodies affect the stability of the outer membrane and increase its permeability, thereby increasing entry of antibiotics into the cell. (2) A large number of anti-AbOMV antibodies bind to efflux-associated channel proteins, thereby occluding the efflux pump channel, consuming efflux pump energy, competing for the efflux binding sites of antibiotics, or interfering with efflux pump protein components and thus reducing the efflux of antibiotics through this channel (Imamura et al., 2005; Al-Hamad et al., 2011). (3) Anti-AbOMV antibodies regulate the expression levels of bacterial OMPs or interfere with bacterial metabolism

(McClelland et al., 2010). Moreover, with the help of antibiotics, vaccines or antibodies can improve AbOMV antibody-mediated opsonophagocytosis and promote the bactericidal effects of active and passive immunity (Ramos-Sevillano et al., 2012).

In summary, the bactericidal effect of the combined use of antibodies and antibiotics is mutually reinforcing and will be a promising technological platform for combating drug-resistant *A. baumannii* infections. In follow-up study, we will continue to study the mechanisms of anti-AbOMV antibodies. First, we will analyze the effects of each individual antibody or antibody combination on bacterial membrane permeability and the efflux pump level, and then RNA-seq will be used to analyze the effects of antibodies on bacterial resistance-related signaling pathways. Thus, the mechanism of anti-AbOMV antibodies in bacterial resistance will be elucidated step by step. We believe that identification of a key target or signaling pathway that regulates bacterial resistance will be of great value for the development of an “antibody-antibiotic” combination drug against PDR *A. baumannii*.

CONCLUSION

This study suggested that binding of anti-AbOMV antibodies to the OMPs of drug-resistant *A. baumannii* could increase the intracellular accumulation of antibiotics, thereby reversing the drug resistance of PDR *A. baumannii* and increasing its susceptibility to antibiotics.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript.

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ETHICS STATEMENT

The animal experimental procedures were approved by the Ethics Committee of Animal Care and Welfare, Institute of Medical Biology, CAMS (Permit Number: SYXK (dian) 2010-0007), in accordance with the animal ethics guidelines of the Chinese National Health and Medical Research Council (NHMRC) and the Office of the Laboratory Animal Management of Yunnan Province, China. All efforts were made to minimize animal suffering. All participants submitted a signed informed consent form to participate in the study. The protocol complied with the Helsinki Declaration and was approved by the Institutional Review Boards of the Institute of Medical Biology, Chinese Academy of Medical Sciences and Peking Union Medical College.

AUTHOR CONTRIBUTIONS

WH and QZ designed the topics, experiments, and charts, performed the statistical analysis, and wrote the manuscript. WL and YC assisted with the supplemental experiments and data analysis. CS, QL, and JZ bred the mice, collected the strains, and performed the electron microscopy techniques. CY, HB, WS, and XY provided the experimental support. YM provided the financial support for the study and proposed the amendments.

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Gene Targets in Ocular Pathogenic *Escherichia coli* for Mitigation of Biofilm Formation to Overcome Antibiotic Resistance

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The present work is an attempt to establish the functionality of genes involved in biofilm formation and antibiotic resistance in an ocular strain of *Escherichia coli* (L-1216/2010) which was isolated and characterized from the Vitreous fluid of a patient with Endophthalmitis. For this purpose, seven separate gene-specific knockout mutants were generated by homologous recombination in ocular *E. coli*. The genes that were mutated included three transmembrane genes *ytfR* (ABC transporter ATP-binding protein), *mdtO* (multidrug efflux system) and *tolA* (inner membrane protein), *ryfA* coding for non-coding RNA and three metabolic genes *mhpA* (3-hydroxyphenylpropionate 1,2-dioxygenase), *mhpB* (2,3-di hydroxyphenylpropionate 1,2-dioxygenase), and *bdcR* (regulatory gene of *bdcA*). Mutants were validated by sequencing and Reverse transcription-PCR and monitored for biofilm formation by XTT method and confocal microscopy. The antibiotic susceptibility of the mutants was also ascertained. The results indicated that biofilm formation was inhibited in five mutants ($\Delta bdcR$, $\Delta mhpA$, $\Delta mhpB$, $\Delta ryfA$, and $\Delta tolA$) and the thickness of biofilm reduced from 17.2 μm in the wildtype to 1.5 to 4.8 μm in the mutants. Mutants $\Delta ytfR$ and $\Delta mdtO$ retained the potential to form biofilm. Complementation of the mutants with the wild type gene restored biofilm formation potential in all mutants except in $\Delta mhpB$. The 5 mutants which lost their ability to form biofilm ($\Delta bdcR$, $\Delta mhpA$, $\Delta mhpB$, $\Delta tolA$, and $\Delta ryfA$) did not exhibit any change in their susceptibility to Ceftazidime, Cefuroxime, Ciprofloxacin, Gentamicin, Cefotaxime, Sulfamethoxazole, Imipenem, Erythromycin, and Streptomycin in the planktonic phase compared to wild type ocular *E. coli*. But $\Delta mdtO$ was the only mutant with altered MIC to Sulfamethoxazole, Imipenem, Erythromycin, and Streptomycin both in the planktonic and biofilm phase. This is the first report demonstrating the involvement of the metabolic genes *mhpA* and *mhpB* and *bdcR* (regulatory gene of *bdcA*) in biofilm formation in ocular *E. coli*. In addition we provide evidence that *tolA* and *ryfA* are required for biofilm formation while *ytfR* and *mdtO* are not required. Mitigation of biofilm formation to overcome antibiotic resistance could be achieved by targeting the genes *bdcR*, *mhpA*, *mhpB*, *ryfA*, and *tolA*.

Keywords: antimicrobial resistance, biofilm, ocular, *E. coli*, endophthalmitis, mutation

INTRODUCTION

Antimicrobial resistance (AMR) refers to the ability of microorganisms (bacteria, fungi, viruses, and parasites) to resist the inhibitory effect of an antimicrobial agent at the minimum inhibitory concentration (MIC) of the antimicrobial agent. AMR is a global phenomenon and bacteria, fungi and viruses are known to exhibit AMR. Various factors intrinsic to the microbe are also known to facilitate AMR such as their ability to inactivate the antimicrobial agent, to prevent its entry, to activate its efflux, to modify the antibiotic or the target, acquire resistance genes, and the ability to form a biofilm. Over the years with the development of the crystal violet method, XTT method, confocal microscopy and scanning electron microscopy it has been possible to monitor the dynamics of biofilm formation, and evaluate simultaneously the extent of AMR. These studies have clearly established that biofilm confers AMR to microorganisms (Hoyle et al., 1992; Elder et al., 1995; Ranjith et al., 2017). Several ocular pathogens like *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *S. epidermidis*, *Micrococcus luteus*, *Serratia marcescens*, *Neisseria* spp., *Moraxella* spp., *Bacillus* spp., *E. coli*, *Proteus mirabilis*, *Enterobacter agglomerans*, and *Klebsiella* spp., also exhibited the potential to form biofilms and were resistant to antibiotics (Katiyar et al., 2012). Recently we demonstrated that ocular *E. coli* from patients with conjunctivitis, keratitis or endophthalmitis were resistant to one or more of the antibiotics tested and majority of the isolates were positive for biofilm formation. *E. coli* L-1216/2010, was the best biofilm forming ocular isolate, and in the biofilm phase it was 100 times more resistant to the 8 antibiotics tested compared to planktonic phase (Ranjith et al., 2017). In the same study it was also demonstrated by DNA microarray analysis that ocular *E. coli* L-1216/2010 in the biofilm phase over-expressed several genes (Ranjith et al., 2017). But, comparison of the expression pattern of specific genes in ocular *E. coli* L-1216/2010 and in pathogenic *E. coli* ABU strain 83972 (Hancock and Klemm, 2007) and in *E. coli* UPEC strain CFT073 (Schembri et al., 2003) were distinctly different though both are pathogens. For instance, *fim* genes coding for type 1 fimbriae, genes coding for the LPS lipid A moiety (*waaB*, *waaP*, *waaJ*, and *waaR*), *yhjN* coding for cellulose synthase regulator protein, were up regulated in ocular *E. coli* L-1216/2010 but not in *E. coli* ABU strain 83972 (Hancock and Klemm, 2007) and in *E. coli* UPEC strain CFT073 (Schembri et al., 2003). Further the 22 genes (c2418 to c2440) that constitute the pathogenicity island and *rfaH* a virulence regulator, which regulates expression of several virulent genes in *E. coli* were up regulated during biofilm formation in several pathogenic strains but not in ocular *E. coli* L-1216/2010 (Schembri et al., 2003; Ranjith et al., 2017). It was also observed that ocular *E. coli* in biofilm phase did not exhibit up regulation of the stress response encoding genes (such as *cspG*, *cspH*, *pphA*, *ibpA*, *ibp*, *soxS*, *hha*, and *yfiD*) as observed in ABU strains of *E. coli*. These results indicate differences between the ocular and the non-ocular pathogenic *E. coli* in expression of genes in the biofilm phase.

In the present study a few genes viz., *mhpA* [coding for 3-(3-hydroxyphenyl) propanoate hydroxylase], *mhpB* (coding

for 2,3-dihydroxyphenylpropionate 1,2-), *ryfA* (coding for non-coding RNA), *tolA* (coding for the inner membrane protein -cell envelope integrity), *bdcR* (coding for the regulatory gene of *bdcA*), *ytfR* (coding for ABC transporter ATP-binding protein), and *mdtO* (coding for multidrug efflux system component) known to be up regulated in expression during biofilm formation in ocular *E. coli* L-1216/2010 (Ranjith et al., 2017) were mutated by homologous recombination based on the procedure of Datsenko and Wanner (2000) to validate the function of the above genes in biofilm formation in ocular pathogenic *E. coli*. The choice of the seven genes for gene specific mutation was based on the following criteria:

(a) genes known to be required for biofilm formation like *ryfA* (Bak et al., 2015) in *E. coli* and *tolA* in *P. aeruginosa* (Whiteley et al., 2001) and *E. coli* (Vianney et al., 2005) and (b) genes up regulated in expression during biofilm phase like *bdcR*, *mdtO*, *mhpA*, *mhpB*, and *ytfR* (Ranjith et al., 2017) but whose specific role in biofilm formation has not yet been demonstrated.

Such studies would help to identify genes involved in biofilm formation in ocular *E. coli* directly, establish whether genes known to be involved in biofilm formation in other organisms have a similar function in ocular *E. coli* and may also establish if there is a relationship between biofilm formation and resistance to antimicrobial agents and vice versa.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Ocular *E. coli* L-1216/2010 which was isolated and characterized from the Vitreous fluid of a patient with Endophthalmitis and resistant to ciprofloxacin and positive for biofilm formation was used in the present study (Ranjith et al., 2017). The bacterium was cultured in LB medium at 37°C. Several mutant strains of this bacterium (Table 1) were generated by homologous recombination (see below). Growth was monitored spectrophotometrically at 600 nm for 24 h.

Biofilm Detection by XTT Method

Culture (200 µl of 1:10 dilution of 0.5 McF culture) was added to a 96 well plate and incubated for 72 h at 37°C. XTT [2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide] (50 µl of 1 mg/ml) (Sigma, United States) and 4.2 µl (0.5 mM) Menadione and 145.8 µl of 1X PBS was added to each well and incubated in the dark for 3 h and quantified using a spectrophotometer (SpectroMax M3, Molecular Devices, CA, United States) at 490 nm. Wells without culture served as the control and the control OD at 490 nm was <0.12.

Visualization of Biofilm by Confocal Laser Scanning Microscopy (CLSM)

Escherichia coli L-1216/2010 and the mutants were cultured on µ- chamber slide 8 well (ibidi, GmbH, Germany) for 72 h as above and the biofilms were rinsed twice gently with autoclaved water and fixed with 4% formaldehyde (Himedia-Secunderabad, India) for 45 min. Fixed biofilms were then washed twice as above

TABLE 1 | Bacterial strains of *E. coli* L-1216/2010, plasmids and PCR primers used in the study.

Plasmids and isolates used in this study		
pKD3	Amp ^r , Chlm ^r	CDFD*
pKD46	Amp ^r	CDFD*
pET28a	Amp ^r	UoH [#]
<i>E. coli</i> DH5α		LVPEI ^{\$}
Ocular <i>E. coli</i>	Biofilm forming ocular <i>E. coli</i> isolate L-1216/2010	This study
Primers for generating mutants*		Amplicon size (bp)
<i>ΔmhpA</i>	F-ATGGCAATACAACACCCTGACATCCAGCCTGCGTGTAGGCTGGA GCTGCTTCGAAGTTCC R-TCAGGCTACCTTTTCGACAGAAACGTCGGCATCAGGCATATGAAT ATCCTCCTTAGTTCC	1082
<i>ΔmhpB</i>	F- ATGCACGCTTATCTTCACTGTCTTTCCACTCGCCGGTGTAGGCT GGAGCTGCTTCGAAGTTCC R- TCAGTTCTCTGTTCTGGCGCTTAACGAGCCAAATCCCATATGAAT ATCCTCCTTAGTTCC	1086
<i>ΔbdcR</i>	F- ATGGTCACTAAAAACAATCTCGCGTTCCAGGTCGTCCTCCGTGA GGCTGGAGCTGCTTCGAAGTTCC R- TCACTCCTCAAGAATAGTTTTATCGCTTCCCCCGCCATATGAAT ATCCTCCTTAGTTCC	1089
<i>ΔtolA</i>	F- GTGTCAAAGGCAACCGAACAACGACAAGCTCAAGCGGGTGT AGGCTGGAGCTGCTTCGAAGTTCC R- TTACGGTTTGAAGTCCAATGGCGGTTTTGAACACCATATGAAT ATCCTCCTTAGTTCC	1089
<i>ΔrfyA</i>	F- GCGGCCCTTTCCGCCGTCTCGCAAACGGCGCGTGTAGGCTGGA GCTGCTTCGAAGTTCC R- AAAATATGCCGCCCAAAGGGCGGCATTCCGGCCATATGAATATC CTCCTTAGTTCC	1078
<i>ΔmdtO</i>	F- ATGAGCGCGCTCAACTCCCTGCCATTACCGGTGGTGTGTAGGC TGGAGCTGCTTCGAAGTTCC R-TCATTGCGTGGCTCCTTGTGCTGTTCCGTGGCGGGCATATGAAT ATCCTCCTTAGTTCC	1086
<i>ΔytfR</i>	F- ATGACGACCGACCAACACCAGGAGATCCTCCGCACCGTGTAGGC TGGAGCTGCTTCGAAGTTCC R- TTACGCCGCAATGGCGTTTCATGATCGCCGGAACGACATATGAA TATCCTCCTTAGTTCC	1086
Primers for complementation		Amplicon size
<i>pmhpA-F</i>	F- AAGCTT ATGGCAATACAACACCCTGACATCC R-ACGGAGCTTATTTGTTGTTGCGCAGATCCAGC	6675
<i>pmhpB-F</i>	F- AAGCTT ATGCACGCTTATCTTCACTGTCTTTCC R-ACGGAGCTTATTTGTTGTTGCGCAGATCCAGC	5465
<i>pbdcR</i>	F-AGCCATATGGTCACTAAAAACAATCTCG R-ACGGAGCTCTCACTCCTCAAGAATAGTTTTATCG	605
<i>ptolA</i>	F-AGCCATGTGTCAAAGGCAACCGAACAACG R-ACGGAGCTCTTACGGTTTGAAGTCCAATGGCGCG	1276
<i>prfyA</i>	F-AGCCATGCGGCCCTTTCCGCCGTCTCGCAAACG R-ACGGAGCTCAAATATGCCGCCCAAAGGGCGGC	315
Primers for Reverse transcription-PCR (validation of mutants)		Amplicon size
Rt- <i>mhpA</i>	F- TGGCGATCGCTGGTGCCGCGCCGG R- CGATCAACTTATCGAGTTTCTCC	126
Rt- <i>mhpB</i>	F- GGTATGTCGACCCGGCGCAAGAGG R- AAAAGCCGTTGTAGTGATCTGG	135
Rt- <i>bdcR</i>	F- CCCAGACGTTTCGCTCCTGAGCAGGC R- TTAATACCAAGATAATCAGTAAC	98
Rt- <i>tolA</i>	F- GTGCTGCATGTCATCTTATTTGCGG R- CCGCACCTGAATCAACCATGACAGCG	116
Rt- <i>rfyA</i>	F- AGGAAAGGATGTTCCGTGGCCG R- TCTTCATTGTGCGGTCCATGC	132
Rt- <i>mdtO</i>	F- ATCCCTTTTGTGGCGTTACTG R- AACAGGCTGGCGATTCCAGC	106
Rt- <i>ytfR</i>	F- CCGGCGTCAAAGCGTTAGACAACG R- GATGGTGCCGCGATCGGCGTGG	119
Primers for sequencing (validation of mutants)		Amplicon size
Seq- <i>mhpA</i>	F- TTCCGGCCACCAGAATAGCC (-200 bp from ATG of <i>mhpA</i>) R- TGCTTAATGAATACAACAGTACTGCG	375
Seq- <i>mhpB</i>	F- GCCATTCGCAAAACCCTGGGCAAGACCC (-200 region from ATG of <i>mhpB</i> gene) R- TGCTTAATGAATACAACAGTACTGCG	375
Seq- <i>bdcR</i>	F-CGTACGATAGCGGCACCGATACC (-198 bp from ATG of <i>bdcR</i>) R- TGCTTAATGAATACAACAGTACTGCG	373

(Continued)

TABLE 1 | Continued

Plasmids and isolates used in this study		
Seq- <i>tolA</i>	F- ACCCGAAACGGTCTTCTGATCGG (-180bp from GTG of <i>tolA</i>) R- TGCTTAATGAATACAACAGTACTGCG	353
Seq- <i>rlyA</i>	F-GGCGGTTTTTGTGCTGGTCCGG (-176 bp from GCG of <i>rlyA</i>) R- TGCTTAATGAATACAACAGTACTGCG	349
Seq- <i>mdtO</i>	F- GAGGGTAAAGTGGATTGATTGGC (-189 from ATG of <i>mdtO</i> gene) R- TGCTTAATGAATACAACAGTACTGCG	364
Seq- <i>ytfR</i>	F-TCCGGCGATGGTGCCTGAAGCC (-186 from ATG of <i>ytfR</i> gene) R- TGCTTAATGAATACAACAGTACTGCG	361

All the hybrid primers in this group contain 36 nucleotides of the target gene (either downstream or upstream sequence depending on whether it is a forward or reverse primer) plus primer for the antibiotic cassette such that the PCR amplicon would contain 36 nucleotides of the target gene from either end plus the antibiotic cassette gene. *CDFD, center for DNA fingerprinting and diagnostics. #UoH, University of Hyderabad. \$LVPEI-L V Prasad Eye Institute.

and stained with 200 μ l of 1.67 μ M Syto[®]9 a nuclear fluorescent dye (Invitrogen, United States) for 30 min. Stained biofilms were washed again with autoclaved water. Confocal images were taken using Zeiss confocal laser scanning microscope (Carl Zeiss LSM 880, Jena, Germany). Argon Laser was excited at 450–490 nm and a 40 $\times$ objective was used set at Zoom 2.

EPS Production Using Calcofluor White Staining

After staining of the cells in the biofilm with Syto 9, the biofilms were stained in the dark with 0.025% Calcofluor white M2R (Sigma Chemical Co., St. Louis, MO, United States) for 30 min. This dye binds to β -linked polysaccharides and fluoresces under long-wave UV light (Wood, 1980) and biofilm could be visualized (blue) using confocal microscopy. Calcofluor white has been used to study exopolysaccharides (EPSs) involved in biofilm formation in a variety of organisms (Zogaj et al., 2001; Ledebor and Jones, 2005). Calcofluor white was excited at 363-nm using a 455/30 band-pass filter (Cowan et al., 2000).

Production of Curli and Cellulose Nanofibers by *E. coli* Strains by the Congo Red-Binding Assay

Curli and cellulose nanofibers production in *E. coli* L-1216/2010 and the mutants was monitored by the Congo red-binding assay method (Arita-Morioka et al., 2015) using Congo red-containing YESCA agar plates. *E. coli* L-1216/2010 and the mutants were cultured overnight and then 10 μ l of the culture were spotted on YESCA media plates containing 10 g/l casamino acids, 10 g/ml Coomassie brilliant blue G-250, 1 g/l yeast extract, and 20 g/L agar. The plates were incubated at 30°C for 3 days and the colonies were photographed. Colonies positive for curli and cellulose nanofibers production were red and colonies positive only for cellulose nanofibers were pink in color and could be easily differentiated.

Quantification of the Attachment of Cells to the Substratum by SYTO9 Staining

Adhesion of cells to the substratum was assessed using the procedure of Schmidt-Emrich et al. (2016). Overnight culture was adjusted to 0.5 McFarland units, diluted 100-fold with the medium and 100 μ l of the diluted inoculum was dispensed into a single well of a 96 well plate (Thermo Fisher Scientific, NunclonTM, Denmark) containing 100 μ l of fresh medium. The plate was incubated at 37 °C for 4 h. The broth was then discarded

by inverting the plate and gently tapping it after which it was washed thrice with 200 μ l of phosphate buffered saline (PBS, Sigma-Aldrich Corporation, St. Louis, MO, United States) and allowed to dry for 30 min. The bacteria in the biofilm adhering to the plate were then stained with 200 μ l of 2.5 μ M SYTO9 in dark and incubated for 15 min at room temperature on a shaker and quantified using a spectrophotometer (SpectroMax M3, Molecular Devices, CA, United States) at 490 nm. Wells without culture served as the control.

Motility of Ocular *E. coli* L-1216/2010 and the Mutants by Monitoring Swimming and Swarming of Cells

Swimming motility is an individual and random-directional movement in liquid medium, and essential for initial attachment to develop biofilm. Swarming motility is a coordinated bacterial social movement across the top of a solid surface, accompanied by hyper-flagellation critical for surface colonization after initial attachment. Swimming and swarming motilities were investigated (Bak et al., 2015) on specific agar plates as indicated below. For this purpose 1 μ l of 0.5 mcF ($\sim 5 \times 10^4$ cells) of each mutant was inoculated with a pipette tip onto swim agar (0.3% Bacto Agar, 1% tryptone and 0.5% NaCl) or swarm agar (0.6% Eiken Agar, 0.5% glucose, 1% tryptone, 0.5% yeast extract and 0.5% NaCl). The swimming assay was performed at 30 °C for 12 h, and swarming assay at 37 °C for 16 h, respectively. The diameter of the swimming circle or the diameter of swarming area was measured and normalized against that of a wild type strain (Bak et al., 2015).

Antibiotic Susceptibility of Ocular *E. coli* L-1216/2010 and the Mutants

The minimum inhibitory concentration (MIC) of the antibiotic was determined by the micro-dilution method as described by the European Committee on Antimicrobial susceptibility Testing (CLSI, 2012). Antibiotics were obtained from commercial sources and each concentration was tested thrice. The susceptibility of the strains was also determined after the formation of the biofilm. For this purpose, the cultures were incubated as above in the 96 well plate at 37°C for 48 h for biofilm formation. Planktonic cells were discarded, bound cells washed with milliQ water and then the antibiotic dissolved in BHI medium (HiMedia, Mumbai, India) was added to the biofilm and incubated for 16 h. Biofilm formation was then monitored by the crystal violet method. The concentration of the antibiotic that inhibited the formation of

the biofilm was determined. *E. coli* isolate L-1216/2010 in the planktonic phase (where in antibiotics were added after 24 h of growth) was used as controls for this experiment.

Isolation of Plasmids, Transformation by CaCl₂ Method, Preparation of Electro-Competent Cells, and Electroporation

Plasmids pKD3 and pKD46 were maintained in DH5 α in LB medium with Ampicillin (100 μ g/ml) (HiMedia, Mumbai, India) and the plasmids were isolated from an overnight 5 ml culture using Qiagen plasmid mini kit (Qiagen, Heilden, Germany). pKD46 plasmid containing the lambda red recombinase (plasmid gifted by CDFD, Hyderabad, India) gene was transformed into ocular *E. coli* by CaCl₂ heat shock method (42°C for 45 s) and was plated on to LB agar plates supplemented with Ampicillin (100 μ g/ml) and incubated at 30°C overnight. Colonies were then selected and grown in the same medium for future use.

Electro-competent cells of *E. coli* pKD46 were prepared using a standard protocol. An overnight culture diluted 1:100 in LB medium was inoculated into fresh LB medium and incubated at 30°C until the O.D₆₀₀ reached 0.1. L-arabinose (10 mM, final concentration) was added to the culture and incubated further to OD₆₀₀ of 0.6 to induce the expression of recombinase gene. The cells were then pelleted by centrifugation at 4000 rpm, washed thrice with autoclaved chilled MilliQ water, and finally resuspended in MilliQ water. The prepared electro-competent cells were stored at –80°C until use.

Construction of Mutants in Biofilm Forming Ocular *E. coli* Isolate L-1216/2010

Mutant strains of *E. coli* were generated by homologous recombination based on the procedure of Datsenko and Wanner (2000). *E. coli* L-1216/2010 was first transformed by the CaCl₂ heat shock method with pKD46 which expresses the Recombinase gene (*E. coli* pKD46) to facilitate homologous recombination). Subsequently, electro-competent cells of *E. coli* pKD46 were electroporated with a linear DNA fragment of the target gene using the Gene-Pulser-Xcelle electroporator (BioRad, Osaka, Japan), at 2.5 kV, 200 Ohm, and 25 μ F. For this purpose the electro-competent ocular *E. coli* pKD46 cells were transferred to pre-chilled electroporation cuvettes (in ice for 10 min) and 5 μ g of the PCR amplified linear DNA fragment was added and electroporated. LB medium (1 ml) (0.5% Yeast extract, 1% Peptone, 1% sodium chloride, and 1.5% Agar) was added immediately to the cells after applying the pulse and the electroporated cells were incubated at 37°C for 2 h and plated on an LB plate containing chloramphenicol (25 μ g/ml) (HiMedia). Transformants were then selected on chloramphenicol plates and mutants of the target gene were validated by sequencing and Reverse transcription-PCR. Prior to electroporation the linear fragment was obtained by using hybrid primers (FH and RH). FH consisted of 36 nucleotides of the 5' end of the gene of interest along with the forward primer of the cassette and RH contained 36 nucleotides of the 3' end of the gene of interest along with

the reverse primer of the cassette. When these primers were used they amplified a linear fragment from pKD3 which would contain 36 nucleotides of the target gene from either end plus the FRT (Flippase Recognition Target) region, the chloramphenicol resistance gene cassette and the FRT minimal region (1100 bp). This linear fragment was amplified (Table 1) for 35 cycles (each cycle: 94°C for 5 min, 94°C for 1 min, 63°C for 90 s, 72°C for 2 min, and finally 72°C for 15 min) using *pfu* polymerase enzyme (Thermo Fisher Scientific, MA, United States).

Complementation of Mutants Using pET28a(+) Vector

(i) Amplification of Gene From Ocular *E. coli* Genome

For complementation studies the genes *bdcR*, *mhpA*, *mhpB*, *ryfA*, and *tolA* were amplified along with 200 bp upstream region using *pfu* polymerase (Table 1). Primers were designed based on the whole genome sequence of *E. coli* MG1655. Amplification was done for 35 PCR cycles as follows: 94°C for 5 min, 35 $\times$ (94°C for 1 min, 56°C for 60 s, 72°C for 1.30 min), 72°C for 15 min. The PCR amplified products were then purified using Nucleospin Gel and PCR cleanup kit (Nucleospin, Duren, Germany) and then digested using Hind III/*NdeI* and XhoI/*SacI* restriction enzymes for 30 min at 37°C. These digested products would be ligated to pET28a(+) vector (as below).

(ii) Ligation of Gene Product From Dtep (i) With pET28a(+) Vector

Plasmid pET28a(+) (gift from J S SPakash, University of Hyderabad, India) was isolated using plasmid isolation kit (Qiagen, Hilden, Germany) and 1 μ g of pET28a(+) was digested with 1 μ l of HindIII/*NdeI* and 1 μ l of XhoI/*SacI* (NEB, Massachusetts, United Kingdom) for 30 min at 37°C. Later the digested vector was purified using Nucleospin Gel and PCR cleanup kit (Duren, Germany) and then ligated to *NdeI* and *SacI* digested target gene using T4DNA ligase (Thermo Fisher Scientific, MA, United States). Ligation was performed using final concentration of 0.06 pmol of the digested PCR amplified product and 0.02 pmol of the digested vector at 22°C for 1 h. The ligation reaction mixture was inactivated at 65°C for 10 min and used for transforming DH5 α to increase the copy number of the plasmid. Subsequently it was used to transform the respective competent mutant ocular *E. coli* cells by heat shock method.

Semi Quantitative PCR for Complement Strains

Reverse transcription-PCR was performed to validate the expression of genes in the mutants and complemented strains using primers as in Table 1. Amplification was done for 40 cycles as follows: 94°C for 5 min, 40 $\times$ (94°C for 30 s, 60°C for 30 s).

STRING Functional Analysis

The predicted functional interactions among genes *bdcR*, *mdtO*, *mhpA*, *mhpB*, *tolA*, and *ytfR* was ascertained using STRING¹, Search Tool for the Retrieval of Interacting Genes/Proteins) analysis.

¹<http://string-db.org/>

RESULTS

In an earlier study we had demonstrated that *E. coli* L-1216/2010 isolated from the vitreous of an endophthalmitis patient exhibited very good ability to form biofilm and in the biofilm phase it was 100 fold more resistant to several antibiotics (Ranjith et al., 2017). We had also identified several genes that were up-regulated in expression during biofilm phase (Ranjith et al., 2017). In the present study, a few of the genes up-regulated in expression during biofilm phase viz., *bdcR*, *mdtO*, *mhpA*, *mhpB*, *ryfA*, *tolA*, and *ytfR* (Ranjith et al., 2017) were mutated by homologous recombination (Datsenko and Wanner, 2000) to validate the function of the above genes in biofilm formation in ocular pathogenic *E. coli*. Following homologous recombination the mutants were validated by PCR and Reverse transcription-PCR. PCR confirmed the insertion of the antibiotic cassette in the target gene such that the PCR amplicon on sequencing revealed that the antibiotic cassette was intact and 36 bp of the target was present upstream and downstream of the antibiotic cassette. Further Reverse transcription-PCR also indicated that the gene is not expressed in the mutant (Supplementary Figure 1).

Biofilm Formation and Growth of the Seven Ocular *E. coli* Mutants

Five ($\Delta bdcR$, $\Delta mhpA$, $\Delta mhpB$, $\Delta ryfA$, and $\Delta tolA$) of the seven mutants showed significant decrease in biofilm formation ($p < 0.05$) as monitored by XTT method. In all the five mutants biofilm formation by the XTT method appeared to be as in *E. coli* ATCC 25922 which served as the negative control. In contrast, the mutants $\Delta mdtO$ and $\Delta ytfR$ retained their ability to form biofilm, similar to wild type *E. coli* L-1216/2010 ($p > 0.05$) (Figure 1A). The inability to form the biofilm in the above five mutants was not dependent on its growth which was not affected compared to the control (Figure 1B). Even the growth curves of the mutants compared to *E. coli* L-1216/2010 was monitored and no significant difference was observed (Figure 1C).

Biofilm Formation in the Seven Ocular *E. coli* Mutants by Confocal Laser Scanning Microscopy (CLSM)

Confocal laser scanning microscopy also confirmed that the luxuriant biofilm that formed after 72 h in *E. coli* L-1216/2010 was significantly decreased in $\Delta bdcR$, $\Delta mhpA$, $\Delta mhpB$, $\Delta ryfA$, and $\Delta tolA$ and was unaffected in $\Delta ytfR$ and $\Delta mdtO$ (Figure 2). The thickness of the biofilm reduced significantly from 16.8 μm in the wild type to as low as 1.5 μm in $\Delta tolA$.

Biofilm Formation of the Ocular *E. coli* Mutants and the Complemented Strains by CLSM and XTT Method

Confocal laser scanning microscopy indicated that in four of the five mutants following complementation with the respective gene ($\Delta bdcR + pbdcR$, $\Delta mhpA + pmhpA$, $\Delta ryfA + pryfA$, and $\Delta tolA + ptolA$) biofilm formation was restored as in the control (Figure 2). Complementation also increased the thickness of the biofilm (range 13.9–16.3 μm) as observed in wild type ocular

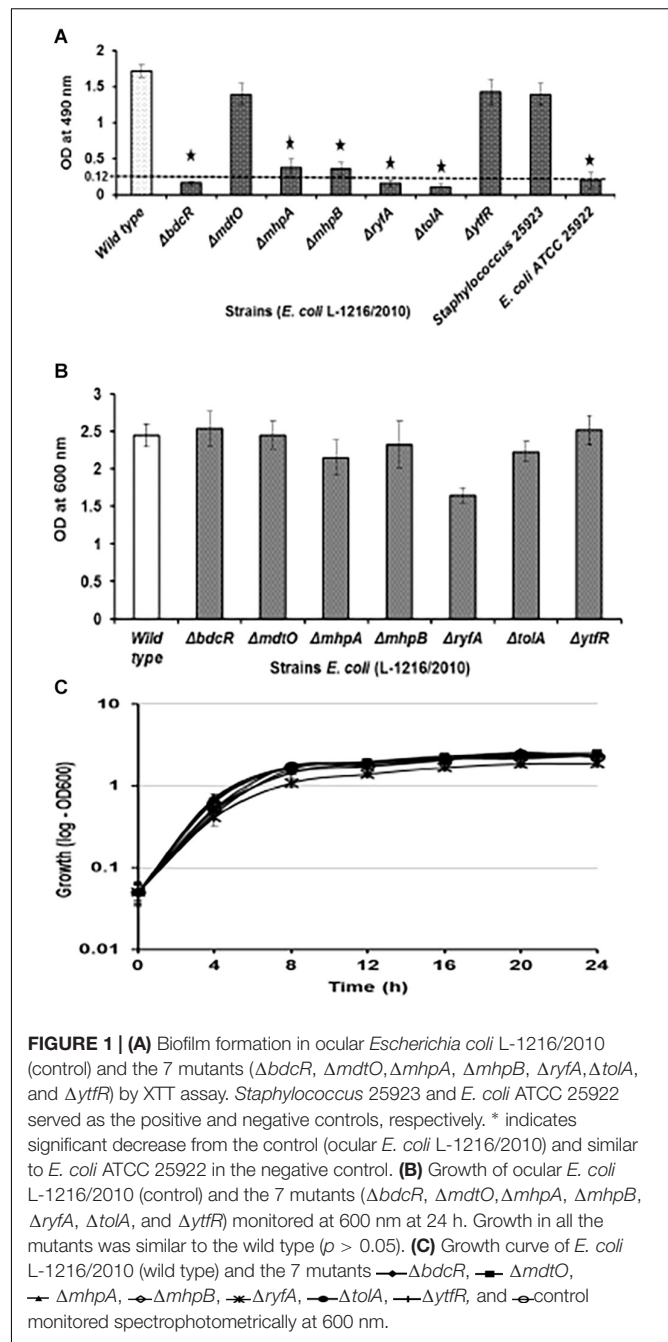


FIGURE 1 | (A) Biofilm formation in ocular *Escherichia coli* L-1216/2010 (control) and the 7 mutants ($\Delta bdcR$, $\Delta mdtO$, $\Delta mhpA$, $\Delta mhpB$, $\Delta ryfA$, $\Delta tolA$, and $\Delta ytfR$) by XTT assay. *Staphylococcus* 25923 and *E. coli* ATCC 25922 served as the positive and negative controls, respectively. * indicates significant decrease from the control (ocular *E. coli* L-1216/2010) and similar to *E. coli* ATCC 25922 in the negative control. **(B)** Growth of ocular *E. coli* L-1216/2010 (control) and the 7 mutants ($\Delta bdcR$, $\Delta mdtO$, $\Delta mhpA$, $\Delta mhpB$, $\Delta ryfA$, $\Delta tolA$, and $\Delta ytfR$) monitored at 600 nm at 24 h. Growth in all the mutants was similar to the wild type ($p > 0.05$). **(C)** Growth curve of *E. coli* L-1216/2010 (wild type) and the 7 mutants $\Delta bdcR$, $\Delta mdtO$, $\Delta mhpA$, $\Delta mhpB$, $\Delta ryfA$, $\Delta tolA$, $\Delta ytfR$, and control monitored spectrophotometrically at 600 nm.

E. coli (16.8 $\pm$ 2.2 μm) ($p > 0.05$). But, complementation did not restore the biofilm in $\Delta mhpB + pmhpB$ (Figure 2). Quantitation of the biofilm by the XTT method also confirmed that except for the mutant $\Delta mhpB$ complementation was efficient in restoring biofilm potential in the remaining four mutants (Figure 3).

EPS Production in the Ocular *E. coli* L-1216/2010 and the Mutants Using Calcofluor White Staining

Using a dual staining strategy with Syto 9 for cells (green) and Calcofluor white for EPS (blue) it was observed that in the

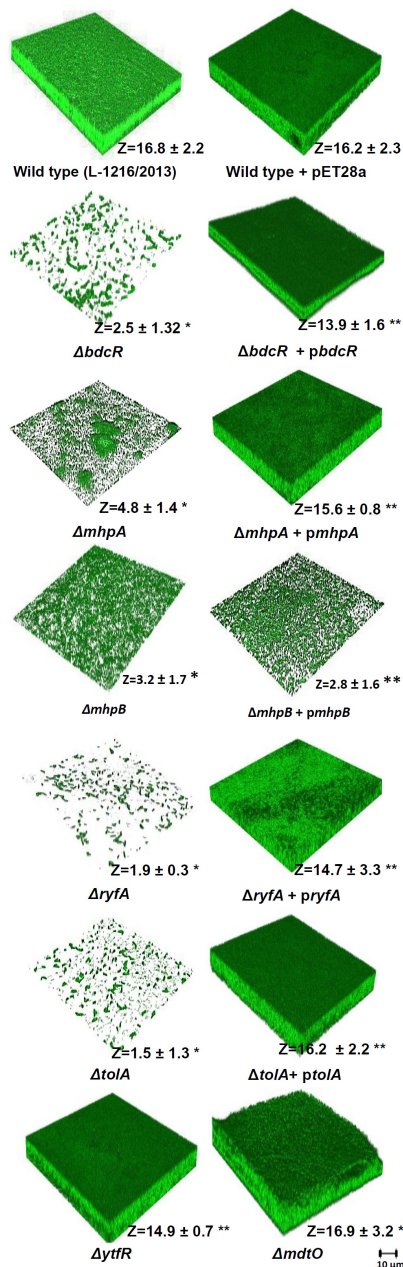


FIGURE 2 | Biofilm formation in ocular *E. coli* L-1216/2010 (wild type) and the 7 mutants ($\Delta bdcR$, $\Delta mhpA$, $\Delta mhpB$, $\Delta ryfA$, $\Delta tolA$, $\Delta ytfR$, and $\Delta mdtO$) and the mutant complemented strains of $\Delta bdcR + pbdcR$, $\Delta mhpA + pmhpA$, $\Delta mhpB + pmhpB$, $\Delta ryfA + pryfA$, and $\Delta tolA + ptolA$. The image of *E. coli* L-1216/2010 + pET28A served as control for all the complemented strains. *Indicates significant decrease in thickness compared to *E. coli* L-1216/2010 (control) ($p \leq 0.05$). ** indicates no significant decrease in thickness compared to *E. coli* L-1216/2010 (control) ($p \geq 0.05$). Z values are expressed in μm . All images were recorded at the same magnification. The scale bar is shown in the figure.

wild type cells EPS thickness increased from 2.53 μm at 4 h to 32.80 μm by 96 h. Maximum thickness was observed at 72 h. In contrast all the 5 mutants showed a significant decrease

in the thickness of EPS. In 3 mutants, namely $\Delta bdcR$, $\Delta mhpA$ and $\Delta mhpB$ EPS thickness increased up to 24 h but remained unchanged subsequently except $\Delta bdcR$ which showed substantial decrease in thickness from 72 to 96 h. The other two mutants $\Delta tolA$ and $\Delta ryfA$ did not show any significant increase in EPS between 4 to 96 h. We also observed that cells of $\Delta mhpA$ and $\Delta mhpB$ had reduced EPS production and appeared to aggregate in to clumps between 48–96 h (Figure 4A).

Production of Curli Fimbriae and Cellulose Nanofibers by Congo Red-Binding Assay in *E. coli* L-1216/2010 and the Mutants

It was observed using Congo red-containing YESCA agar plates that out of 7 mutants, three were red in color, dry and either smooth ($\Delta mdtO$) or rough ($\Delta mhpA$ and $\Delta tolA$), two ($\Delta mhpB$ and $\Delta ytfR$) were pink, dry and smooth, and the remaining two ($\Delta bdcR$ and $\Delta ryfA$) were cream in color, dry and smooth. The wild type was also observed to be red, dry and smooth. These morphological features indicated that the wild type and 3 mutants that were dry and red are positive for curli fimbriae and cellulose nanofibers production (Römling, 2005; Milanov et al., 2015), the 2 mutants that were pink and smooth were positive for cellulose nanofibers production (Zogaj et al., 2003), and the 2 mutants that were cream colored were negative for both curli fimbriae and cellulose nanofibers production (Figure 4B).

Swimming, Swarming, and Adhesion of Ocular *E. coli* L-1216/2010 and the Mutants

Swimming and swarming of the wild type and mutant cells was monitored overnight and the results indicated that compared to *E. coli* L-1216/2010 swimming was significantly reduced in two mutants ($\Delta ryfA$ and $\Delta tolA$, $p < 0.05$) (Figure 5A). In contrast swarming was significantly reduced only in *tolA* ($p < 0.05$) (Figure 5B). Results of adhesion indicated that the number of cells attached to the substratum appeared to be similar in all except $\Delta tolA$ where less cells appeared to be attached (Figure 5C).

Antibiotic Sensitivity of the Ocular *E. coli* Mutants and the Complemented Strains

The five mutants which did not form the biofilm ($\Delta bdcR$, $\Delta mhpA$, $\Delta mhpB$, $\Delta ryfA$, and $\Delta tolA$) following exposure to 9 different antibiotics in the planktonic phase exhibited MICs similar to that observed for the wild type *E. coli* L-1216/2010 (Table 2). Mutant $\Delta ytfR$ which retained its ability to form a biofilm also had a similar MIC compared to the wild type *E. coli* in the planktonic phase. But, mutant $\Delta mdtO$ which retained its ability to form a biofilm showed reduced MIC to 4 out of the 9 antibiotics tested (viz., Sulfamethoxazole, Imipenem, Streptomycin and Erythromycin) (Table 2) in the planktonic phase. We also checked the MIC of the mutants $\Delta ytfR$ and $\Delta mdtO$ in response to the above four antibiotics in the their biofilm phase and observed that the MIC compared to the

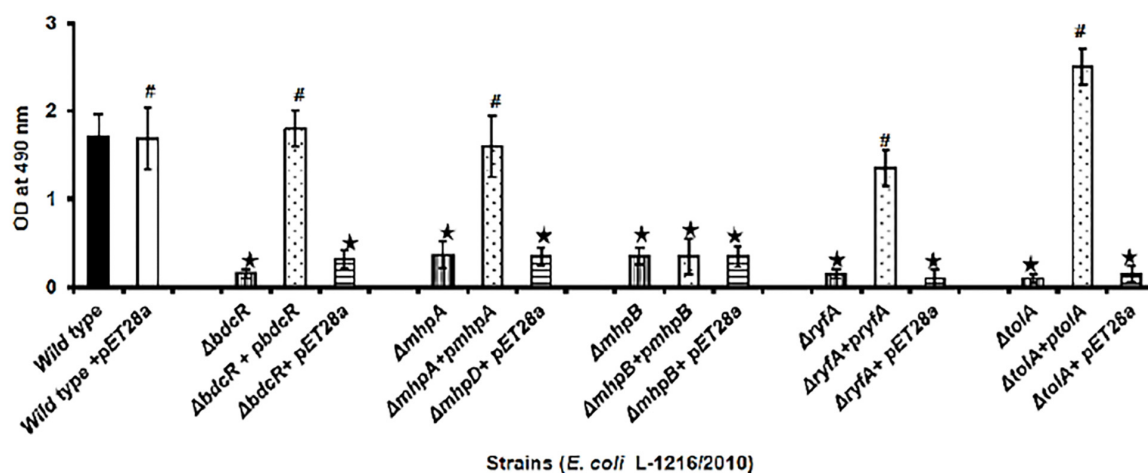


FIGURE 3 | Quantification of biofilm formation by the XTT method in ocular *E. coli* L-1216/2010 (control), the 5 mutants ($\Delta bdcR$, $\Delta mhpA$, $\Delta mhpB$, $\Delta ryfA$, and $\Delta tolA$) and the 5 mutant complemented strains ($\Delta bdcR + pbdcR$, $\Delta mhpA + pmhpA$, $\Delta mhpB + pmhpB$, $\Delta ryfA + pryfA$, and $\Delta tolA + ptolA$). Control and mutants complemented with pET28A served as control for the complemented strains. * indicates significant decrease in biofilm formation compared to *E. coli* L-1216/2010 (control) ($p \leq 0.05$). # indicates no significant decrease in biofilm formation compared to *E. coli* L-1216/2010 (control) ($p > 0.05$).

control was altered only in $\Delta mdtO$ in the biofilm phase implying that the mutation had not affected its ability to form biofilm but has altered its sensitivity to antibiotics (Table 3). Finally we also checked the antibiotic sensitivity of the complemented strains in the biofilm phase and the results indicated that the complemented strains $\Delta bdcR + pbdcR$, $\Delta mhpA + pmhpA$, and $\Delta mhpB + pmhpB$ did not differ in their sensitivity to all the 9 tested antibiotics compared to the wild type cells whereas $\Delta tolA + ptolA$ appeared to be more resistant to 2 of the 9 antibiotics (namely Gentamycin and Erythromycin) (Table 4).

Interaction of the Mutated Genes in *E. coli* L-1216/2010

STRING network analysis indicated that genes *bdcR*, *mdtO*, *mhpA*, *mhpB*, *tolA*, and *ytfR* were organized in to two functional arms namely microbial metabolism and transmembrane transporter activity. Genes *bdcR*, *mhpA*, *mhpB*, and *tolA* were part of the microbial metabolism arm and interacted with genes involved in pyruvate metabolism (genes *mhpF*, *plfB*, *tdcE*, and *adhE*), 3-(3-hydroxy) phenyl propionate catabolic process (genes *mhpA* and *mhpB*), acetaldehyde dehydrogenase activity (genes *mhpF* and *adhE*) and genes involved in biofilm such as c-di-GMP-binding biofilm dispersal mediator protein (*bdcR*). The remaining 2 genes *mdtO* and *ytfR* were part of the transmembrane transport activity and interacted with genes involved in transport such as protein transport genes (*tolA*, *tolB*, *tolQ*, and *tolR*) and transmembrane transport genes (*ompF*, *tolC*, *acrD*, *acrA*, JW0451, *macB*, *emrA*, *macA*, *mdtP*, *mdtO*, *mdtN*, *hrsA*, *ytfT*, *ytfF*, and *ytfR*) (Figure 6). The interaction between the genes was mediated by *ompR* encoding for DNA-binding transcriptional dual regulator, *ompF* encoding for outer membrane porin 1a, *adhE* and *mhpF* encoding for aldehyde dehydrogenase, *tolC* encoding for transport channel and *tolA* encoding for inner membrane protein member of Tol-Pal

system. The network also included genes coding for the “two component signal transduction system” *ompR* and *rcsA*.

DISCUSSION

Understanding the molecular basis of biofilm formation is very crucial in identifying targets to hack biofilms so that virulent and pathogenic bacteria could be controlled. Several studies have demonstrated that in *E. coli* in the biofilm phase several hundreds of genes are differentially expressed (Schembri et al., 2003; Beloin et al., 2004; Ren et al., 2004; Domka et al., 2006; Hancock and Klemm, 2007; Ranjith et al., 2017). In a recent study we demonstrated that in ocular *E. coli* L-1216/2010 also several genes were differentially expressed in the biofilm phase (Ranjith et al., 2017). Further, ocular *E. coli* could be differentiated from *E. coli* K12, a laboratory non-pathogenic strain of *E. coli*, with respect to expression of genes involved in cell cycle control, mitosis and meiosis, cell motility, intracellular trafficking, secretion, vesicular transport and defense mechanisms (Ranjith et al., 2017) implying that ocular *E. coli* is probably more like a pathogen. In fact this may indeed be so because the ocular *E. coli* L-1216/2010 and ABU *E. coli* (Ranjith et al., 2017) a pathogenic strain, were similar with respect to differential regulation of genes involved in intracellular trafficking, secretion and vesicular transport, defense mechanisms and replication, recombination, and repair which are normally associated with pathogenic bacteria. In the present study the question addressed is whether ocular pathogen are similar to other pathogens with respect to genes involved in biofilm formation and associated drug resistance.

Over the years it has been reasonably well established that genes involved in motility (Wood et al., 2006), that facilitate attachment to substratum (Prigent-Combaret et al., 2000; Ren et al., 2005), that code for production of extracellular polymers

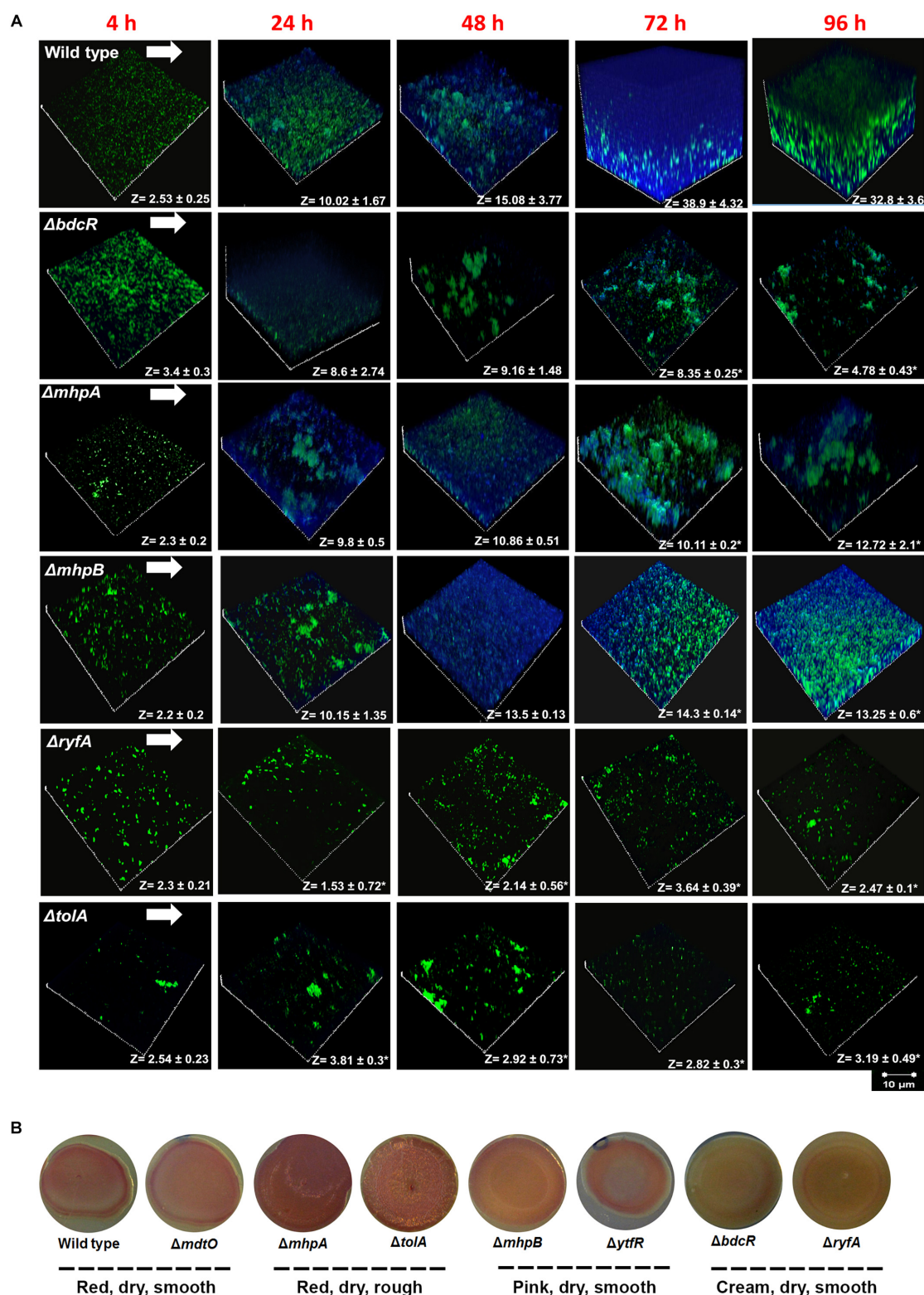
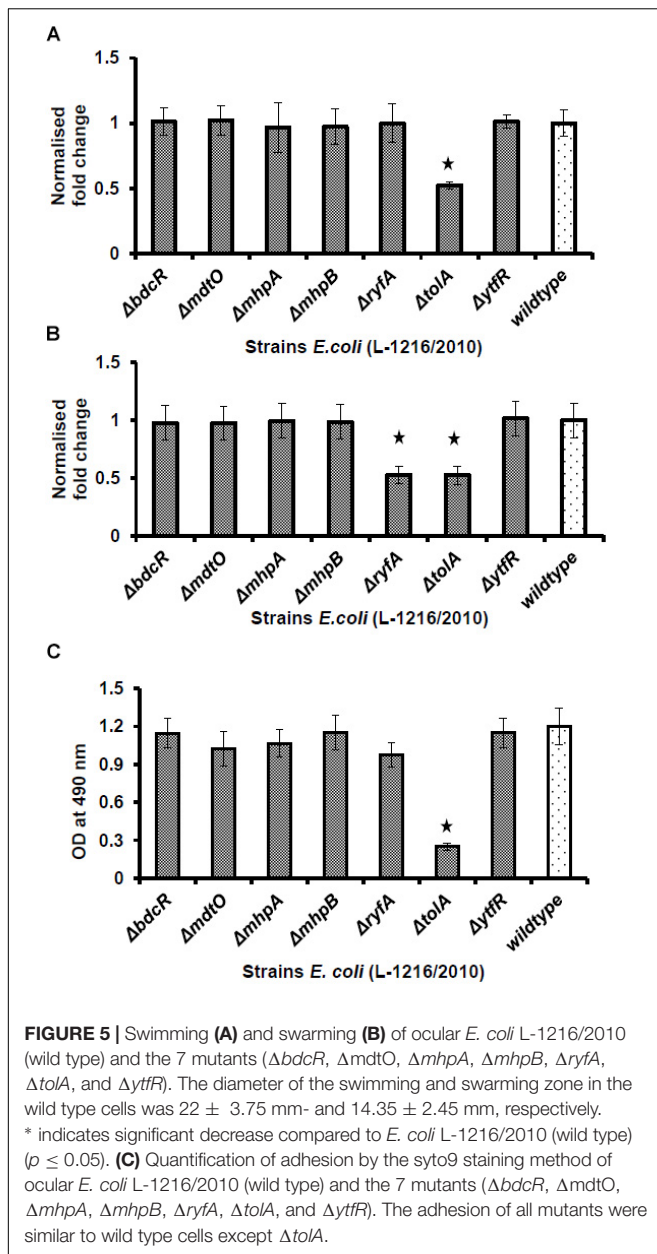


FIGURE 4 | (A) EPS production in ocular *E. coli* L-1216/2010 (wild type I) and the 5 mutants ($\Delta bdcR$, $\Delta mhpA$, $\Delta mhpB$, $\Delta ryfA$, and $\Delta tolA$). Z values are expressed in μ m. All images were recorded at the same magnification. The scale bar is shown in the figure. * indicates significant decrease in thickness compared to *E. coli* L-1216/2010 (wild type) ($p \leq 0.05$). **(B)** Production of curli and cellulose nanofibers in ocular *E. coli* L-1216/2010 (wild type) and the 7 mutants ($\Delta bdcR$, $\Delta mdtO$, $\Delta mhpA$, $\Delta mhpB$, $\Delta ryfA$, $\Delta tolA$, and $\Delta ytfR$).



and adhesive factors (Danese et al., 2000; Schembri et al., 2004), that help in formation and maturation of micro-colonies and in cell signaling (quorum sensing) (Reisner et al., 2003) are associated with biofilm formation. A few of the genes such as *tolA*, *ryfA* (Whiteley et al., 2001), *ymdB* (Kim et al., 2013), *csrA* (Jackson et al., 2002), *spoT* and *relA* (de la Fuente-Nunez et al., 2014), and *ydgG* (Herzberg et al., 2006) have been shown to be required for biofilm formation by specific knock out of the gene (s) either in *P. aeruginosa* or in *E. coli* (Vianney et al., 2005). In the present study seven gene knock outs were generated in ocular *E. coli* L-1216/2010 to ascertain the role of these genes (*bdcR*, *mdtO*, *mhpA*, *mhpB*, *tolA*, *ryfA*, and *ytfR*) in biofilm formation. Earlier studies had indicated that knockout mutants of two genes *ryfA* and *tolA* had lost the potential to form biofilm

(Whiteley et al., 2001; Vianney et al., 2005). In this study we confirm that in ocular *E. coli*, *ryfA* which codes for a small RNA (Bak et al., 2015) following deletion exhibits a decrease in biofilm development probably due to the observed decrease in EPS production, cellulose nanofibers and curli production. The mutant also exhibited significant decrease in swarming. Earlier studies had indicated that curli are important for biofilm formation by facilitating initial attachment to a surface and cell-to-cell cohesion (Pratt and Kolter, 1999). In an earlier study it was demonstrated that in *P. aeruginosa*, *tolA* gene was activated in biofilms (Whiteley et al., 2001) and mutation of the gene in *E. coli* MG1655 and two clinical strains of *E. coli* PHL881 and PHL885 led to a decrease in biofilm formation implying the importance of *tolA* in biofilm formation (Vianney et al., 2005). They also attributed the decrease in biofilm formation to the observation that in the *tol* mutants the adherence of cells to the substratum was lowered. The present study confirms both the observations that in $\Delta tolA$ mutants of ocular *E. coli* biofilm formation is decreased and it could be attributed to a decrease in adhesion of cells, swimming, swarming and production of EPS. Interestingly it was observed that in the mutant, both curli and cellulose nanofibers production was not affected implying that curli is not mandatory for biofilm formation. The reports on the requirement of curli for biofilm formation are contradictory with a few indicating that curli and cellulose nanofibers are not required for biofilm formation (Niba et al., 2007; Cegelski et al., 2009) unlike the report of Arita-Morioka et al. (2015). It also appears that since *tolA* interacts with other *tol* genes (*tolB*, *tolQ*, and *tolR*), with *ompF* involved in bacteriocin (*tolA*, *tolB*, *tolQ*, and *tolR*) and drug transport (*acrD*, *acrA*, *JW0451*, *macB*, *emrA*, *mdtP*, *mdtO*, and *mdtN*) and *tolC* which regulates genes involved in transmembrane transport activity (Figure 6) it may contribute to drug resistance. But mutants did not show any change in susceptibility to 9 antibiotics tested implying that *tolA* may not be directly involved in drug resistance.

We had earlier shown that many genes encoding dehydrogenases (*mhpA*, *mhpB*, *mhpF*, *yiaY*, *yajO/ydbK*, and *yjjN*) were up regulated during biofilm formation in ocular *E. coli* (Ranjith et al., 2017) implying their likely role in biofilm formation. But none of these genes were demonstrated to be required for biofilm formation. In this study we demonstrate that biofilm formation was decreased substantially in separate knockout mutants of *mhpA* and *mhpB* implying that these two genes are required for biofilm formation. *mhpA* and *mhpB* are specifically involved in 3-(3-hydroxy) phenyl propionate catabolic process and thus it is likely that these two genes may be associated with a metabolic function. In fact, String network analysis also indicated a close interaction between *mhpA* and *mhpB* with other microbial metabolic genes such as genes involved in pyruvate metabolism (genes *mhpF*, *plfB*, *tdcE*, and *adhE*) and genes coding for acetaldehyde dehydrogenases (genes *mhpF* and *adhE*) and could thus be involved in microbial metabolism, drug metabolism and support drug resistance a phenomenon associated with biofilm formation. But their role in drug resistance is unlikely since it was observed that the mutants susceptibility to the nine antibiotics tested remained unchanged. When we investigated the phenotypic characteristics

TABLE 2 | Antibiotic sensitivity of the ocular *E. coli* L-1216/2010 and the 7 mutants to 9 different antibiotics.

Antibiotic	MIC of antibiotic ($\mu\text{g/ml}$)*							
	Wild	$\Delta bdcR$	$\Delta mdtO$	$\Delta mhpA$	$\Delta mhpB$	$\Delta rylA$	$\Delta tolA$	$\Delta ytfR$
Cefotaxime	16	16	16	16	16	16	16	16
Ceftazidime	12	12	12	12	12	12	12	12
Cefuroxime	28	28	28	28	28	28	28	28
Ciprofloxacin	12	12	12	12	12	12	12	12
Erythromycin	32	32	16	32	32	32	32	32
Gentamycin	12	12	12	12	12	12	12	12
Imipenem	32	32	16	32	32	32	32	32
Streptomycin	24	24	12	24	24	24	24	24
Sulfamethoxazole	24	24	16	24	24	24	24	24

*Antibiotic susceptibility test was ascertained by the drug dilution method and repeated thrice.

TABLE 3 | Antibiotic susceptibility of ocular *E. coli* L-1216/2010 and mutants $\Delta ytfR$ and $\Delta mdtO$ in the biofilm phase to 4 different antibiotics.

	MIC of antibiotic ($\mu\text{g/ml}$)*					
	Wild type		$\Delta mdtO$		$\Delta ytfR$	
Antibiotic ($\mu\text{g/ml}$)	Planktonic	Biofilm	Planktonic	Biofilm	Planktonic	Biofilm
Erythromycin	32	512	16	64	16	512
Imipenem	32	512	16	256	32	512
Streptomycin	24	1024	12	128	24	1024
Sulfamethoxazole	24	1024	16	32	24	1024

*Antibiotic susceptibility test was ascertained by the drug dilution method and repeated thrice.

TABLE 4 | Antibiotic susceptibility of ocular *E. coli* L-1216/2010 and mutants complemented with their respective genes in the biofilm phase to 9 different antibiotics.

Antibiotic	MIC of antibiotic ($\mu\text{g/ml}$)*				
	Wild	$\Delta bdcR$ + p_{bdcR}	$\Delta mhpA$ + p_{mhpA}	$\Delta rylA$ + p_{rylA}	$\Delta tolA$ + p_{tolA}
Cefotaxime	256	256	256	256	256
Ceftazidime	1024	1024	1024	1024	1024
Cefuroxime	512	512	512	512	512
Ciprofloxacin	512	512	512	512	512
Erythromycin	512	512	512	512	1024
Gentamycin	512	512	512	512	> 1024
Imipenem	512	512	512	512	512
Streptomycin	1024	1024	1024	1024	1024
Sulfamethoxazole	1024	1024	1024	1024	1024

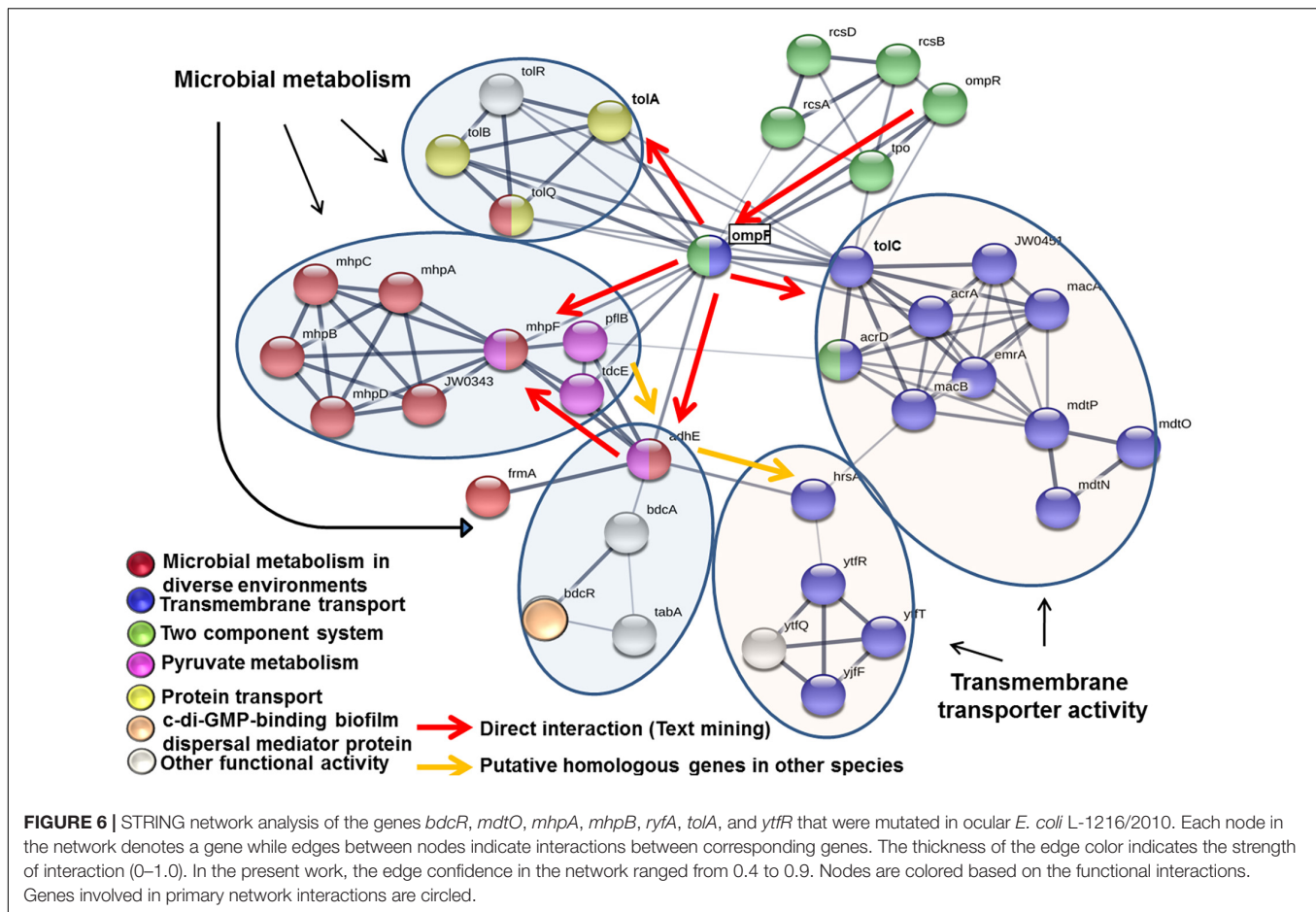
*Antibiotic susceptibility test was ascertained by the drug dilution method and repeated thrice.

it was observed that both the mutants ($\Delta mhpA$ and $\Delta mhpB$) ability to swim, swarm and attachment remained unaffected. In addition it was observed that curli and nanofiber production was not affected in $mhpA$ but in contrast, $\Delta mhpB$ only curli production was affected.

In ocular *E. coli* it was demonstrated that the genes *bdcA* and *bdcR* were upregulated more than 20 fold in the biofilm phase implying their need for biofilm formation (Ranjith et al., 2017). It has been demonstrated that *bdcA* deletion reduces biofilm dispersal by decreasing motility and thus does not affect biofilm formation (Ma et al., 2011). In accordance with the above observation in the present study it was observed that knockout mutants of *bdcR*, a negative regulator of *bdcA*, and showed

reduced biofilm formation. But the mutants ability to swim, swarm and attach was not altered but EPS, curli and cellulose nanofiber production was significantly decreased. Thus it appears that *bdcR* is required for biofilm formation in ocular *E. coli*. It was also observed that unlike the wild type cells, knockout mutants of *mhpA*, *mhpB*, and *bdcR* showed clumping of cells at some time points during the biofilm phase.

Genes coding for ABC transporter ATP-binding protein and belonging to the ATP-binding cassette (ABC) transporter superfamily have been implicated in biofilm formation. But their involvement in biofilm formation appears to vary from organism to organism. For instance in *Rhizobium leguminosarum* mutation in the ATP-binding protein of an uncharacterized



ABC transporter operon significantly reduced the number of viable cells and simultaneously inhibited biofilm formation suggesting that a functional transporter is essential for normal biofilm formation (Vanderlinde et al., 2010). In contrast, (Zhu et al., 2008; Ceruso et al., 2014) observed that in *Listeria monocytogenes*, inactivation of the putative ABC transporter or the permease component, caused enhanced biofilm-formation compared to the wild type, indicating that *LMOF* 2365_1875 negatively regulates biofilm formation. At the same time (Ceruso et al., 2014) also observed that *LMOF* 2365_1877 another mutant in the ABC transporter gene did not have any effect on biofilm formation. In the present study we observed that deletion of *ytfR* (a gene that codes for ABC transporter ATP-binding protein) in ocular *E. coli* did not alter its biofilm formation potential as observed in the above studies and also did not affect the motility, initial attachment and cellulose nanofiber production but curli production was affected. It is possible that the function of *ytfR* is compensated by other genes of the ABC transporter superfamily. In fact, String analysis does indicate that *ytfR* closely interacts with other members of the ABC transporter superfamily like *ytfT*, *yjfF*, *ytfQ*, and *hrsA* which may functionally compensate for its deletion. Recently it was demonstrated by Pinweha et al. (2018) that in *Burkholderia pseudomallei*, ABC transporter mutant *bpsl1039-1040* showed reduced biofilm

formation as compared with the wild-type strain ($P = 0.027$) when cultured in LB medium supplemented with nitrate under anaerobic growth conditions. But this reduction in biofilm formation was not noticeable under aerobic conditions. Thus overall it would appear that ABC transporter gene function with respect to biofilm formation varies not only from organism to organism but also under the physiological condition it is tested.

Among the various genes that were upregulated in ocular *E. coli* we also noticed that genes involved in multidrug resistance like *mdtO* (synonym *yjcQ*) were also up regulated in their biofilm phase in ocular *E. coli*. This is of interest since biofilm formation is related to resistance to antibiotics (Kaplan, 2011) but the correlation appears to be inconsistent and the results are conflicting. For instance in *P. aeruginosa* and *Acinetobacter baumannii* biofilm production was significantly higher in multidrug resistant (MDR) isolates (Abidi et al., 2013; Gurung et al., 2013; Qi et al., 2016). But it was not so in MDR *S. aureus* (Eyoh et al., 2014). In this study we observed that in *mdtO* deletion mutant of ocular *E. coli* formation of biofilm was unaltered and all the attributes of a biofilm forming bacterium such as swimming, swarming, attachment and production of curli, and cellulose nanofibers were unaltered. But the susceptibility of the *mdtO* deletion mutant to Sulfamethoxazole, Imipenem, Erythromycin, and

Streptomycin was increased as judged by the lower MICs. Thus the observations emphasize a role for the gene exclusively in drug resistance.

In the current study, out of the five deletion mutants ($\Delta bdcR$, $\Delta mhpA$, $\Delta mhpB$, $\Delta tolA$, and $\Delta ryfA$) complementation was successful in all the mutants except $\Delta mhpB$ in which the biofilm formation potential was not restored. Further the plasmid used for complementation had no effect on the growth. In two mutants of ABC transporter in *Rhizobium leguminosarum* attempts to restore wild-type phenotype of biofilm formation by complementation was also not successful (Vanderlinde et al., 2010) and was attributed to a dominant-negative effect as observed earlier for other ABC transporters (Bliss et al., 1996; Miyamoto et al., 2002; Eyoh et al., 2014). In the present case it needs to be established.

The STRING network analysis of the deleted genes in *E. coli* appeared to be involved in two functional arms namely “microbial metabolism” and “transmembrane transporter activity.” Genes *mhpA*, *mhpB*, *bdcR*, and *tolA* constituted the metabolism arm and deletion of these genes decreased biofilm formation but their susceptibility to antibiotics in the planktonic phase was unaltered. It appears that these genes involved in microbial metabolism are essential for biofilm formation and supports a previous study that acetate metabolism is related to the formation of biofilms in *E. coli* (Pruss et al., 2010). The four genes *bdcR*, *mhpA*, *mhpB*, and *tolA* of the metabolic arm also interacted with other metabolic genes of pyruvate metabolism, 3-(3-hydroxy) phenyl propionate catabolic process and acetaldehyde dehydrogenase activity regulated either directly or indirectly by their interaction with *ompF* (outer membrane porin). In contrast, *E. coli* cells mutated for genes *ytfR* and *mdtO* were implicated in “transmembrane transporter activity” and deletion of these genes did not alter their ability to produce biofilm as in wild type cells. But, one of the mutants $\Delta mdtO$ showed more sensitivity to Sulfamethoxazole, Imipenem, Streptomycin, and Erythromycin both in the planktonic and biofilm phase. Thus these genes appear to be more related to drug resistance. *ompF* also regulated this arm by indirectly interacting with *mdtO* through *tolC* and with *ytfR* through *adhE*. This arm also included genes involved in drug transport (*ompF*, *acrD*, *acrA*, *JW0451*, *macB*, *emrA*, *mdtP*, *mdtO*, and *mdtN*) and sugar transport genes (*hrsA*, *ytfT*, *ytfE*, and *ytfR*). It is also observed that genes coding for the “two component signal transduction system” *ompR* and *rcaA* (Pruss, 2017) were central to regulating the two arms through *ompF*. Thus it is possible that genes of the “microbial metabolism” arm are required for biofilm formation whereas genes of the “transmembrane transporter activity” are required for drug resistance and not essential for biofilm formation.

CONCLUSION

In the present study we mutated a total of seven genes in ocular *E. coli* L-1216/2010 out of which in five mutants ($\Delta bdcR$, $\Delta mhpA$, $\Delta mhpB$, $\Delta ryfA$, and $\Delta tolA$) biofilm formation was

inhibited. In three of the mutants ($\Delta bdcR$, $\Delta mhpA$, and $\Delta mhpB$) swimming, swarming and adhesion were not affected. In $\Delta tolA$ swimming, swarming and adhesion were affected. where as in $\Delta ryfA$ only swarming was affected. In addition, all the five mutants exhibited decrease in EPS production and in two of the mutants ($\Delta bdcR$ and $\Delta ryfA$) curli and cellulose nanofibers production was also inhibited. Thus the only feature that appears to be shared by all the five mutants is their inability to produce EPS. In the remaining two mutants ($\Delta mdtO$ and $\Delta ytfR$) biofilm formation was unaltered. Out of these five genes which are demonstrated to be required for biofilm formation *ryfA* and *tolA* were earlier demonstrated to be required for biofilm formation (Vianney et al., 2005; Bak et al., 2015). Thus we report for the first time that genes *bdcR*, $\Delta mhpA$, and $\Delta mhpB$ are required for biofilm formation in ocular *E. coli*. We also report that gene *mdtO*, a transmembrane protein involved in the multi-drug transport of sulphanilamide drugs in planktonic phase, when mutated did not affect the biofilm formation in ocular *E. coli* L-1216/2010 but the mutant was more sensitive to Sulfamethoxazole, Imipenem, Erythromycin, and Streptomycin both in the planktonic and biofilm phase. Thus mitigation of biofilm formation to overcome antibiotic resistance could be achieved by targeting the genes *bdcR*, *mhpA*, *mhpB*, *tolA*, and *ryfA*.

AUTHOR CONTRIBUTIONS

KR performed the experiments and participated in the data analysis. JR and JP helped in generating knockout and complementation and provided the logistic support. KA helped in string analysis. SShi participated in manuscript writing and editing. SSha conceived the project, executed the project, and helped in manuscript writing and finalization. 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/fmicb.2019.01308/full#supplementary-material>

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Antifungal Activity and Potential Mechanism of N-Butylphthalide Alone and in Combination With Fluconazole Against *Candida albicans*

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Candida albicans is a common opportunistic fungal pathogen that may cause nosocomial fungal infections. The resistance of *Candida albicans* to traditional antifungal drugs has been increasing rapidly in recent years, and it brings a great challenge in clinical treatment. N-butylphthalide is originally extracted from the seed of *Apium graveolens* and is currently used for the treatment of ischemic stroke in the clinic. This study demonstrated that n-butylphthalide exhibited antifungal activity against *Candida albicans* with minimum inhibitory concentrations of 128 µg/ml; moreover, n-butylphthalide combined with fluconazole showed synergistic antifungal effects against resistant *Candida albicans*, resulting in a decrease in the minimum inhibitory concentrations of fluconazole from >512 to 0.25–1 µg/ml. Time-killing curves verified the antifungal activity in dynamic. Besides, n-butylphthalide exhibited anti-biofilm activity against *Candida albicans*, biofilms preformed <12 h with sessile minimum inhibitory concentrations of 128–256 µg/ml and synergism was observed when n-butylphthalide combined with fluconazole against resistant *Candida albicans* biofilms preformed <12 h, resulting in a decrease in the sessile minimum inhibitory concentrations of fluconazole from >1,024 to 0.5–8 µg/ml. Furthermore, *in vitro* antifungal effects of n-butylphthalide were confirmed *in vivo*. N-butylphthalide prolonged survival rate of larvae infected by *Candida albicans*, reduced the fungal burden in larvae and caused less damage to larval tissues. Notably, n-butylphthalide inhibited hyphal growth and induced intracellular reactive oxygen species accumulation and a loss in mitochondrial membrane potential, which was a potential antifungal mechanism. Besides, the synergistic effects between n-butylphthalide and fluconazole potentially relied on the mechanism that n-butylphthalide significantly promoted drug uptake, and suppressed drug efflux via down-regulating the drug transporter encoding genes *CDR1* and *CDR2*. These findings demonstrated the antifungal effects and mechanisms of n-butylphthalide against *Candida albicans* for the first time, which might provide broad prospects for the identification of new potential antifungal targets.

Keywords: antifungal activity, *Candida albicans*, fluconazole, n-butylphthalide, potential mechanism

INTRODUCTION

Due to the extensive application of broad-spectrum antibiotics, immunosuppressive agents, and medical implant devices, the incidence of fungal infections has increased rapidly in the last few decades (Suleyman and Alangaden, 2016). The leading *Candida* species, *Candida albicans* (*C. albicans*), is the most common fungal pathogen that may cause epidermal and potentially life-threatening invasive infections, especially in immunocompromised patients (Dimopoulos et al., 2007). Fluconazole (FLC), a kind of azoles, is the most frequently used antifungal drug for prevention and treatment of *C. albicans* infections due to the high efficacy and low toxicity. However, drug resistance to antifungals, especially to FLC among *C. albicans* species, increased sharply along with long-term use of it (Whaley et al., 2016). Furthermore, biofilms adhered on the abiotic and biotic surfaces act as the natural barrier to the dispersion of antifungal drugs and are inherently resistant to most antifungal drugs (Bonhomme and d'Enfert, 2013; Desai et al., 2014). Thus, there is an urgent need to develop therapeutic strategies to combat drug resistance of *C. albicans*.

Natural products, especially extracted from traditional Chinese herbal medicine, provide a huge treasure pool for drug discovery by serving as compounds with metabolic activity in their natural form or synthetic modification (Mishra and Tiwari, 2011). It is worth noting that phytocompounds exhibit prominent potential as antifungal agents or as synergistic agents with FLC, particularly against *Candida* spp. (Lu et al., 2017). For example, Shao et al. confirmed that sodium houttuynfonate revealed relatively strong antifungal potential against *C. albicans* (Huang et al., 2015; Shao et al., 2017; Da et al., 2019). N-butylphthalide (NBP) (Figure 1A) is originally extracted from the seed of *Apium graveolens*, and it is a new drug that has been independently researched and developed in China (Zhao et al., 2014). NBP has a wide range of pharmacological effects on cerebrovascular diseases: resisting cerebral ischemia, improving brain cell energy metabolism, and inhibiting thrombosis. Currently, NBP is widely used in the clinic for the treatment of ischemic stroke because of its low toxicity and good safety (Zhao et al., 2014; Abdoulaye and Guo, 2016). Furthermore, it has been confirmed

that compound1 (Figure 1B) and compound2 (Figure 1C) are the structural analogues of NBP and are also extracted from *Apium graveolens* seeds, have antifungal activity against *C. albicans* (Momin et al., 2000; Momin and Nair, 2001). However, at present, there are no reports investigating the antifungal activity of NBP, alone and combined with FLC, against *C. albicans*.

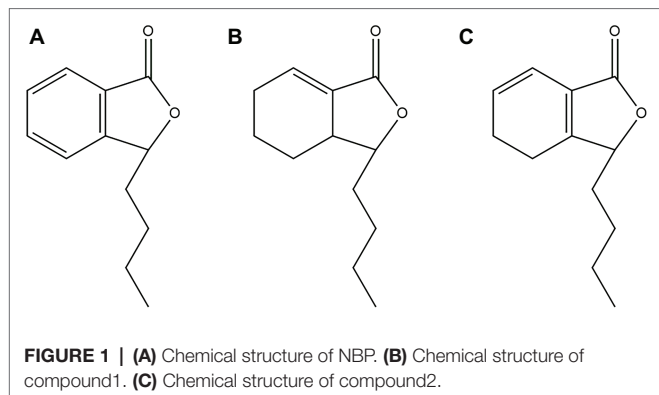
In this study, the *in vitro* antifungal effects of NBP alone and in combinations with FLC against planktonic *C. albicans* and biofilms in different stages (4, 8, 12, and 24 h) were evaluated. The dynamical antifungal effects of NBP were demonstrated by time-killing curves. In addition, *Galleria mellonella* (*G. mellonella*)-*C. albicans* infection model was established and the survival rate, fungal burden, and histopathology were used to evaluate the effects of NBP *in vivo*. For the exploration of underlying mechanism, we investigated the effects of NBP on hyphal growth, the levels of intracellular reactive oxygen species (ROS) and mitochondrial membrane potential ($\Delta\psi_m$). Furthermore, to explore the potential synergistic mechanism of NBP combined with FLC, we conducted rhodamine 6G assays to detect the effects of NBP on drug uptake and efflux of resistant strains. We also carried out real-time quantitative PCR assays (RT-PCR) to determine the gene expression levels of *CDR1*, *CDR2*, and *MDR1*, which encode efflux pump proteins.

MATERIALS AND METHODS

Strains, Culture, and Agents

Six *C. albicans* strains were used in this study, including two FLC-susceptible strains (CA4 and 8) and four FLC-resistant strains (CA10, 16, 103, and 632). CA4, 8, 10, and 16 were collected from the clinical laboratory at Qianfoshan Hospital Affiliated to Shandong University (Jinan, China), and CA103 and 632 were kindly provided by Professor Changzhong Wang (School of integrated traditional and western medicine, Anhui University of traditional Chinese medicine, Hefei, China). Their susceptibilities were determined according to Clinical and Laboratory Standards Institute (CLSI) document M27-A3 (Institute CaLS, 2008a,b). *C. albicans* ATCC 10231, kindly provided by the Institute of Pharmacology, School of Pharmacy, Shandong University (Jinan, China), was used as the quality control strain. CA10 was used as a representative strain for time-killing test, *in vivo* experiment and mechanism exploration. Strains were refreshed from the frozen stocks at -80°C and inoculated at least twice onto sabouraud solid medium for 18 h at 35°C before all experiments. RPMI 1640 (pH 7.0) was used as the liquid medium for diluting drugs and strains.

All drugs (NBP, penicillin sodium and FLC) were purchased from Dalian Meilun Biotech Co. Ltd., China. Stock solution of NBP was dissolved in absolute ethyl alcohol with 0.5% tween80 at a final concentration of 12,800 $\mu\text{g/ml}$. Stock solutions of penicillin sodium and FLC were prepared in sterile distilled water to a final concentration of 2560 $\mu\text{g/ml}$. All stock solutions were stored at -20°C until use.



Determination of Minimum Inhibitory Concentrations of Planktonic Cells

The minimum inhibitory concentrations (MICs) of NBP alone and in combination with FLC against *C. albicans* isolates were determined with a broth microdilution method as described by the CLSI guidelines (Institute CaLS, 2008a,b). The tests were performed in 96-well flat-bottomed microtiter plates. The final concentration of fungal suspension in RPMI 1640 medium was 10^3 CFU/ml, the final concentration of NBP ranged from 4 to 256 μ g/ml and the final concentration of FLC ranged from 0.125 to 64 μ g/ml. All of the wells were filled with RPMI 1640 to a final volume of 200 μ l. A drug-free well served as a growth control, and wells containing RPMI 1640 medium only were set as negative controls. Plates were incubated at 35°C for 24 h. The growth inhibition was determined both by visual reading and by measuring the optical density at 492 nm using a microplate reader. MIC₈₀ was defined as the lowest concentration of drug, alone and in combination that inhibited the growth of yeast by 80% compared with the control group (Lewis et al., 2002; Li et al., 2011; Khan and Ahmad, 2012). The *in vitro* interaction of the drug combination was interpreted in terms of the fractional inhibitory concentration index (FICI) (Odds, 2003). The FICI model was expressed as follows: $FICI = FIC_{FLC} + FIC_{NBP} = (MIC_{80} \text{ of FLC in combination} / MIC_{80} \text{ of FLC alone}) + (MIC_{80} \text{ of NBP in combination} / MIC_{80} \text{ of NBP alone})$. The interpretation of the FICI was defined as FICI of ≤ 0.5 for synergy, FICI > 4.0 for antagonism and $0.5 < FICI \leq 4.0$ for no interaction.

Determination of Sessile Minimum Inhibitory Concentrations of *C. albicans* Biofilms

Sessile MICs (SMICs) of NBP, alone and combined with FLC against *C. albicans* (CA4, 10 and 16), were evaluated as described by Ramage and Lopez-Ribot (2005) with moderate modifications. In brief, biofilms were formed by adding 200 μ l cell suspension (10^3 CFU/ml) into 96-well flat-bottomed microtiter plates over four time intervals (4, 8, 12, and 24 h) at 35°C. At each time point, each well was washed with 200 μ l PBS three times to remove the planktonic and nonadherent cells. Subsequently, drugs of different concentrations were added and the plates were incubated for another 24 h at 35°C. The final concentration of FLC and NBP in wells ranged from 0.125 to 64 μ g/ml and from 4 to 256 μ g/ml, respectively. A metabolic assay based on the reduction of 2,3-bis (2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) was carried out to determine the sMICs. SMIC₈₀ referred to the lowest concentrations, where there was an 80% reduction in the XTT-colorimetric readings compared with the drug-free control (Prazynska and Gospodarek, 2014; Zhong et al., 2017). Colorimetric absorbance was measured at 492 nm in a microtiter plate reader. The FICI model was used to illustrate the interaction between NBP and FLC against *C. albicans* biofilms as described above.

Time-Killing Curve Assay

Groups containing NBP (64, 128, and 256 μ g/ml, respectively), FLC (1 μ g/ml), NBP/FLC (128 and 1 μ g/ml, respectively) and 10^5 CFU/ml of *C. albicans* suspension were then incubated at 35°C with constant shaking (200 rpm). The group with no drug was served as a control growth group. At prearranged time points (0, 6, 12, 24, and 48 h) after incubation, the amount of living cells was then measured by colony counting methods (Li et al., 2008, 2015; Shrestha et al., 2015). For judgment of the interaction between NBP and FLC, synergism was defined as a ≥ 2 lg10 decrease in CFU/ml and indifference as a < 2 lg10 decrease in CFU/ml compared to the most active drug, and antagonism as a ≥ 2 lg10 increase in CFU/ml compared to the least active drug (Li et al., 2014b).

Determination of *in vivo* Antifungal Effects by *G. mellonella* Infection Model

Three *in vivo* experiments, survival assay, fungal burden determination and histological study, were carried out. The initial steps of each experiment were identical. *G. mellonella* larvae during the last instar of the larval development were selected to be absent of dark spots and similar in size (approximately 0.25 ± 0.02 g). Each group contained 20 randomly chosen larvae and they were placed in perish dishes at 35°C overnight before experiments. About 10 μ l of *C. albicans* suspension (10^8 CFU/ml) was inoculated directly to the last left pro-leg. Before injection, the area was swabbed by ethanol for disinfection. After 2 h injection, where four groups of the larvae were injected *via* the last right pro-leg with 10 μ l of sterile PBS, NBP (40 μ g/ml), FLC (160 μ g/ml), and NBP + FLC (40 + 160 μ g/ml), all groups of larvae were incubated at 35°C in the dark (Frenkel et al., 2016; Lukowska-Chojnacka et al., 2016; Li et al., 2017).

For survival assay, four groups of larvae were pretreated as described above. Survival was recorded every day for 4 days. Larva was considered dead if they gave no response to slight touch with forceps.

For fungal burden determination, another four groups of larvae were pretreated as described above. Three larvae were randomly taken from per group daily over 4 days and then homogenized in 3 ml sterile PBS/penicillin sodium using a homogenizer. Subsequently, fungal burden of each group was determined by colony counting methods (Krezdorn et al., 2014).

For histological study, four groups of larvae were pretreated as described above and a group of larvae was treated as the blank group without injectant. After 2 days of incubation, two larvae were taken randomly from each group and then were immersed in 4% paraformaldehyde fixative overnight. Subsequently, larvae were fixed in tissue OCT-freeze medium and cut into 14 μ m tissue sections using a freezing microtome (Gu et al., 2016). The tissue sections then were stained with Periodic acid Schiff (PAS) and were observed under a microscope.

Hyphal Growth Assay

Hyphal growth assay was performed in hypha-inducing media, RPMI1640 and spider medium in the well-plate (Li et al., 2014a;

Haque et al., 2016). *C. albicans* suspension (2×10^5 CFU/ml) was treated with different concentrations of NBP (32, 64, and 128 μ g/ml) at 35°C for 4 h. The group treated without NBP was served as a control group. The cell suspension was then aspirated and each well was washed with 200 μ l PBS to remove the nonadherent cells. The samples were examined under bright field using 20X objective lens by TH4-200 fluorescence microscope (Olympus, Japan) and photographed.

Measurement of Reactive Oxygen Species Levels Assay

The levels of ROS produced by *C. albicans* treated with different concentrations of NBP were measured using the DCFH-DA (MedChem Express, USA). *C. albicans* suspension (5×10^5 CFU/ml) was treated with NBP (32, 64, and 128 μ g/ml) for 4 h and the group treated without NBP was set as a control group. The cells were then washed with PBS, incubated with 40 μ M DCFH-DA in the dark for 30 min and detected by a BD FACS Aria II flow cytometer (Becton Dickinson, USA) with an excitation wavelength at 488 nm and emission wavelength at 530 nm.

Analysis of $\Delta\psi_m$

Rhodamine123 (Rh123, Sigma, USA) was used to examine the effect of NBP on the *C. albicans* $\Delta\psi_m$ in this study (Zheng et al., 2018). The yeast cells were pretreated with different concentrations of NBP as described in “Measurement of ROS levels assay.” The cells were stained then with 15 μ M Rh123 for 30 min in the dark and detected by the flow cytometer with an excitation wavelength at 488 nm and emission wavelength at 530 nm.

Rh6G Uptake and Efflux Assay

The drug uptake and efflux of *C. albicans* were measured by Rh6G assay due to both Rh6G and FLC are substrates of drug transporters (Pina-Vaz et al., 2005). *C. albicans* suspension (10^7 CFU/ml) was first de-energized for 1 h in PBS (without glucose), collected, and resuspended again to obtain the concentration as above.

For Rh6G uptake assay, final concentrations of 10 μ M Rh6G and 32 μ g/ml NBP were added to the de-energized cells simultaneously, and cells without NBP were served as the control group. The mean fluorescence intensity (MFI) of intracellular Rh6G was measured every 10 min for a total of 60 min by the flow cytometer with excitation wavelength at 488 nm and emission wavelength at 530 nm.

For Rh6G efflux assay, Rh6G was added to the de-energized cells suspension at a final concentration of 10 mM. The samples were incubated in a shaking incubator at 35°C for 1 h and later transferred to an ice-water bath for 30 min to stop the uptake of Rh6G. Then cells were collected, washed and resuspended in glucose/PBS (5%). At the same time, NBP at a final concentration of 32 μ g/ml was added and Rh6G alone served as the control group. At special time intervals (0.40, 80, 120, 160, and 200 min), the MFI of intracellular Rh6G was measured using a flow cytometer with excitation wavelength at 488 nm and emission wavelength at 530 nm (Peralta et al., 2012).

TABLE 1 | Primers used in this study.

Genes	Primer sequences (5'→3')
ACT1	F: TGGACGGTGAAGAAGTTGCT R: TTGGATTGGGCTTCATCACCA
CDR1	F: CCATGACTCCTGCTACCGTG R: CCATCGAGACCAACCCACAA
CDR2	F: TGCTGAACCGACAGACTCAG R: GACCAGCCAATACCCACAA
MDR1	F: AGTTGCTTGGGGTAGTCCG R: TGCTCTCAACTTTGGTCCGT

Real-Time Quantitative Polymerase Chain Reaction

C. albicans suspension (5×10^5 CFU/ml) was treated with 32 μ g/ml NBP diluted with sabouraud liquid medium for 16–18 h and the group treated without NBP was set as a control group. *C. albicans* cells were collected, washed, and total RNA was isolated by the E.Z.N.A Yeast RNA kit (e9080, OMEGA). Diluted RNA was then treated with PrimeScript RT reagent kit (RR047A, TaKaRa Biotechnology) to obtain cDNA through a reverse transcription reaction. The thermal cycling condition was 95°C for 30 s as an initial denaturation step, followed by 40 cycles of 95°C for 10 s and 60°C for 34 s and ended by the melting conditions of 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. The expression of each gene was normalized to that of the *ACT1* gene. Drug transporter encoding genes, *CDR1*, *CDR2*, and *MDR1* were determined by the RT-PCR assay as mentioned above. Sequences of the primers are listed in **Table 1**. The result was calculated using the $2^{-(\Delta\Delta Ct)}$ method (Pfaffl, 2001).

Statistics

All experiments were performed at least three times independently. Graphs and statistical analyses were performed with GraphPad Prism 7 (GraphPad, La Jolla, CA) and IBM SPSS Statistics 22 (SPSS, Chicago, IL). All the experimental data measuring by the flow cytometer were analyzed by BD FACSDiva v6.1.3 and FlowJo v7.10.1 software. Fungal burden, rhodamine 6G uptake and efflux and relative expression levels of genes was analyzed using an unpaired *t*-test. The levels of ROS and $\Delta\psi_m$ were analyzed using one-way analysis of variance (ANOVA). *p* < 0.05 was considered significant.

RESULTS

Minimum Inhibitory Concentrations of N-Butylphthalide Alone and in Combination With Fluconazole Against *C. albicans*

The MICs of NBP and FLC, alone and in combination against the six tested *C. albicans* isolates, were listed in **Table 2**. NBP exhibited antifungal activity against *C. albicans* with MICs of 128 μ g/ml, and also exhibited synergistic effects combined

with FLC against resistant *C. albicans* with FICIs of 0.25, resulting in a decrease in the MICs of NBP from 128 to 32 µg/ml and the MICs of FLC from >512 to 0.25–1 µg/ml. Besides, although no synergism was observed with FICIs of >0.5 when NBP combined with FLC against susceptible *C. albicans*, the MICs of NBP could decrease from 128 to 8–64 µg/ml and the MICs of FLC could decrease from 0.5–1 to 0.25–0.5 µg/ml.

Sessile Minimum Inhibitory Concentrations of N-Butylphthalide Alone and Combined With Fluconazole Against *C. albicans* Biofilms

The sMICs of NBP and FLC, alone and in combination against the biofilms formed by *C. albicans* isolates, CA4, 10,

and 16, were listed in **Table 3**. NBP exhibited anti-biofilm activity against *C. albicans* biofilms pre-formed <12 h with sMICs of 128–256 µg/ml, and also exhibited synergistic effects combined with FLC against resistant *C. albicans* biofilms pre-formed <12 h with FICIs <0.5, resulting in a decrease in the sMICs of NBP from 128–256 to 32–64 µg/ml and the sMICs of FLC from >1,024 to 0.5–8 µg/ml. Furthermore, although no synergism was observed when NBP combined with FLC against susceptible *C. albicans* biofilms pre-formed <12 h with FICIs of >0.5, the sMICs of NBP could decrease from 128 to 64 µg/ml and the sMICs of FLC could decrease from >1,024 to 0.5–4 µg/ml. In addition, NBP alone or combined with FLC hardly inhibited mature biofilms pre-formed over more than 24 h, demonstrating the limitation of NBP; being that it only has antifungal activity against immature biofilms.

TABLE 2 | Drugs interactions of NBP and FLC against *C. albicans* *in vitro*.

Strain ^a	MIC ₈₀ (µg/ml) ^b				FICI ^b	Interpretation ^c
	Alone		Combined			
	NBP	FLC	NBP _{comb}	FLC _{comb}		
CA4	128	0.5	8	0.25	0.56	NI
CA8	128	1	64	0.5	1	NI
CA10	128	>512	32	0.25	0.25	SYN
CA16	128	>512	32	1	0.25	SYN
CA103	128	>512	32	0.25	0.25	SYN
CA632	128	>512	32	0.25	0.25	SYN

^aCA, *Candida albicans*.

^bNBP, *n*-butylphthalide; FLC, fluconazole; FICI, fractional inhibitory concentration index.

^cSYN, synergism; NI, no interaction.

MIC₈₀ denotes minimum inhibitory concentration of drug that inhibited fungal growth by 80% compared with the growth control.

MIC₈₀ values and FICIs are shown as the median of three independent experiments.

TABLE 3 | Drugs interactions of NBP and FLC against preformed *C. albicans* biofilms *in vitro*.

Strain ^a	Time(h) ^b	sMIC ₈₀ (µg/ml) ^c				FICI ^c	Interpretation ^d
		Alone		Combined			
		NBP	FLC	NBP	FLC		
CA4	4	128	>1,024	64	0.5	>0.5	NI
	8	128	>1,024	64	0.5	>0.5	NI
	12	128	>1,024	64	4	>0.5	NI
	24	>512	>1,024	>512	>256	1.3	NI
CA10	4	128	>1,024	32	0.5	0.25	SYN
	8	128	>1,024	32	4	0.26	SYN
	12	256	>1,024	64	8	0.27	SYN
	24	>512	>1,024	>512	>1,024	2	NI
CA16	4	128	>1,024	32	0.5	0.25	SYN
	8	128	>1,024	32	2	0.25	SYN
	12	256	>1,024	64	4	0.26	SYN
	24	>512	>1,024	>512	>1,024	2	NI

^aCA, *Candida albicans*.

^bTime indicates incubation period of preformed biofilms.

^cNBP, *n*-butylphthalide; FLC, fluconazole; FICI, fractional inhibitory concentration index.

^dSYN, synergism; NI, no interaction.

sMIC₈₀ denotes sessile minimum inhibitory concentration of drug that produced an 80% reduction of biofilms metabolic activity compared with the growth control. sMIC₈₀ values and FICIs are shown as the median of three independent experiments.

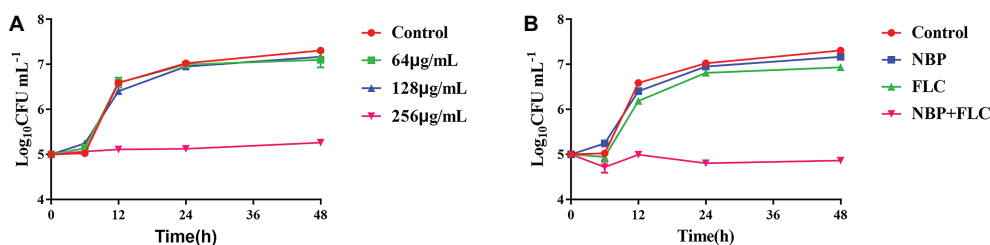


FIGURE 2 | Time-killing curves of NBP alone (A) and in combination with FLC (B) against *C. albicans*. The initial yeast concentrations were adjusted to 10^6CFU/mL . (A) The concentrations of NBP were 64, 128, and 256 $\mu\text{g/mL}$, respectively. (B) The concentrations of NBP were 128 $\mu\text{g/mL}$ when combined with FLC (1 $\mu\text{g/mL}$). Values represent the means $\pm$ standard deviation of three replicates.

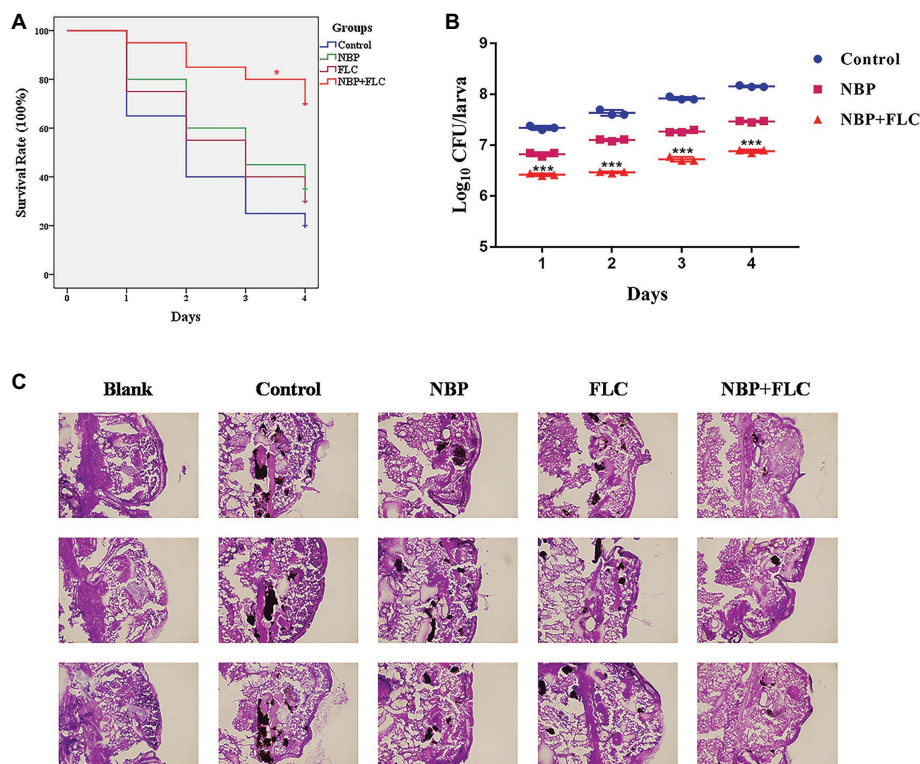


FIGURE 3 | Effect of NBP in combination with FLC during *G. mellonella* infection with *C. albicans*. Larvae were injected with the inoculum of a cell concentration of 10^6CFU/larva and then treated with PBS (control), FLC (160 $\mu\text{g/mL}$), NBP (40 $\mu\text{g/mL}$), or FLC (160 $\mu\text{g/mL}$) combined with NBP (40 $\mu\text{g/mL}$). (A) Survival curve of *G. mellonella* infected with *C. albicans*. Values represent the means $\pm$ standard deviation of three replicates. * $p < 0.05$ compared with the NBP-treated group (Kaplan-Meier test). (B) Fungal burden of *G. mellonella* infected with *C. albicans*. Data for the treatment of FLC monotherapy group were not showed because the data were similar with that of the NBP group. Values represent the means $\pm$ standard deviation of three replicates. *** $p < 0.001$ compared with the NBP-treated group (unpaired *t*-test). (C) Histopathology of *G. mellonella* infected with *C. albicans*. Larvae of the blank group were treated with neither *C. albicans* nor drugs. Melanized nodules containing yeast clusters and filaments could be observed except in blank group. The photographs were collected from three independent experiments.

Time-Killing Curves

The results showed that, in the presence of 256 $\mu\text{g/mL}$ NBP, a significant enhancement in the degree of antifungal activity was observed after 6 h, and there was a $1.91/2.05 \text{ log}_{10} \text{CFU mL}^{-1}$ decrease at 24/48 h time point compared with the control group (Figure 2A). For the drug combination experiment, a fungal growth delay could be seen in the FLC alone group, however, it was more evident in the combination group and there was a $2.01/2.07 \text{ log}_{10} \text{CFU mL}^{-1}$ decrease at 24/48 h time

point compared with the FLC alone group (Figure 2B), indicating a synergistic antifungal effect in dynamic.

Antifungal Effects of N-Butylphthalide Against *C. albicans* in vivo

The survival rate is the most important index to evaluate the effect of drugs *in vivo* with *G. mellonella* infection model. After 4 days of incubation, the control group showed a survival rate of 20%, while NBP group showed a survival rate of 35%,

which was higher than the control group. The combinations of NBP and FLC significantly enhanced the survival rate to 70% compared with the NBP group ($p < 0.05$) (Figure 3A).

The fungal burden analysis suggested that the larval fungal burden increased gradually over 4 days after injection in all groups. Treatment with NBP slightly decreased fungal burden than the control group and data of FLC monotherapy group were not shown in figure because the data were similar with those of the NBP monotherapy group. The combination of NBP and FLC significantly reduced fungal burden compared with the NBP group (p 's of all 4 days was <0.001), especially in the last 3 days (Figure 3B).

Histopathology studies (Figure 3C) revealed that in comparison with the blank group, the *C. albicans* cells mainly existed in the form of filamentous clusters in larval tissues after infection. In the drug monotherapy groups, although the amounts of melanized nodules were similar with the control group, the size of these melanized nodules of these two groups was smaller than those of the control group. However, only few small melanized nodules were discovered in the drug combination group.

Effects of N-Butylphthalide on *C. albicans* Hyphal Growth

C. albicans hyphae induced by RPMI 1640 medium and spider medium were shown in Figures 4, 5, respectively. As shown,

C. albicans could form long and interlaced hyphae in both RPMI 1640 and spider medium, and NBP inhibited *C. albicans* hyphal growth in both media tested in a dose dependent manner. 32 $\mu\text{g/ml}$ NBP could lead to form slightly shorter hyphae than the control group. 64 $\mu\text{g/ml}$ NBP could induce to form loose and patchy hyphae, while when the concentration increased to 128 $\mu\text{g/ml}$, cells were mainly maintained as yeasts and few filament could be observed in the field of vision.

Effects of N-Butylphthalide on Reactive Oxygen Species Production

The generation of excessive ROS is considered as a potential fungicidal mechanism and we measured the levels of intracellular ROS after treated with NBP. The results showed that NBP significantly induced intracellular ROS accumulation of *C. albicans* in a dose-dependent manner ($p < 0.001$) (Figure 6). The levels of ROS resulted in approximately 165, 245, and 283% increases in MFI after treated with 32, 64, and 128 $\mu\text{g/ml}$ NBP, respectively, compared with that of control group.

Effects of N-Butylphthalide on $\Delta\psi_m$

To determine whether the NBP induced ROS accumulation was involved in the metabolic state of mitochondria, $\Delta\psi_m$ treated with NBP was measured. As shown in Figures 7A–D, NBP significantly reduced the number of *C. albicans* cells with an intact $\Delta\psi_m$ and increased the number of cells with

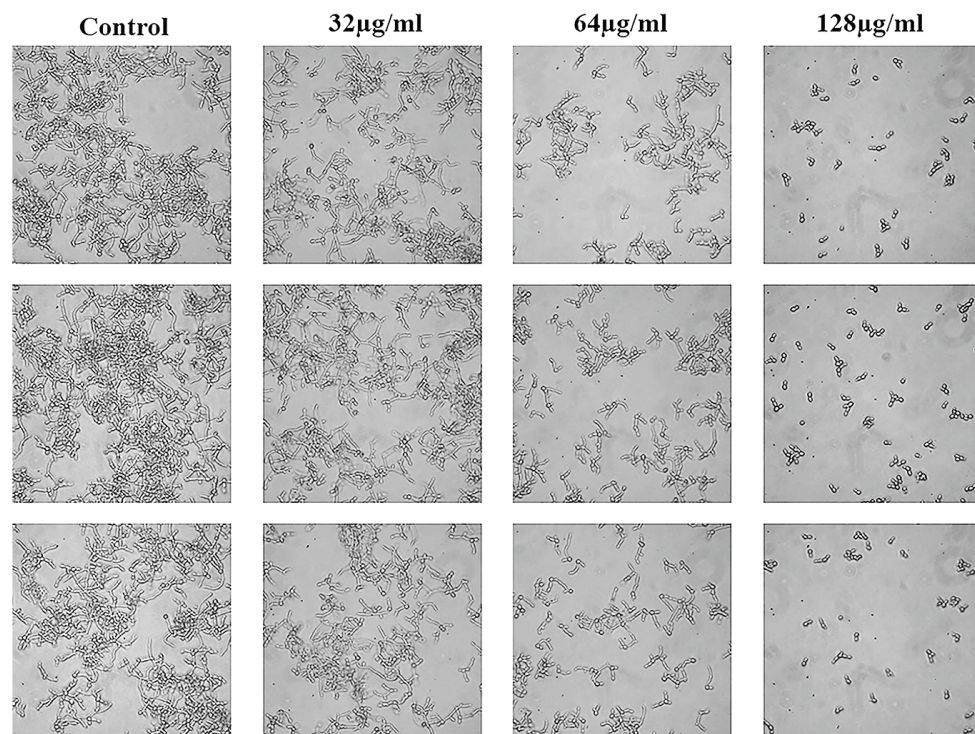


FIGURE 4 | Effect of NBP on *C. albicans* hyphal growth induced by RPMI 1640 medium. NBP was diluted in hyphae-inducing media, RPMI 1640 medium, at a final concentration of 0, 32, 64, and 128 $\mu\text{g/ml}$, respectively. The cellular morphology was photographed after incubation at 37°C for 4 h. The photographs were collected from three independent experiments.

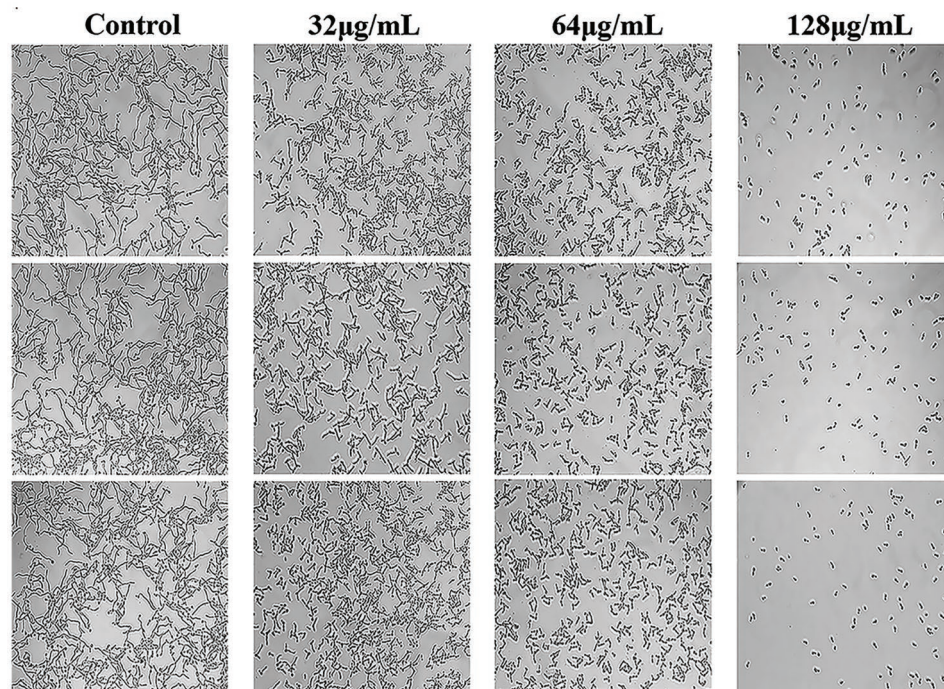


FIGURE 5 | Effect of NBP on *C. albicans* hyphal growth induced by spider medium. NBP was diluted in hyphae-inducing media, spider medium, at a final concentration of 0, 32, 64, and 128 $\mu\text{g}/\text{mL}$, respectively. The cellular morphology was photographed after incubation at 37°C for 4 h. The photographs were collected from three independent experiments.

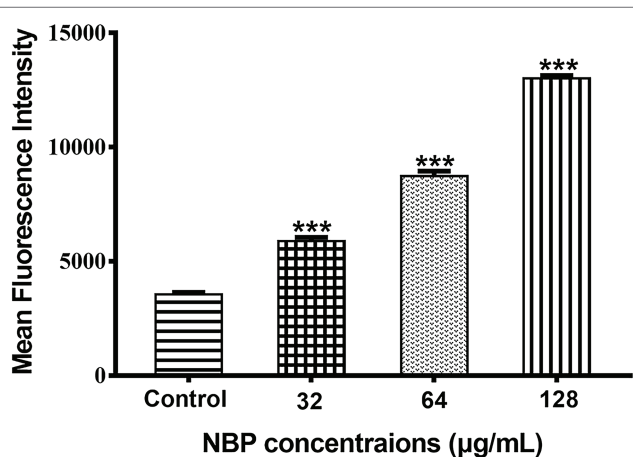


FIGURE 6 | Effect of NBP on the levels of ROS in *C. albicans*. Yeast cells pretreated with diverse concentrations of NBP (0, 32, 64, and 128 $\mu\text{g}/\text{mL}$) for 4 h were incubated with 40 μM DCFH-DA and then analyzed by a flow cytometer. Values represent the means $\pm$ standard deviation of three replicates. *** $p < 0.001$ compared with the control group (one-way ANOVA).

low $\Delta\psi_m$ in a dose dependent manner. The percentage of *C. albicans* with reduced $\Delta\psi_m$ increased from 2.49% in the control group to 8.93, 23.5, and 25.5% at 32, 64, and 128 $\mu\text{g}/\text{mL}$ NBP, respectively. Besides, NBP obviously reduced intracellular Rh123 MFI of *C. albicans* in a dose dependent manner (Figure 7E), also suggesting a decrease in $\Delta\psi_m$. Thus, NBP

induced a loss in $\Delta\psi_m$ of *C. albicans* and consequently caused mitochondrial dysfunction.

Effects of N-Butylphthalide on Drug Uptake and Efflux of *C. albicans* and Drug Transporters Genes

In the drug uptake experiment, NBP significantly increased drug absorption after 20 min and cells in the NBP-treated group absorbed higher concentrations of Rh6G than the control group ($p < 0.001$) (Figure 8A). In the drug efflux experiment, after treated with glucose solution, the MFI both in the control group and NBP-treated group showed a downward trend. However, NBP evidently suppressed the decrease especially after 120 min ($p < 0.001$) and cells treated with NBP pumped out much lower concentration of Rh6G compared with the control group in 200 min (Figure 8B). Subsequently, gene expression experiments confirmed that NBP significantly down-regulated the expression levels of genes, *CDR1* and *CDR2*, that encode *C. albicans* drug resistant protein (Cdrp), however, for the expression level of *MDR1*, there was no difference between the NBP-treated group and the control group (Figure 9). In a word, the results of Rh6G assay and RT-PCR method suggest that the synergistic antifungal effect of NBP and FLC was related to promote drug uptake and reverse drug efflux *via* down-regulating drug transporters genes, *CDR1* and *CDR2*.

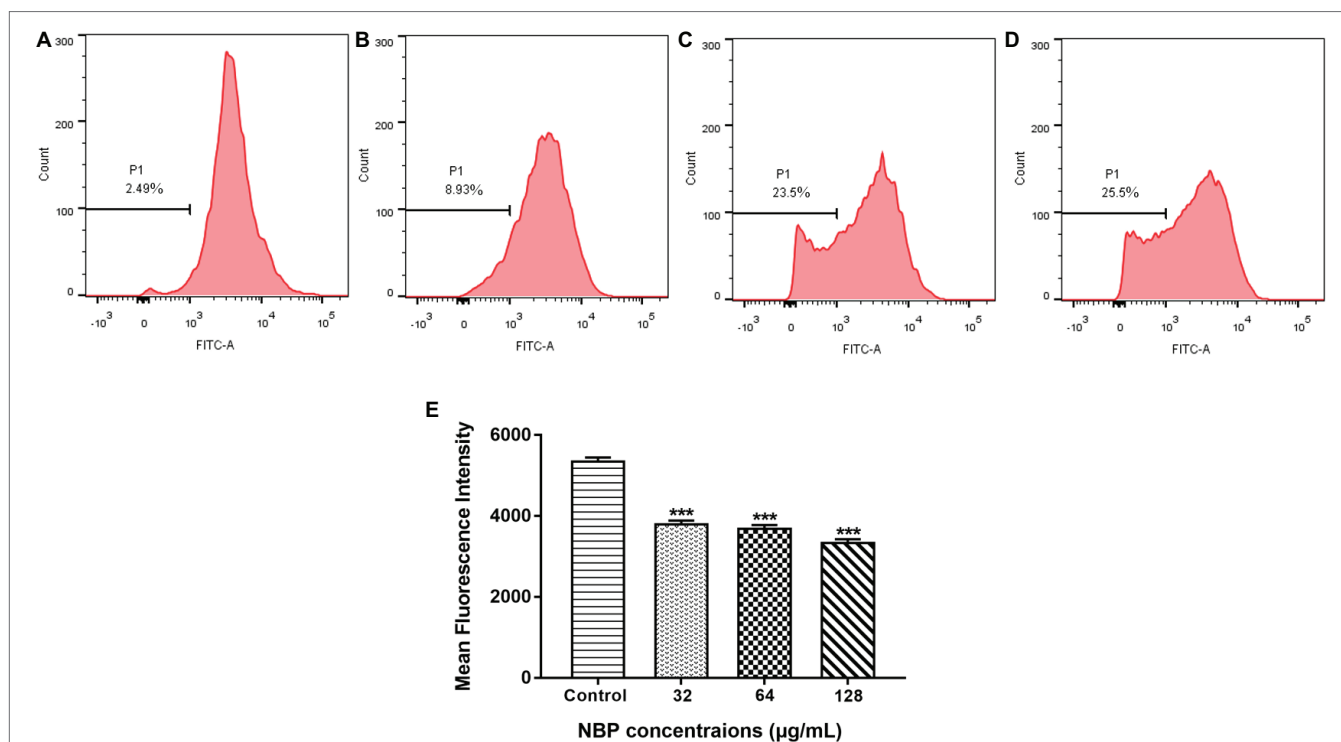


FIGURE 7 | Effect of NBP on the $\Delta\psi_m$ of *C. albicans*. Yeast cells pretreated with diverse concentrations of NBP (0, 32, 64, and 128 $\mu\text{g/ml}$) for 4 h were incubated with 15 μM Rh123 and then analyzed by a flow cytometer. (A–D) Effect of different doses of NBP on $\Delta\psi_m$ loss in *C. albicans*. (A) Negative control, (B) 64, (C) 128, (D) 256 $\mu\text{g/ml}$. Representative images from three independent experiments are shown. The percentages of cells in the left section of the histogram refer to the number of cells with depolarized mitochondria. (E) Effect of NBP on the mean fluorescence intensity of Rh123-stained *C. albicans*. *** $p < 0.001$ compared with the control group (one-way ANOVA).

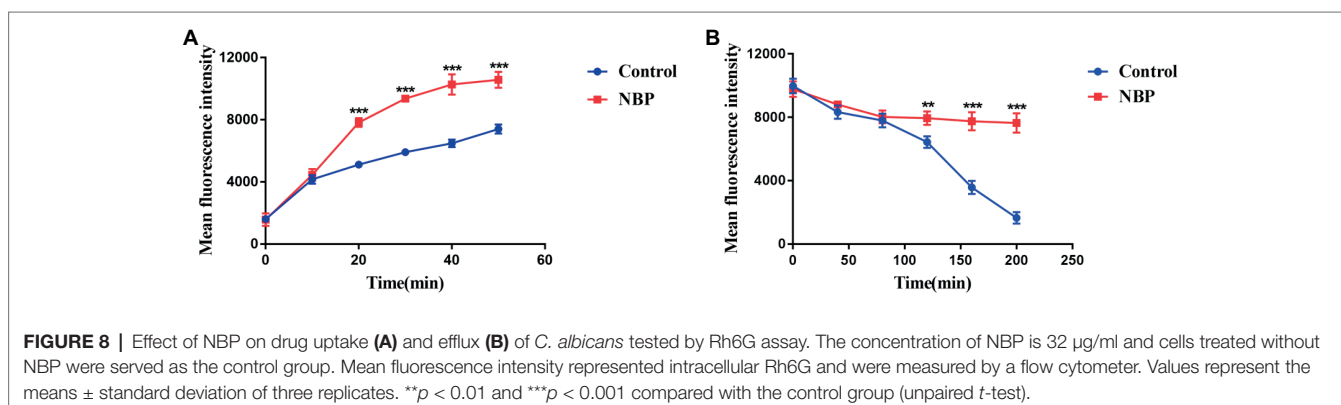
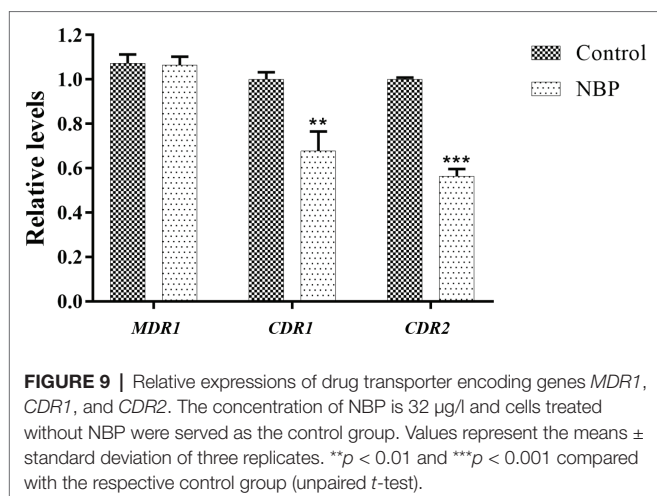


FIGURE 8 | Effect of NBP on drug uptake (A) and efflux (B) of *C. albicans* tested by Rh6G assay. The concentration of NBP is 32 $\mu\text{g/ml}$ and cells treated without NBP were served as the control group. Mean fluorescence intensity represented intracellular Rh6G and were measured by a flow cytometer. Values represent the means $\pm$ standard deviation of three replicates. ** $p < 0.01$ and *** $p < 0.001$ compared with the control group (unpaired t -test).

DISCUSSION

Treatment of fungal infections, especially candidiasis, is still a challenging problem due to the rising isolation rates of resistant strains (Pfaffer and Diekema, 2007). In our previous studies, we focused on studying the mechanism of drug resistance in *C. albicans* and exploring more non-antifungal drugs with antifungal activity to overcome drug resistance of *C. albicans*, especially sensitizers of traditional antifungal drugs, such as antibiotics, glucocorticoid and calcium channel blockers (Liu et al., 2016; Sun et al., 2017; Lu et al., 2018).

In this study, we demonstrated NBP, a component of *Apium graveolens* seeds and currently used for the treatment of ischemic stroke in clinic, exerted antifungal activity against *C. albicans* with MICs of 128 $\mu\text{g/ml}$ and NBP combined with FLC showed synergistic effects against resistant *C. albicans*, leading to a decrease in the MICs of FLC from >512 to 0.25–1 $\mu\text{g/ml}$. Besides, time-killing curves provided figures that described dynamic antifungal effects of NBP alone and synergistically combined with FLC on *C. albicans*, which was in accordance with those from broth microdilution assays.



Biofilms, consisting of complicated communities of microorganisms embedded in cells-derived matrix, build a heterogeneous and natural drug-tolerant environment (Bonhomme and d'Enfert, 2013). *C. albicans* biofilms, especially formed on medical implants, are common during *C. albicans* infections and pose a great challenge to clinical treatment due to the intrinsic resistance to most antifungal drugs (Mathe and Van Dijck, 2013). We demonstrated that NBP exerted anti-biofilm activity against *C. albicans* biofilms pre-formed <12 h with sMICs of 128–256 µg/ml and NBP combined with FLC showed synergistic effects against resistant *C. albicans* biofilms pre-formed <12 h, leading to a decrease in the sMICs of FLC from >1,024 to 0.5–8 µg/ml. These results indicated that NBP has broad prospects in prevention and treatment of immature *C. albicans* biofilms related infections.

The *G. mellonella* model, whose immune response is similar with that of mammals, is used as an infection model host and *Galleria mellonella*-*Candida albicans* infection model is widely applicable to rapidly evaluate the efficacy of drugs against *C. albicans* infections *in vivo* (Li et al., 2013; Aneja et al., 2016). Compared with mammalian model, *G. mellonella* model has many advantages, such as significant ethical, economical, accessible and easy manipulative (Mylonakis et al., 2007; Vilcinskis, 2011). Besides, the *G. mellonella* larvae infection model belongs to invertebrate and does not require ethical approval. In the present study, we found that NBP monotherapy enhanced survival rate of *C. albicans* infected larvae, cleared more *C. albicans* cells in larvae and caused less damage to larval tissues compared with the control group, while the combined treatment of NBP and FLC exhibited a better therapeutic effects. Acute toxicity test indicated that median lethal dose of NBP to mice was much higher than the effective dose; moreover, chronic toxicity test excluded chronic adverse events during long-term application of NBP (Tian et al., 2016). The doses (0.4 µg/larva for NBP and 1.6 µg/larva for FLC) used to treat larvae was converted from those used to treated human beings. Thus, in consideration of therapeutic effects on *G. mellonella* infection model and good safety of NBP, it is promising for application of NBP combined with antifungal

drugs against *C. albicans* infections, while more researches are needed to be carried out in future.

For the potential mechanisms exploration, we first measured the effects of NBP on hyphal growth. As a dimorphic fungus, *C. albicans* proliferated in either a yeast form or a hypha form (Wilson et al., 2016). Hyphae are crucial components of *C. albicans* biofilms and required for virulence and pathogenicity, contributing to adhesion and invasion of host cells (Whiteway and Bachewich, 2007). The results showed that the morphological characteristics of *C. albicans* hyphae, induced by both RPMI 1640 and spider medium, were similar and NBP inhibited *C. albicans* hyphal growth in a dose-dependent manner. Hyphae induced by 64 µg/ml NBP were shorter and looser than that of control group, while 128 µg/ml NBP obviously inhibited the yeast-to-hypha morphological transition and *C. albicans* were mainly maintained in yeast form. Thus, NBP exhibited antifungal activity *via* inhibiting hyphal growth, attenuating virulence factors and reducing invasiveness and pathogenicity.

Generation of ROS regulates the process of apoptosis in eukaryotes, leading to enzyme inactivation, cells dysfunction and subsequent cell death (Perrone et al., 2008; Chen et al., 2013). Mitochondria is a major subcellular source of intracellular ROS. $\Delta\Psi_m$ plays a coupling role in mitochondrial oxidative phosphorylation and its stability is beneficial to maintain regular physiological functions of cells (Dai et al., 2015; Zhu et al., 2015). In this study, NBP significantly induced *C. albicans* intracellular ROS accumulation and a loss in *C. albicans* $\Delta\Psi_m$ in a dose-dependent manner. Recent studies showed the induction of excessive ROS in *C. albicans* was a critical factor in cell death induced by many antifungal drugs (Chen et al., 2013; Chang et al., 2017). Depolarization of the mitochondrial membrane indicates the change of $\Delta\Psi_m$ and the subsequent flow of the outer membrane is also a critical stage in the intrinsic apoptosis pathway (Chen et al., 2015; Wang et al., 2015). Thus, the results suggested NBP induced *C. albicans* cells death was probably triggered by stimulated intracellular ROS accumulation and dysfunction of mitochondrion, finally leading to cell death through mediation of apoptosis.

It is extensively accepted that resistance of *C. albicans* to most antifungal drugs is partly mediated by the efflux mechanism. The non-specificity of drug efflux pump transporters possibly explains the phenomenon of cross-resistance between diverse antifungal drugs (Pina-Vaz et al., 2005). Generous studies demonstrated that inhibiting the activity of drug efflux pumps might be a non-negligible mechanism to illustrate the synergism of interactions between non-antifungal drugs and conventional antifungal drugs (Pina-Vaz et al., 2005; Li et al., 2017). In this study, the results suggested that NBP significantly promoted drug uptake of *C. albicans*. Indeed, *C. albicans* could agglomerate and adhere to form biofilms that are important biological barrier for drug diffusion and inherent resistant to most antifungal therapy. With the formation of biofilms, cell viscosity increases, and drug diffusion slows down. NBP could inhibit the *C. albicans* biofilms, which may be one of the reasons why NBP promotes drug uptake by destroying the physical barrier of biofilms. Subsequently, it was demonstrated that NBP suppressed drug efflux, led to high levels of FLC concentrations

in fungal cells and then possibly increased the susceptibility of *C. albicans* to FLC. RT-PCR method further confirmed that NBP down-regulated the expressions of drug transporters genes *CDR1* and *CDR2*. Thus, the synergistic effect of NBP and FLC relied on promoting drug uptake and reversing the mechanism of drug efflux targeting drug transporters genes *CDR1* and *CDR2*.

In summary, NBP alone exhibited antifungal activity against both planktonic *C. albicans* and biofilms. Strong synergism was observed when NBP combined with FLC against resistant *C. albicans* and biofilms pre-formed by resistant strains. Time-killing curves confirmed antifungal effects of NBP in dynamic. The antifungal activity of NBP was further confirmed *in vivo* with *G. mellonella* infection model. Mechanism researches showed that NBP could inhibit the hyphal growth, induced intracellular ROS accumulation and caused mitochondrial dysfunction. Besides, the mechanism of synergism between NBP and FLC relied on promotion of drug uptake, suppression drug efflux by down-regulating drug transporters genes *CDR1* and *CDR2*. To the best of our knowledge, this study is the first to elucidate the antifungal activity of NBP both *in vitro* and *in vivo* and explore the potential mechanisms. These findings might provide insights into the possible therapeutic application of NBP as antifungal agents or sensitizers of traditional antifungal drugs in the future.

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AUTHOR CONTRIBUTIONS

YG, WL, and SS designed the experiments. YG and YL performed the experiments. YG, XH, and LH interpreted the data. YG and SS wrote the manuscript. All authors approved the manuscript for publication.

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Variability of the Ability of Complex Microbial Communities to Exclude Microbes Carrying Antibiotic Resistance Genes in Rabbits

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Reducing antibiotic use is a necessary step toward less antibiotic resistance in livestock, but many antibiotic resistance genes can persist for years, even in an antibiotic-free environment. In this study, we investigated the potential of three fecal complex microbial communities from antibiotic-naïve does to drive the microbiota of kits from antibiotic-exposed dams and outcompete bacteria-carrying antibiotic-resistant genes. The fecal complex microbial communities were either orally delivered or simply added as fresh fecal pellets in four to five nests that were kept clean from maternal feces. Additionally, four nests were cleaned for the maternal feces and five nests were handled according to the common farm practice (i.e., cleaning once a week) as controls. At weaning, we measured the relative abundance of 26 antibiotic resistance genes, the proportion of *Enterobacteriaceae* resistant to tetracycline and sulfonamide antibiotics, and the taxonomic composition of the microbiota by sequencing the 16S rRNA genes of one kit per nest. Changing the surrounding microbes of the kits can hinder the transmission of antibiotic resistance genes from one generation to the next, but the three communities widely differed in their ability to orient gut microbes and in their impact on antibiotic resistance genes. The most efficient delivery of the microbial community reduced the proportion of resistant *Enterobacteria* from 93 to 9%, decreased the relative abundance of eight antibiotic resistance genes, and changed the gut microbes of the kits at weaning. The least efficient did not reduce any ARG or modify the bacterial community. In addition, adding fecal pellets was more efficient than the oral inoculation of the anaerobic suspension derived from these fecal pellets. However, we were unable to predict the outcome of the exclusion from the data of the donor does (species composition and abundance of antibiotic resistance genes). In conclusion, we revealed major differences between microbial communities regarding their ability to exclude antibiotic resistance genes, but more work is needed to understand the components leading to the successful exclusion of antibiotic resistance genes from the gut. As a consequence, studies about the impact of competitive exclusion should use several microbial communities in order to draw general conclusions.

Keywords: rabbit, gut microbiota, antibiotic resistance gene, 16S sequencing, competitive exclusion

INTRODUCTION

Microbes exposed to antibiotics in livestock may develop resistance mechanisms, including a variety of targets such as efflux systems, drug modifiers or changes in the target's configuration (Davies and Davies, 2010). As a matter of fact, the abundance of antibiotic resistance genes (ARGs) in the gut of livestock varies between countries. For example, Chinese pigs have more ARGs than their French conspecifics (Xiao et al., 2016). Furthermore, the antibiotic resistance genes found in the human gut seem to coincide with the antibiotics authorized for livestock in Spain and Denmark (Forslund et al., 2013), thereby raising the question of ARGs potentially transferred from bacteria inhabiting the gut of livestock to bacteria inhabiting the human gut.

To make matters worse, livestock harboring antibiotic resistance genes in their gut could represent a constant potential threat to public health despite the constant decrease of antibiotic use. Indeed, some ARGs incur almost no fitness cost for the bacteria. For example, *Campylobacter* spontaneously evolved a resistance against macrolides and was not outcompeted by its sensitive kin in an antibiotic-free environment (Zeitouni et al., 2012). In human volunteers, some ARGs may persist for 2 years after the selection pressure via antibiotics, so that the commensal microbiota might be a major reservoir of ARGs (Jernberg et al., 2007, 2010).

Considering that dams do receive antibiotics, the ARG persistence in the gut microbiota of dams implies that the kits could inherit antibiotic-resistant microbes that persist until slaughter at 63 days, even if the kits are raised without antibiotics. It should be noted that dams are particularly exposed to antibiotics. For example, a French doe during a reproductive cycle receives on average 1.4 ± 0.7 antibiotic treatments per day, while a growing rabbit receives 0.8 ± 0.3 (Fortun-Lamothe et al., 2011) during the fattening period.

Luckily, the sensitivity of the kits' microbiota to their surrounding microbes could also be the key to exclusion-based approaches. The idea of using a complex microbial community to remediate against a bacterial species of interest was first described in 1973 (Nurmi and Rantala, 1973) to outcompete *Salmonella* in broilers. Competitive exclusion was also successfully adapted to the exclusion of antibiotic-resistant *E. coli* in broilers (Hofacre et al., 2002; Nuotio et al., 2013) and in mice after an antibiotic treatment (Ubeda et al., 2013). Today, fecal transplantation using complex microbial communities is also used in hospitals to cure *Clostridium difficile* infections (Chapman et al., 2016) and lyophilization protocols are developed to facilitate its routine use (Staley et al., 2017). However, studies using complex microbial communities for competitive exclusion remain relatively scarce for mammalian livestock and it is difficult to draw general conclusions. For example, *Salmonella* could be outcompeted *in vitro* from communities taken from pigs (Anderson et al., 1999; Genovese et al., 2003) but microbial communities used for competitive exclusion have also been reported to sometimes increase the antibiotic resistance of *E. coli in vivo* (Kim et al., 2005).

In this study, we investigated the potential of three fecal complex microbial communities from antibiotic-naïve does to drive the microbiota of kits from antibiotic-exposed dams and to outcompete bacteria carrying antibiotic resistant genes. The fecal complex microbial communities were either orally delivered or simply added as fresh fecal pellets in the nest. We measured the antibiotic resistance genes, the *Enterobacteriaceae* resistant to antibiotics, and the taxonomic composition of the microbiota of the kits at weaning.

MATERIALS AND METHODS

Animals

The kits for which the competitive exclusion was performed came from 37 gravid dams (labeled D1 to D40) that were acquired from a French rabbit farm for which the antibiotic history was available. The dams were selected so that they all received diclazuril (coccidiostat), tilmicosin (macrolide), sulfadimethoxine (sulfonamide), oxytetracycline and trimethoprim. They also received a subcutaneous injection of 23 mg/kg of sulfadimethoxine and 5 mg/kg trimethoprim twice a day for three days and 100 mg/kg oxytetracycline by a single subcutaneous injection 25 days before their due date. They arrived in the animal facility of the French National Institute for Agricultural Research (INRA) 18 days before their parturition. In contrast, the three non-gravid "donor" does (D41, D43 and D44) producing the competitive microbial communities were from farms that have banned the use of antibiotics for at least 5 years.

Suspensions of the Competitive Microbial Communities From Source Does

The fecal suspensions tested for competitive exclusion were prepared less than 2 h before their oral administration: for each donor doe, five fecal pellets (approx. 5 g) were added to a bottle containing 5 g of 1-mm glass beads and 40 mL 0.9% NaCl. After the bottle was sealed with a septum, oxygen was purged through a 0.1-mm needle via five cycles of pumping at -700 mbar and filling with 500 mbar N_2 . The content was then homogenized using vigorous shaking.

Growth Environment of the Animals, Competitive Microbial Exposure and Sampling

Gravid dams were fed with a standard diet (Aliment Lactation, Hycole, France) until weaning. They were raised in cages measuring $100 \times 42 \times 60$ cm that included 24×42 cm nests that could be closed by a sliding door. Suckling was allowed from 8 to 8:30 am for the first 21 days. From Day 1 to 21, dams had no access to their kits other than during the suckling window, which is common practice until Day 7 for meat rabbit farming in France. The litters were equilibrated to 6–8 kits per litter at Day 1. The 37 dams that had already given birth (Day 0) were divided into eight groups. The kits in the control group were raised as usual; the kits from the "No Feces" control group

(i.e., “Control NF”) were raised as usual except that we removed all maternal fecal pellets after suckling up to Day 21. For the kits of the six treated groups, we also removed the maternal fecal pellets after suckling but we put them in contact with the fecal microbes of a “donor” Doe (D41, D43 or D44) until Day 29. Each donor doe was kept individually in cages located in different rooms for the whole duration of the experiment. The groups I41, I43, and I44 (Inoculation from D41, D43, and D44, respectively) received the microbial suspensions prepared as described above by letting them suck 100 μ l from a syringe every second day between 10 and 11 am from Day 2 to 29. Gloves and syringes were changed between each cage to avoid cross-contamination. For the groups P41, P43, and P44 (fecal pellets from D41, D43, and D44, respectively), we put five fresh fecal pellets from the relevant donor doe in the nest containing the kits. At Day 21, the nests were opened and kits could freely roam in the whole cage and interact with the dam until Day 36. At Day 35, one kit per cage was anesthetized via an intramuscular injection of 80 mg ketamine/kg (Chlorketam 1000, Vétquinol, France) followed by an intracardiac injection of 182 mg pentobarbital/kg (Doletal, Vétquinol, France), causing death. The experiments were conducted under the agreement number APAFIS-2018021215568441 for animal experimentation from the French Ministry of Agriculture (Ethics Committee C2EA-86). The fecal pellets were immediately recovered from the digestive tract and a 100-mg subsample was placed in dry ice and then stored at -80°C for DNA extraction. Another 500-mg subsample was mixed with 3 mL of sterile phosphate-buffered peptone water containing 30% w/w glycerol and immediately mixed with a pestle by hand. The resulting homogenate was plated on MacConkey medium to isolate *Enterobacteriaceae*, and 10 colonies per sample were stored at -80°C in order to later test their resistance to antibiotics. At Day 3, 17, and 35, fresh fecal pellets from maternal and the three donor does were recovered and placed in dry ice. A 2-g subsample was homogenized with a bagmixer (90 s, setting 8) for isolation and storage of *Enterobacteriaceae*, as previously described for kit fecal samples. At Day 35, a fecal pellet from each doe was immediately stored at -80°C for DNA extraction.

Testing Antibiotic Resistance of *Enterobacteriaceae* Isolates

The antibiotic susceptibility of *Enterobacteriaceae* isolates collected from the does or the kits was tested by plating a 24-h pure culture onto Mueller-Hilton agar supplemented with 16 $\mu\text{g/mL}$ tetracycline or 128 $\mu\text{g/mL}$ sulfamethoxazole according to the ECOFF *E. coli* epidemiological cut-off values defined by Eucast (eucast, 14/05/2015). Each test included a resistant and sensitive control strain to confirm that the antibiotic was active and that the incubation conditions allowed growth. The average percentage of antibiotic-resistant *Enterobacteriaceae* isolates was calculated for each lactating doe (maximum of 30 isolates with 10 isolates per sampling day) and for their nest (maximum of 30 isolates with 10 isolates for one kit per nest). When the number of isolates was too low to calculate an average percentage ($n < 10$ for the doe or the nest) and/or when the average percentage

of antibiotic-resistant *Enterobacteriaceae* of the lactating doe was less than 50%, we excluded the maternal doe/kit couple from the analysis.

DNA Extraction and Detection of Antibiotic Resistance Genes

The microbial DNA was extracted at weaning from the three donors, the 37 dams and the 37 kits using the ZR-96 Fungal/Bacterial DNA KitTM in which 80 mg of frozen feces were processed according to the manufacturer's instructions (Zymo Research Corp, Irvine, CA, United States). The microbial DNA was also extracted from the three donor does at 16 days for ARG detection. The DNA concentration was measured using the Quant-iTTM PicoGreenTM dsDNA Assay Kit according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, United States) and subsequently diluted to 10 ng/ μ l.

High throughput real-time qPCR was performed using the Biomark microfluidic system from Fluidigm (San Francisco, CA, United States) using a 96.96 Dynamic ArrayTM Integrated Fluidic Circuit (IFC). Pre-amplification of the samples, chip loading and qPCR reactions in nanoliter volumes were performed according to the manufacturer's protocol. A pre-amplification step was applied to all samples for all primer sets except the *Zhu16S* primer set. Briefly, 13 ng of total DNA were submitted to 14 PCR cycles using the PreAmp Master Mix (Fluidigm) and a mix of primers (50 nM final concentration). Pre-amplified samples were 5-fold diluted after an exonuclease treatment. The diluted pre-amplified samples and the primer sets were loaded in a 96.96 IFC using an IFC Controller HX (Fluidigm). The Biomark thermal protocol was as follows: a thermal mix step (50°C , 2 min; 70°C , 30 min; 25°C , 10 min), a hot start (50°C , 2 min; 95°C , 10 min), 35 cycles of PCR (95°C , 15 s; 60°C , 60 s), and a final melting phase (60°C to 95°C). The list of primers can be found in **Supplementary Table S1**. The relative quantities for each gene was interpolated using a generated standard curve build from serial dilutions with Fluidigm real-time PCR analysis software (v4.3.1) **Supplementary Figure S9** and compared to the negative control. The abundances of each ARG relative to the 16S rRNA genes were then used for further analysis.

Microbial Community Analysis by 16S rRNA Gene Sequencing

The V3V4 region was amplified from the 77 purified genomic DNA with the primers F343 and R784 (**Supplementary Table S1**) using 30 amplification cycles with an annealing temperature of 65°C . Because MiSeq (chemistry v3) enables paired 250-bp reads, the ends of each read are overlapped and can be stitched together to generate extremely high-quality, full-length reads of the entire V3 and V4 region in a single run. Single multiplexing was performed using 6 bp indexes developed in-house, which were added to R784 during a second PCR with 12 cycles using indexing forward primer and reverse primer. The resulting PCR products were purified and loaded onto the Illumina MiSeq cartridge according to the manufacturer's instructions. The quality of the run was checked internally using PhiX, and then each pair-end sequence was assigned to its

sample with the help of the previously integrated index. Each pair-end sequence was assembled using Flash software (Magoc and Salzberg, 2011) with an overlap of at least 10 bp between the forward and reverse sequences, allowing 10% of mismatch. The lack of contamination was checked with a negative control during the PCR (water was used as the template). The quality of the stitching procedure was controlled using four bacterial samples that are run routinely in the sequencing facility in parallel to the current samples. The resulting sequences were stored in Genbank (Bioproject SRP100061). Sequences longer than 300 bp were checked for chimeras and clusterized at 0.03 and taxonomically assigned with USEARCH v8.1.1861 (Edgar, 2013) using rdp trainset 16, yielding 15334 ± 3018 sequences per sample. The table was rarefied with the R package phyloseq1.16.2 (McMurdie and Holmes, 2013).

Statistical Analysis

The statistical analysis was performed with R 3.3.1 (R Development Core Team, 2008). The significance between the relative abundances of antibiotic-resistant genes in lactating dams vs. donor does was tested using the Mann–Whitney–Wilcoxon test for two groups with the Benjamini–Hochberg correction for multiple testing. This was performed with the `wilcox.test` and the `p.adjust` functions. The significant confidence level was set at 0.05.

The difference in the abundances of ARGs between the dams in the eight groups was tested using the Kruskal–Wallis test with the Benjamini–Hochberg correction of the two-tailed Dunn's test for multiple testing since ARGs could increase or decrease. The efficiency of fecal pellets vs. the corresponding suspension in decreasing ARGs was tested using a paired *t*-test. In other words, the decrease observed in the fecal pellets was compared to the decrease observed in the suspension for each ARG. The difference of the percentage of antibiotic-resistant *Enterobacteriaceae* between the different groups of lactating dams or groups of kits was also tested using the Kruskal–Wallis test, but the multiple testing was performed with the `pgirmess` package (version 1.6.9) in order to obtain a two-tailed *p*-value since we assume that the percentage of antibiotic-resistant genes decreases.

The richness was estimated by the Chao1 estimate using the estimateR function (R package vegan 2.4-2), which was then analyzed by a one-way analysis of variance (ANOVA) to detect an impact of the group. The significance of the separation observed on the nMDS plot using the Bray–Curtis dissimilarity was tested using pairwise permutational multivariate analysis of variance with distance matrices on the square-root-transformed abundances of the operational taxonomic units (OTUs) with the `adonis` function, after confirming the homogeneity of the variance through the `betadis` function (R package vegan 2.4-2). Two samples out of 37 were ignored because of aberrant profiles (kit10 and kit28).

In order to quantify the immigration rates under the assumptions of the neutral model, we used the script provided by Burns et al. (2016).

The linear and the non-linear relationships between the OTUs and the ARGs were characterized by the maximal information coefficient (MIC) index (Reshef et al., 2011). Briefly, this index

compares a *X*- and an *Y*- vector of values by quantifying the non-randomness of points in a two-dimensional space. This index was calculated on all the OTUs with more than 10 counts on average, which represented 104 OTUs. A relationship was considered significant if its MIC value was observed in less than 1% of the 37950 MIC values across 100 randomized datasets. In our case, the MIC threshold values were 0.66365 for the kits and 0.65429 for the dams.

RESULTS

Comparison of the ARGs in the Feces From the Lactating Dams and the Donor Does

As expected, many antibiotic resistance genes are reduced in does from farms that have banned antibiotics for several years (referred to as “donor” does below). Based on the 37 samples from the lactating dams and the six samples from the donor does, half of the ARGs (15 out of 26, **Supplementary Table S2**) are significantly reduced if we consider the three donor does as a group, despite their heterogeneity in ARGs. Most of the ARGs (21 out of 26, **Table 1**) were still detectable in the feces of all the donor does, suggesting that ARGs might persist for years even in an antibiotic-free environment.

Both Inoculation of Suspensions and Contact With Fecal Pellets Decrease the Total Carriage of Antibiotic-Resistant Genes in Kit Gut Microbiota

When inoculating the kits with a microbial suspension every second day between day 2 and 29, suspension I43 reduced eight antibiotic resistance genes (*aac6Im*, *aadE*, *aph2Ib*, *aphA3*, *CblA1*, *ermB*, *ermG*, and *tet40*), whereas suspension I44 reduced the abundance of *ermG* by half (**Table 1**). It should be noted that the inoculation can also add an antibiotic resistance gene if that gene is abundant in the donor doe. For example, donor Doe41 exhibited an unexpectedly high amount of *mefA* (macrolide efflux pump), which explains why kits fed with suspension I41 had 312-fold more *mefA* than the control kits.

Adding the fecal pellets to the nest is slightly more efficient than oral ingestion to drive the competitive exclusion. Indeed, six ARGs decreased in both kits exposed to suspension I43 and to the fecal pellets of Doe43, but the decrease was more drastic for kits exposed to pellets (72% vs. 59%, $p = 0.027$). In addition, the fecal pellets from Doe44 decreased the carriage of three different ARGs (*aac6Im*, *aph2Ib*, and *cblA1*), whereas the corresponding suspension only changed *ermG*.

While adding fecal pellets from antibiotic-naïve donor does generally decreases the ARG transmission from a dam to its kits, the relative success could not always be simply inferred from the individual levels of the antibiotic resistance genes in the donor does (**Table 1** and **Supplementary Figure S1**). For example, adding fecal pellets of Doe43 decreased *tet40* abundance in the kits by 68%, but adding fecal pellets from Doe44 had no effect, even though the latter had a lower abundance of *tet40* (**Table 1**).

TABLE 1 | Ratios of relative ARGs abundances in kits and in the three donor does.

		ARG ratio of kits vs. control kits (<i>n</i> = 37 litters, one kit per litter)							ARG ratio of donor does vs. lactating dams (<i>n</i> = 37)		
	ARG	ControlNF/ Control	I41/ Control	P41/ Control	I43/ Control	P43/ Control	I44/ Control	P44/ Control	donor Doe41/Lactating Dams	donor Doe43/Lactating Dams	donor Doe44/Lactating Dams
Aminoglycoside	<i>aac6lm</i>				0.38			0.4	0.47	0.16	0.11
	<i>aacA.aphD</i>								0.15	0.00	0.01
	<i>aadE</i>				0.45	0.49			0.57	0.29	0.51
	<i>ant6lb</i>					0.42			0.44	0.23	0.16
	<i>aph2lb</i>				0.40	0.28		0.4	0.51	0.06	0.13
	<i>aph3lb</i>								2.10	0.05	0.02
	<i>aphA3</i>				0.49	0.32			0.44	0.31	0.31
	<i>MGaph</i>								0.03	0.02	0.00
	<i>strB</i>								2.40	0.02	0.00
Beta lactam	<i>CblA1</i>				0.04			0.04	0.11	0.19	0.04
	<i>cepA29</i>								2.25	0.26	0.01
Macrolide	<i>ermB</i>				0.47	0.19			0.43	0.09	0.03
	<i>ermG</i>				0.21	0.11	0.5		0.18	0.08	0.13
	<i>lnuC</i>								1.01	0.38	0.12
	<i>metA</i>		311						15.70	1.05	0.01
Phenicol	<i>floR</i>								1.73	2.46	0.15
Sulfamide	<i>sul2</i>								48.50	0.00	0.00
Tetracycline	<i>tet32</i>		1.7						0.46	0.31	1.10
	<i>tet33</i>				9.20			11.8	4.82	1.31	0.08
	<i>tet40_1</i>				0.45	0.32			0.63	0.44	0.27
	<i>tetM</i>								0.84	0.15	0.70
	<i>tetO</i>								0.35	0.86	0.18
	<i>tetQ</i>								0.32	0.63	0.37
	<i>tetY</i>								0.00	0.00	0.00
	<i>dfrD</i>								0.88	0.00	0.00
Trimethoprim									0.84	1.34	2.27
Vancomycin	<i>vanTG</i>										
Significance of	<i>p</i> -values	0.62	0.5	0.2	0.08	0.02*	0.2	0.02*			
separation of the	<i>R</i> ²	0.12	0.10	0.14	0.13	0.16	0.12	0.16			
communities from the											
controls based on 16S											
data											

The kits are compared to the kits of the control group at weaning. The donor does are compared to the lactating dams. The value of ARGs in the donor does was highlighted when it was lower than the ARG abundance in the lactating dams. The ratios are reported only when the kits exposed to the microbial communities were significantly different from the control kits. The ratios higher than 1 are in red (see **Supplementary Table S3** for values before ratio). The pairwise *p*-values of the group separation of the microbial communities from the controls based on 16S rRNA sequences are also reported. *Indicates statistical significance.

This was confirmed by a second pair of primers targeting *tet40* (0.24 vs. 0.40 arbitrary units, **Supplementary Table S2**). In other words, the competition does not simply occur between a microbe carrying the ARG and its relative lack thereof.

Inoculation of Suspensions and Contact With Fecal Pellets Also Decrease the Carriage of Antibiotic-Resistant *Enterobacteria*

The higher efficiency of fecal pellets for competitive exclusion is also suggested when considering the exclusion of *Enterobacteria* resistant to tetracycline estimated from 10 isolates per kit (**Figure 1** with four or five kits per group at D35). Indeed, the fecal pellets from Doe44 significantly excluded these bacteria ($p = 0.02$), while suspension I44 shows a trend that only becomes significant when performing a second trial (**Supplementary Table S7** and **Supplementary Figure S2**). In contrast, the ratio of tetracycline-resistant *Enterobacteria* in the “No Feces” controls was similar to the ratio observed in the lactating does, so that

removing the feces potentially left by the lactating doe is not enough to stop the transmission of antibiotic-resistant bacteria. It should be noted that 14 out of 59 *Enterobacteriaceae* isolates were resistant to tetracycline in donor Doe41, four out of 50 in Doe 43, whereas no isolates were resistant in Doe44.

Inoculation of Suspensions and Contact With Fecal Pellets Impact the Total Microbial Community of the Kits

We generated 1.4 M of 16S rRNA sequences across 4023 OTUs after rarefaction at 5423 sequences per sample. The richness was not impacted (681 ± 145 OTUs) but the fecal communities of the kits in contact with fecal pellets from Doe43 and Doe44 differed from the communities observed in the control kits, unlike the fecal communities of kits in contact with fecal pellets from Doe41 (p -values from ADONIS = 0.004, 0.008, and 0.3, respectively, **Table 1**). This impact is illustrated by the non-metric dimensional scaling (nMDS) representation, which shows the similarity of the impact of the oral ingestion of the suspension

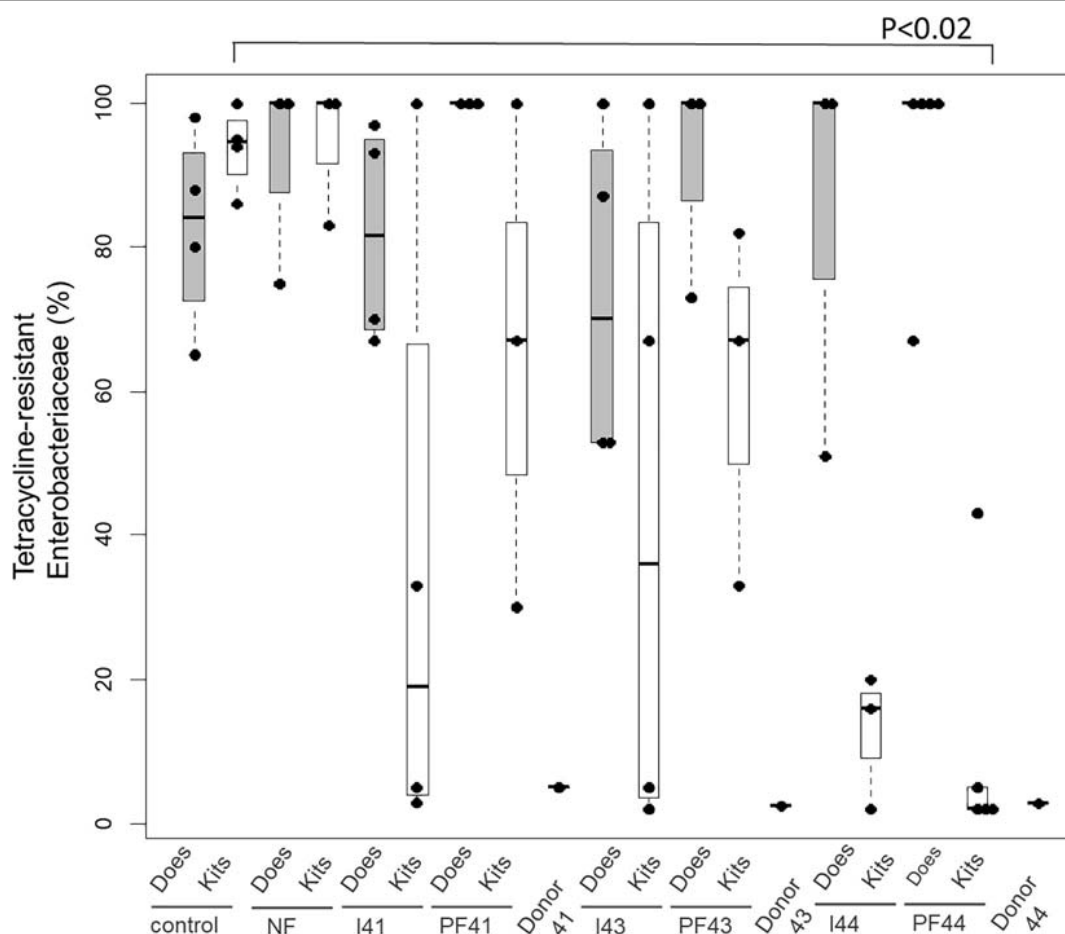
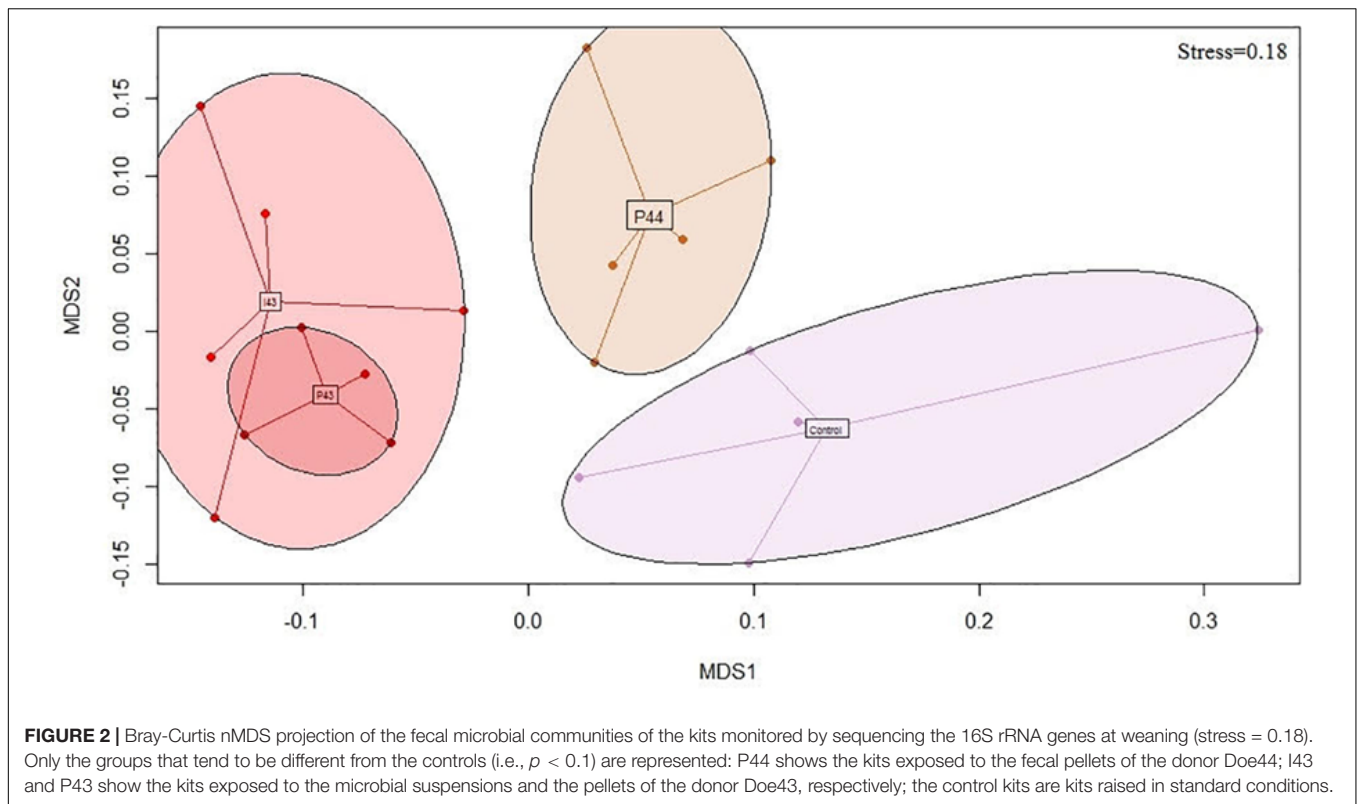


FIGURE 1 | Proportion of *Enterobacteriaceae* isolates resistant to tetracycline in the eight groups: controls (C), the no-feces controls (NF), Inoculum41 (I41) and fecal pellets of Doe41 (P41), Inoculum43 (I43) and fecal pellets of Doe43 (P43), Inoculum44 (I44) and fecal pellets of Doe44 (P44). The gray bars indicate the proportion of resistant *Enterobacteria* in the dams at weaning; the white bars show the resistance in the corresponding kits at weaning. The proportion of *Enterobacteriaceae* isolates in the donor does is also indicated.



vs. adding the fecal pellets in the nest on microbial communities (Figure 2 and Supplementary Figure S3). The microbiota of the lactating does differed from the microbiota of the kits (58% of the OTUs detected in kits were absent from the does, Supplementary Figure S4) but reassuringly did not present a group pattern similar to the pattern observed in the kits ($p = 0.8$, Supplementary Figure S5), which confirms that the separation between the groups is due to the inoculations. As observed for the transmission of ARGs and *Enterobacteria*, simply removing the feces potentially left by the lactating mother had no significant impact on the microbiota of the kits at weaning ($p = 0.62$, Table 1). Consistently with the lack of effect of microbes from Doe41 described above, Suspension I41 and the corresponding fecal pellets also had no significant impact on the microbial community of the weaning ($p = 0.7$ and 0.3 , Table 1). The abundances of the OTUs in the microbial communities of the 37 kits were not directly correlated to their abundance in the microbiota of the corresponding 37 does, even when keeping the mother-kit couple (Supplementary Figure S4). Still, the metacommunity can be estimated from the measurements in the kits within each group. Interestingly, this species abundance distribution revealed that the more immigration there was from the metacommunity, the stronger the impact on the total microbial community of the kits would be. Indeed, the p -value of the impact on the total community correlates negatively to the immigration rate predicted by the neutral model using the distribution of the OTUs across the samples in relation to their relative abundance ($r^2 = 0.4$, Supplementary Table S4 and Supplementary Figure S6).

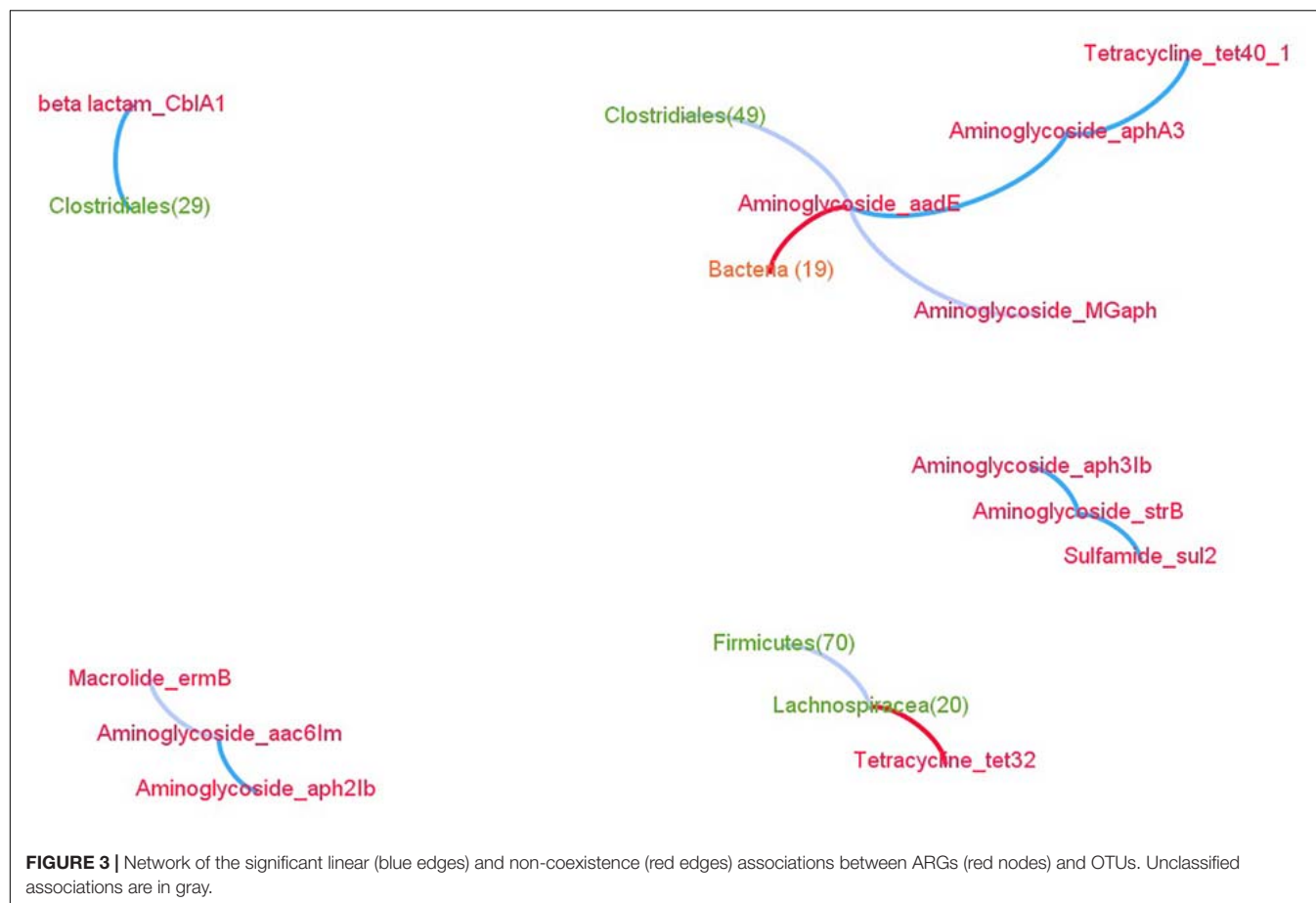
Identification of Clusters of ARGs

By comparing the abundance of the ARGs across the kits, we identified three significant ARG clusters, i.e., ARGs whose abundance are more correlated than chance would predict (Supplementary Table S5 and Supplementary Figure S7): *aph2Ib/aac6Im*, *aph3Ib/strB/sul2*, and *MGaph/aadE/aphA3/tet40* (Figure 3). Interestingly, the latter two contain genes from different classes of antibiotics (namely aminoglycoside and sulfamide or aminoglycoside and tetracycline). Naturally, the abundance of amplicons obtained from primers targeting the same gene also fall into the same clusters, such as *tet40_1* and *tet40_2*. Despite the marked differences in microbiota composition between kits and dams, the *aph2Ib/aac6Im* and *aph3Ib/strB* clusters are also observed in the dams (Supplementary Table S6 and Supplementary Figure S8).

Identification of OTUs That Are Associated With ARGs

In kits, four OTUs were significantly associated with the ARGs. First, *CblA1* was proportional to OTU26, affiliated with the *Clostridiales* order and commonly observed in rabbits (Ley et al., 2008). Second, *MGaph* did not coexist with OTU19 and had a complex relationship with OTU49 (*Clostridiales*). Finally, *tet32* did not coexist with OTU20 belonging to the *Lachnospiraceae* family (Figure 3 and Supplementary Table S4).

In does that were analyzed separately to avoid spurious relationships (Supplementary Table S6), the only significant ARG-OTU relationships were *CblA1* being proportional to



OTU21, affiliated with the *Bacteroides* genus in which this gene was first described [20], and *ermB* (target methylation) associated with OTU95 belonging to the *Clostridiales* order in which *ermB* determinants were previously detected in *Clostridium* (Spigaglia et al., 2005) and in *Ruminococcus* (Kennedy et al., 2018). It should be noted that the closest affiliation of OTU95 was *Ruminococcus*, albeit with a low confidence (0.26).

DISCUSSION

Evidence for Vertical Transmission

In our study, we modified the microbial community that colonizes the gut of the kits using microbial fecal communities from foreign does by either providing them as anaerobic suspensions or as fecal pellets in the nest before weaning. The “donor” does are from farms that have banned antibiotics for at least 2 years. The feces are collected from the kits at weaning, at which point 26 antibiotic-resistant genes are evaluated via quantitative PCR, the gene coding for the small ribosomal subunit is sequenced, and the cultivable *Enterobacteriaceae* are tested for tetracycline and sulfonamide resistance using classic culturing techniques.

The vertical transmission of antibiotic-resistant microbes from mother to kits is confirmed by the control group in

which the ratio of antibiotic-resistant *Enterobacteria* in the feces of the kits mirrors the ratio observed in lactating dams. It should be noted that this ratio does not depend on age, unlike the abundance of the OTUs, as previously described in rabbits (Combes et al., 2014), in humans (Odamaki et al., 2016), and in mice (Langille et al., 2014). This intergenerational transmission might be the reason why ARGs against phenicols are still detectable, even though they have not been used in French rabbits for at least 20 years (Anses editor, 2011), and *Streptomyces*, known to produce phenicols, was undetectable in our samples. Luckily the intergenerational transmission of OTUs and the associated ARGs can be interrupted when using the correct inoculum and delivery method, as described below.

Variability of Ability of the Microbial Communities to Discontinue the Vertical Transmission

The absence of the target is not enough to guarantee a successful exclusion. For example, Doe41 had a low abundance of resistant *Enterobacteria*, yet fails to exclude them. For ARGs as well, the mere absence of the ARG to be outcompeted is not sufficient. For example, Doe43 has more copies of *ermB* than Doe44, yet, paradoxically, only

the microbes from Doe43 decrease the carriage of *ermB* in the kits. Likewise, Doe43 and Doe44 are both free from tetracycline-resistant *E. coli* according to our detection method, yet only microbes from Doe44 significantly decrease the proportion of tetracycline-resistant *Enterobacteria* ($p = 0.02$). Predicting the exclusion of targeted genes from complex communities is a critical milestone that requires additional information about the microbiota performing the exclusion. Indeed, it is challenging to predict the outcome of the exclusion based on the abundance of the ARGs in the donor doe and the lactating dam (Supplementary Figure S1). It should be noted that the outcompeting microbes are not necessarily ARG-free variants that are phylogenetically close: for example, vancomycin-resistant *Enterococcus* belonging to the *Proteobacteria* phylum are outcompeted *in vivo* by the *Barnesiella* genus belonging to the *Bacteroides* (Ubeda et al., 2013). Interspecific exclusion could explain why ARGs increase or decrease in a surprising manner, as mentioned above. Such an interspecific exclusion also implies that exclusion cannot be efficiently understood by investigating the species of interest alone but requires data about the whole microbial community. Indeed, simply replacing the targeted antibiotic-resistant microbes by their antibiotic-sensitive conspecifics is temporary at best. In pigs for example, using antibiotic-sensitive *Megasphaera* only delays the appearance of antibiotic-resistant strains (Stanton and Humphrey, 2011), possibly because of horizontal gene transfer toward the sensitive *Megasphaera*. By acting on the whole community of the gut microbes at once, exclusion with complex communities hinders the vertical transmission of microbes as well as the horizontal transfers of ARGs from ARG-rich communities toward the sensitive microbes, which might occur between microbial species via plasmids, transposons or even phages (Muniesa et al., 2013; Zhu et al., 2013).

Impact of the Mode of Delivery of the Microbial Communities on the Success of Competitive Exclusion

The success of competitive exclusion also depends on the delivery method of the microbes: adding fecal pellets was more efficient than anaerobic suspensions. This is surprising because oxygen may penetrate the fecal pellets, thereby killing 73% of the bacteria, which are primarily anaerobic in the gut (Harris et al., 1976) within minutes (Brusa et al., 1989). Furthermore, competitive experiments confirmed that microbial communities are more likely to contain bacteria excluding the targeted species when they are kept in anaerobic conditions (Ubeda et al., 2013). In contrast, other authors suggest that oxygen exposure could be less crucial than previously thought. Some bacteria form oxygen-tolerant spores specialized for host-to-host transmission, and even non-spore-forming bacteria can remain viable for 2 days upon oxygen exposure (Browne et al., 2016). In addition, oxygen can be used by 23% of the bacteria in the lumen [which are facultative anaerobes (Harris et al., 1976)] and by gut microbes from the mucus (Albenberg et al., 2014), suggesting that oxygen plays a role in the gut. In our experiment, the role of oxygen remains

unclear because microbial respiration from the gut bacteria that tolerate oxygen may limit the actual penetration of oxygen in the fecal pellets. Regardless, we demonstrated the relevance of coprophagy for the intergenerational transmission of microbes and associated ARGs.

Interestingly, coprophagy is found in many animal species including pigs (Orland and Brand, 1991), rabbits (Combes et al., 2014), termites (Rosenberg and Zilber-Rosenberg, 2011), horses and rats (Galef, 1979; Crowell-Davis and Houpt, 1985). For example, piglets reportedly ingest 20 g of their mother's feces daily (Orland and Brand, 1991). Regarding rabbits, the doe defecates in the nest (Kovacs et al., 2006), and the fecal pellets that the kits ingest when they are between 8 and 20 days old change their microbiota at weaning (Combes et al., 2014). This natural behavior probably plays a role in the colonization since when 24-h-old kits are placed in another nest, the colonization of the kit's gut from the neonatal environment provided by the lactating doe overrides the one from the biological mother (Abecia et al., 2007). In contrast, humans do not have this behavior, which might explain why the birth imprint is more important in humans. Indeed the microbiota composition and the occurrence of resistance genes of newborns are linked to the microbes in the vaginal fluids (Dominguez-Bello et al., 2010, 2016; Alicea-Serrano et al., 2012).

However, coprophagy is not the only force driving the microbial colonization of the gut since removing the fecal pellets had no detectable effect when no foreign microbes were added to the nest. Hence, simply removing the maternal pellets is not enough to fight against the transmission of antibiotic-resistant microbes because the contact during suckling is sufficient to insure transmission of the maternal microbes. This could be due to either the microbial contact during the milking process (which lasts 10 min in rabbits) or to small amounts of bacteria transmitted before birth, as observed in human (Moles et al., 2013). Unfortunately we did not measure the microbiota at birth so we cannot decipher between the two hypothesis. We also did not observe the artificially increased diversity that animals in incubators from day 2 after birth might exhibit (Schmidt et al., 2011), confirming that we are closer to the real conditions than the incubator model in which one single inoculation event was sufficient to drive the microbial communities. Together, these results show that coprophagy overrides the other factors behind the microbial communities in kit guts.

From an applied point of view, outcompeting the maternal microbiota by adding foreign fecal pellets could have broad implications at the farm and at the national level. At the farm level, a "donor" doe could be kept free from any antibiotic treatment in order to use its fecal pellets to reintroduce antibiotic-sensitive microbes to the rest of the herd when a disease outbreak requires antibiotic use in does. While completely eliminating the ARGs does not seem possible, competitive exclusion could also be interesting at the country level for specific resistances. Indeed, Finland has less microbes that hydrolyze third-generation cephalosporins than Sweden, even though they have a similar organization for broilers. The main difference is that Finland used a commercially available complex microbial community excluding *Salmonella* for decades, which

probably also decreased the carriage of *E. coli* producing extended spectrum beta-lactamase as a side-effect (Nuotio et al., 2013), suggesting that wide use of competitive exclusion can impact the ARGs at the national level. In this context, clusters of ARGs such as *ant6lb/ermB* could be defined as a priority target since many clusters of ARGs were observed in livestock (Johnson et al., 2016). It should be noted that widely applying competitive exclusion might generate a normalization of the microbial community in livestock, as was observed in patients exposed to fecal microbiota transplantation via oral ingestion of lyophilized microbes (Staley et al., 2017).

There are four limitations of our study: firstly, kits were allowed to roam freely in the area of the dam from Day 21 to 36 in order to mimic the real farming conditions. This contact could have blunted the impacts of the treatments observed at Day 36. Secondly, we sampled only one kit in each 37 litters, which was sufficient to observe that the variability within litter was less than the variability between the treatments, but we could not estimate the exact within-litter variability. Thirdly, 2 out of 3 donor does had unexpected high levels in some ARGs, such as *mefA* for example, possibly because the donor does received a diet favoring a bacteria carrying *mefA* but the exact reasons underpinning the high abundance of *mefA* remains unknown. Whatever the cause, this limits the usefulness of these donors to fight against ARGs unless increasing the abundance of *mefA* is acceptable, and a careful assessment should determine the abundance of the ARGs in the donor does even if the donors were not exposed to antibiotics. Fourthly, we rarefied at 5423 sequences per sample, so the rare microbes were not taken into account in the analysis.

CONCLUSION

In conclusion, our work demonstrates the transmission of ARGs from the lactating does to their kits and paves the way toward a microbiome-based remediation strategy against ARGs in rabbits.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Ethic Committee C2EA-86. The experiments were conducted under the agreement number

APAFIS-2018021215568441 for animal experimentation from the French Ministry of Agriculture (Ethic Committee C2EA-86).

AUTHOR CONTRIBUTIONS

OZ, CSA, VD, and AB-M designed the study. CSA and SS extracted the microbial DNA, chose the primers, and analyzed the microbial sequences. LC uploaded the sequences. CSA and OZ carried out the statistical tests. VD and NA screened the *Enterobacteriaceae* isolates for antibiotic resistance. VD, CSA, OZ, and AB-M drafted the manuscript. All authors edited and approved the 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/fmicb.2019.01503/full#supplementary-material>

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Integrative and Conjugative Elements-Positive *Vibrio parahaemolyticus* Isolated From Aquaculture Shrimp in Jiangsu, China

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The development of multidrug- and toxin-resistant bacteria as a result of increasing industrialization and sustained and intense antimicrobial use in aquaculture results in human health problems through increased incidence of food-borne illnesses. Integrative and conjugative elements (ICEs) are self-transmissible mobile genetic elements that allow bacteria to acquire complex new traits through horizontal gene transfer and encode a wide variety of genetic information, including resistance to antibiotics and heavy metals; however, there is a lack of studies of ICEs of environmental origin in Asia. Here, we determined the prevalence, genotypes, heavy metal resistance and antimicrobial susceptibility of 997 presumptive strains of *Vibrio parahaemolyticus* (*tlh*⁺, *tdh*⁻), a Gram-negative bacterium that causes gastrointestinal illness in humans, isolated from four species of aquaculture shrimp in Jiangsu, China. We found that 59 of the 997 isolates (5.9%) were ICE-positive, and of these, 9 isolates tested positive for all resistance genes. BLAST analysis showed that similarity for the eight strains to *V. parahaemolyticus* was 99%. Tracing the *V. parahaemolyticus* genotypes, showed no significant relevance of genotype among the antimicrobial resistance strains bearing the ICEs or not. Thus, in aquaculture, ICEs are not the major transmission mediators of resistance to antibiotics or heavy metals. We suggest future research to elucidate mechanisms that drive transmission of resistance determinants in *V. parahaemolyticus*.

Keywords: *Vibrio parahaemolyticus*, antimicrobial susceptibility, integrative and conjugative elements, heavy metal resistance, genotypes

INTRODUCTION

Microbes rapidly acquire or donate new genes and phenotypes through the process of horizontal gene transfer between organisms that is a key driver of microbial evolution (Pan et al., 2019). Integrative and conjugative elements (ICEs) are self-transmissible modular mobile genetic elements (MGEs) integrated into a host genome that are passively propagated during chromosomal

replication and cell division, and mediate the acquisition of complex new traits in bacteria (Johnson and Grossman, 2015). Recent studies have shown these MGEs contain cargo genes encoding traits including resistance, virulence, novel carbon source metabolism, and degradation of aromatic compounds, that may benefit the recipient bacteria (Rubio-Cosials et al., 2018; Xu et al., 2018). ICEs tend to be mosaic and modular, ranging from 20 to >500 kb. ICEs are excised from the host chromosome and then transfer to recipients via conserved conjugation machinery in the type IV secretion system (Flores-Ríos et al., 2019), prior to reintegration into the host chromosome.

Vibrio parahaemolyticus is a Gram-negative, halophilic, mesophilic, aerobic bacterium common in warm climate marine and estuarine environments. Pathogenic strains in food cause serious health issues to humans, including gastroenteritis, septicemia, and wound infection (He et al., 2016). Shrimp represents an important reservoir of *V. parahaemolyticus*, particularly in fresh and refrigerated stock, but it is also recorded from frozen stock. China is the world's largest producer of aquatic products (He et al., 2016). However, industrial development and use of antimicrobials in aquaculture have led to increased heavy metal pollution and development of multidrug resistant (MDR) bacteria that are problematic in many aquatic systems as they drive incidence of food-borne illnesses (Lopatek et al., 2018). Previous studies have revealed that bacteria can acquire resistance via conjugation or transformation to induce a wide variety of disease and adapt to the harsh environment (Matyar, 2012). The World Health Organization (WHO) produced a global map of antimicrobial resistance, warning that a "post-antibiotic" world could soon become a reality in April 2014 (WHO, 2014). Recent studies indicated that drugs which were once lifesavers are now worthless, for instance, chloramphenicol, once a physician's first choice against typhoid, is no longer effective in many parts of the world and resistance has spread around the world (Woolhouse and Farrar, 2014). Antimicrobial resistance is a global problem that requires global solutions, better surveillance is essential. Nevertheless, to date, no global approaches were conducted on further demonstrating the characterization of the *V. parahaemolyticus* isolates present in shrimp-production industry, despite their great significance in economy and human health.

The discovery and early studies of ICEs were stimulated by interest in bacterial resistance to antibiotics and heavy metals, and how that resistance was spread. MGEs with ICE-like properties have been described in several species of Gammaproteobacteria, particularly *Vibrio* (Liu et al., 2019). However, few studies report on ICEs in *V. parahaemolyticus* isolates from Asia. Hence, in this study, we focused on analyzing the *V. parahaemolyticus* strains from different shrimp samples in Jiangsu, China to determine the antibiotic and heavy metal resistance of these bacteria and to investigate the relationship between antibiotic and heavy metal. Molecular characterization and phenotypes of antibiotic resistance and heavy metals have been characterized. The information will facilitate the better understanding of this bacterium and facilitate related risk assessment and health management for consuming seafood.

MATERIALS AND METHODS

Sample Collection

Freshwater shrimp (*Procambarus clarkii*, *Macrobrachium nipponense*, *Penaeus vannamei*, and *Macrobrachium rosenbergii*), which are commonly shrimp breeds in China were collected once a month from Xuzhou Kaiming Fish Market, Jiangsu, China from May to October 2016–2018. *P. clarkii* and *M. nipponense* are key economic species in Jiangsu; *P. vannamei* and *M. rosenbergii* are cultured widely in the littoral provinces of southeastern China. We randomly collected samples of each species following a modified version of a standard protocol (Kaysner and DePaola, 2004). Samples were placed in sterile plastic bags (Shanghai Sangon Biological Engineering Technology and Services Co., Ltd., Shanghai, China) and taken to the laboratory on ice for homogenization within 2 h of collection.

V. parahaemolyticus Isolation and Identification

Vibrio parahaemolyticus was isolated and identified as described by the Chinese Government Standard (GB17378-2007) and He et al. (2016). Shrimp (25 g) were rinsed with sterile saline solution then homogenized for 60 s in sterile bags (Stomacher) containing 225 mL of sterile saline. Serial 10-fold dilutions (to 1:10⁴) were prepared, and 100 µl of each dilution were spread on thiosulfate citrate-bile salts-sucrose plates (TCBS; Beijing Land Bridge Technology Company Ltd., Beijing, China) which were incubated for 16 h at 37°C. Putative *V. parahaemolyticus* colonies (which are green on TCBS) were placed separately in wells of 96-well microtiter plates containing 200 µl of sterile alkaline peptone water plus 3% NaCl (pH 8.5 ± 0.2).

Screening and Identification of Virulence and ICE Genes

Presumptive *V. parahaemolyticus* colonies (*N* = 25 per species of shrimp) were selected, screened, and identified using PCR-based screening of the species-specific marker gene thermolabile hemolysin (*tlh*). Table 1 lists primers used in this study. In *tlh*-positive *V. parahaemolyticus* strains, the virulence-associated genes thermostable direct hemolysin (*tdh*) and TDH-related hemolysin (*trh*), SXT/R391-like ICE conserved genes (*int*, *attR*, *traC*, *setR*, and *traI*), and the typical resistance genes for streptomycin (*strA/strB*), trimethoprim (*dfrA1/dfr18*), and sulfamethoxazole/trimethoprim (*sul2*) were identified using primers as described by He et al. (2015a, 2016) and Beker et al. (2018). Strain taxonomy was determined from 16S rRNA gene sequences, obtained using primer pair 27F and 1492R; sequencing was performed by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd., (Shanghai, China) (He et al., 2016). *V. parahaemolyticus* ATCC33846 (*tdh*⁺, *trh*⁺) and ATCC17802 (*tdh*[−], *trh*[−]) were used as positive controls. Genomic DNA was prepared using a MiniBest bacterial genomic DNA extraction kit (v. 2.0; Japan TaKaRa BIO, Dalian, China). DNA was amplified using a

Mastercycler pro PCR thermal cycler (Eppendorf, Hamburg, Germany). DNA sequences were assembled into contigs using ContigExpress software¹. Protein functions were analyzed using BLAST².

Susceptibility to Antimicrobials and Heavy Metals

In vitro susceptibility of isolates to antimicrobial agents according to the guidance of the Performance Standards

¹<http://www.contigexpress.com>

²<http://ncbi.nlm.nih.gov/BLAST>

TABLE 1 | Primers used in this study.

Primer	Nucleotide sequence (5'–3')	Target genes	References
27F	AGAGTTTG ATCCTGGCTCAG	16S rDNA	Weisburg et al., 1991
1492R	GGTTACC TTTTACGACTTG		
L-TLH	AAAGCGGAT TATGCAGAAGCACTG	<i>tlh</i>	Panicker et al., 2004
R-TLH	ACTTTCTAGC ATTTTCTCTGC		
Tdh-1	CCATCTGTCC CTTTCTCTGCC	<i>tdh</i>	Panicker et al., 2004
tdh-4c	CCACTACCA CTCTCATATGC		
VPTRH-L	TTGGCTTCG ATATTTTCAGTATCT	<i>trh</i>	Panicker et al., 2004
VPTRH-R	CATAACAAAC ATATGCCCATTTCCG		
tral-F tral-R	GCAAGT CCTGATCCGCTATC CCAGGGCAT CTCATATGCGT	<i>tral</i>	Bani et al., 2007
traC-F	TTGACGCT GTTTTACCAACG	<i>traC</i>	Bani et al., 2007
traC-R	GGCACGAC CTTTTTCTCCCC		
setR-F	ACGGCGGA GATGTTTTGT	<i>setR</i>	Bani et al., 2007
setR-R	GTGCGCC AATGCTCAGTT		
attR-F	GGTTTAG CCACAGTTGTTC	<i>attR</i>	Song et al., 2013
attR-R	CGTCAG GGTGCGAGAT		
Int-F	GACGCATT TCATCCAGG	<i>int</i>	Song et al., 2013
Int-R	GCAACAG CGGGTAGACA		
SUL2-F	AGGGGGC AGATGTGATCGC	<i>sul2</i>	Falbo et al., 1999
SUL2-B	TGTGCGG ATGAAGTCAGCTCC		
STRA-F	TTGATGT GGTGTCCCAGCAATGC	<i>strA</i>	Dalsgaard et al., 1999
STRA-B	CCAATCG CAGATAGAAGCAA		
strB-F	GGCACCC ATAAGCGTACGCC	<i>strB</i>	Dalsgaard et al., 1999
strB-R	TGCCGAG CACGGCGACTACC		
df1-F	CGAAGAA TGGAGTTATCGGG	<i>df1A1</i>	Iwanaga et al., 2004
df1-B	TGCTGG GGATTTCAGGAAAG		
TMP-F	TGGGTAA GACACTCGTCATGGG	<i>df18</i>	Falbo et al., 1999
TMP-B	ACTGCCG TTTTCGATAATGTGG		

for Antimicrobial Disk Susceptibility Tests of the Clinical and Laboratory Standards Institute (CLSI) (2006, Approved Standard-Ninth Edition, M2-A9, Vol. 26 No. 1) following the approach described by Song et al. (2013) and He et al. (2015a). Mueller-Hinton agar medium (Oxoid, United Kingdom), and the discs (Oxoid, United Kingdom) were used in this study. Examined antimicrobial agents included: 30 µg of chloramphenicol (CHL); 10 µg of gentamicin (CN); 25 µg of sulfamethoxazole/trimethoprim 19:1 (SXT); 5 µg of rifampin (RIF); 30 µg of tetracycline (TET); 10 µg of ampicillin (AMP); 100 µg of spectinomycin (SPT); 30 µg of kanamycin (KAN); 5 µg of trimethoprim (TM); and 10 µg of streptomycin (STR). The assays were performed in triplicate experiments, and reference strain *Escherichia coli* ATCC25922 was purchased from the Institute of Industrial Microbiology (Shanghai, China) and used for quality control. Broth Dilution Testing (microdilution) was used to measure quantitatively the minimal inhibitory concentration (MIC) *in vitro* of the tested heavy metals against the strains, according to the Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically (2006, CLSI, Approved Standard-Seventh Edition, M7-A7, Vol. 26 No. 2). The heavy metals tested were: NiCl₂, CrCl₃, CdCl₂, PbCl₂, CuCl₂, ZnCl₂, BaCl₂, and HgCl₂ (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China); *E. coli* K12 MG1655 strain was used as the control.

Molecular Typing of *V. parahaemolyticus* Strains

Vibrio parahaemolyticus was cultured in Luria-Bertani broth (Beijing Land Bridge Technology Co.) following methods described by He et al. (2015b). Genomic DNA was extracted using a CHEF Bacterial DNA Plug Kit (Bio-Rad Laboratories, Hercules, CA, United States). Agarose plugs were prepared by mixing equal volumes of cell suspension, and each plug was digested using *NotI* (Japan TaKaRa BIO). Electrophoresis was performed at 6 V/cm, 14°C, at a field angle of 120°, using 1% SeaKem Gold agarose (Lonza, Basel, Switzerland). Pulsed-field gel electrophoresis (PFGE) patterns were visualized under 260-nm light; images were recorded using the UVP EC3 Imaging system (UVP LLC), and data were analyzed using NTSYSpc 2.10e software.

RESULTS AND DISCUSSION

V. parahaemolyticus Isolation and Identification

Procambarus clarkii, *M. nipponense*, *P. vannamei*, and *M. rosenbergii* are common species of shrimp consumed in Jiangsu, China, and PCR analysis showed that 997 of 1800 (55.4%) bacterial isolates obtained from them tested positive for the *V. parahaemolyticus*-specific *tlh* gene. There were distinct temporal patterns of *tlh*-positive prevalence (**Figure 1**); over the 3-year study, *tlh* gene abundance was greater during the summer months (July and August) when temperature is highest.

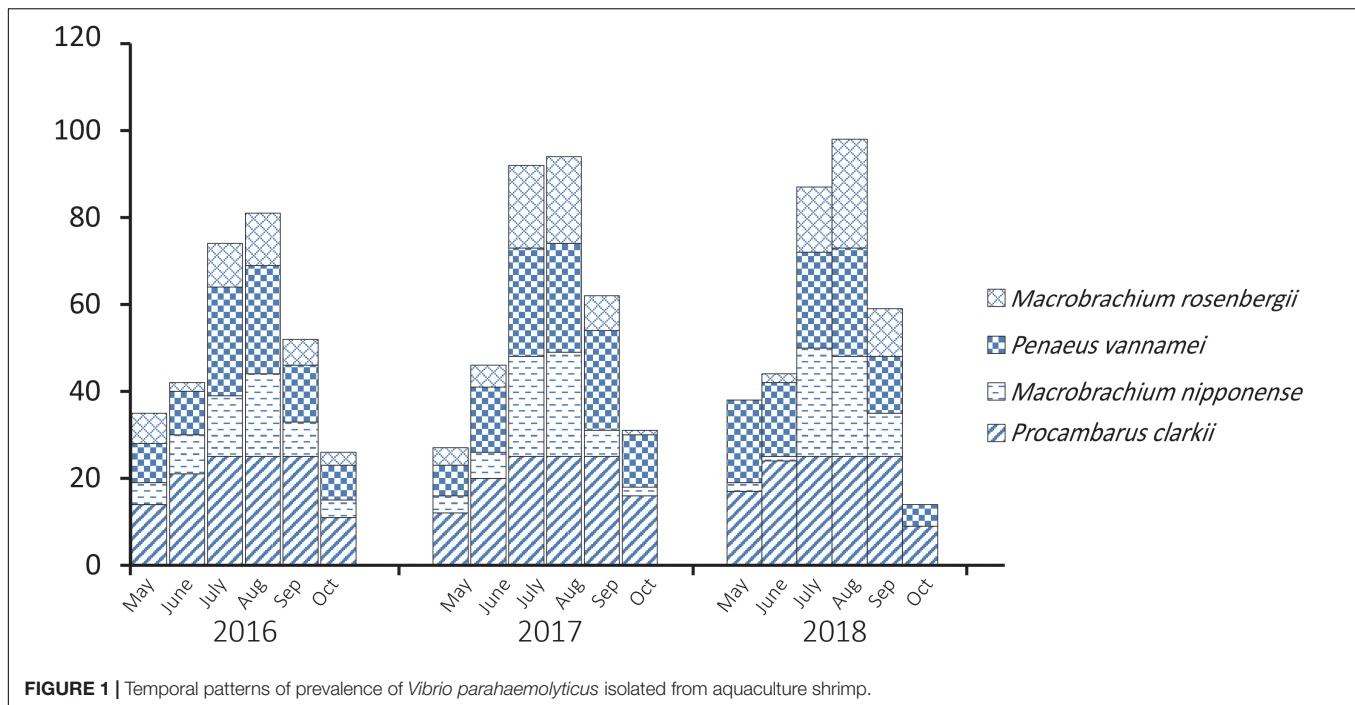


FIGURE 1 | Temporal patterns of prevalence of *Vibrio parahaemolyticus* isolated from aquaculture shrimp.

Prevalence of Virulence Associated-Genes and Conserved ICE Genes

Pathogenic *V. parahaemolyticus* produces two major toxic proteins, TDH and TRH, that are important in the diarrheal diseases caused by this species (Boyd et al., 2008). We tested the 997 *tlh*-positive strains for the presence of virulence-associated toxin genes *tdh* and *trh*, and found that most isolates were considered not virulent; we did not amplify *tdh* from any isolate, and amplified *trh* from only two isolates (obtained from *P. clarkii* in August 2018). Similar very low incidence of pathogenic *V. parahaemolyticus* has been reported in many non-clinical samples (He et al., 2016; Hu and Chen, 2016; Martinez-Urtaza et al., 2016; Lopatek et al., 2018; Zhao et al., 2018; Jiang et al., 2019).

Analysis of the highly conserved core genes of SXT/R391-like ICEs revealed that 59 (5.9%) of the isolates tested positive for all five genes (*int*, *attR*, *traC*, *setR*, and *traI*). Occurrence was highest in isolates recovered from *P. clarkii* (33.9%), followed by *P. vannamei* (32.2%), *M. nipponense* (20.3%), and *M. rosenbergii* (13.6%), and resistance genes *strA/strB*, *dfrA1/dfr18*, and *sul2* were recorded in 25.4, 15.3, and 33.9% of the 59 isolates, respectively, with nine strains testing positive for all three resistance genes (Table 2).

Antimicrobial Susceptibility and Heavy Metal Tolerance

Our results revealed distinct antibiotic-resistance patterns for the 59 isolates that were positive for the highly conserved ICE genes. All isolates were AMP resistant, and resistance to STR, TM, RIF, and SXT was 25.4, 22.0, 18.6, and 16.9%, respectively; resistance

to CHL, SPT, and KAN was 15.3% (Figure 2). We found that approximately 84.7, 81.3, 74.6, and 72.9% of the isolates exhibited intermediate susceptibility to SPT, KAN, STR, and TM, and while 15.3% of isolates showed intermediate patterns of susceptibility to CN and TET, none of the isolates was resistant to these two antibiotics. We found nine isolates tested positive for the three resistance genes *strA/strB*, *dfrA1/dfr18*, *sul2*, and these strains were resistant to CHL, SXT, RIF, AMP, SPT, KAN, TM, and STR, with intermediate susceptibility to CN and TET. BLAST analysis showed that the 16S rRNA gene sequence of isolate VpJHY15 shared 99% similarity with that from *Proteus vulgaris*³, and similarity for the other eight strains (VpJHY4, VpJHY9, VpJHY16, VpJHY35, VpJHY36, VpJHY39, VpJHY41, and VpJHY48) to *V. parahaemolyticus* was 99%.

Therefore, in this study, eight *V. parahaemolyticus* isolates tested positive for SXT/R391-like ICE conserved genes (*int*, *attR*, *traC*, *setR*, *traI*) and associated typical resistance genes (*strA/strB*, *dfrA1/dfr18*, *sul2*). These strains may be described as having MDR phenotypes because they were resistant to at least one agent in ≥ 3 categories of antimicrobial (Thapa Shrestha et al., 2015). These isolates were also examined for susceptibility to heavy metals (Malik and Aleem, 2011), we found minimal inhibitory concentrations (MICs) of 3200 $\mu\text{g/mL}$ for Ni^{2+} , Cr^{3+} , Cd^{2+} , Cu^{2+} , Pb^{2+} , and Mn^{2+} ; 1600 $\mu\text{g/mL}$ for Zn^{2+} ; and 50 $\mu\text{g/mL}$ for Hg^{2+} (Table 3). All strains were resistant to Zn^{2+} and Pb^{2+} , and most also displayed tolerance to Cu^{2+} (87.5%), Cd^{2+} (75%), and Hg^{2+} (62.5%), while a few were resistant to Cr^{3+} (37.5%), Ni^{2+} (25%), and Mn^{2+} (12.5%). *V. parahaemolyticus* isolates derived from the four shrimp species showed tolerance to at least four heavy-metal agents (Table 4). While we found

³<http://rdp.cme.msu.edu/>

TABLE 2 | Genotypes of *V. parahaemolyticus* isolated from shrimp.

Source (species)	Strain no.	Sample date	t1h	Virulence-associated genes		ICEs conserved-core genes					Resistance genes		
				tdh	trh	int	attR	tral	traC	setR	strA/strB	dfrA1/dfr18	sul2
<i>Procambarus clarkii</i>	VpJHY1	July 2016	+	–	–	+	+	+	+	+	–	–	+
	VpJHY2	July 2016	+	–	–	+	+	+	+	+	–	–	–
	VpJHY3	August 2016	+	–	–	+	+	+	+	+	+	–	+
	VpJHY4	August 2016	+	–	–	+	+	+	+	+	+	+	+
	VpJHY5	August 2016	+	–	–	+	+	+	+	+	–	–	–
	VpJHY6	July 2017	+	–	–	+	+	+	+	+	–	–	–
	VpJHY7	August 2017	+	–	+	+	+	+	+	+	+	–	+
	VpJHY8	August 2017	+	–	–	+	+	+	+	+	+	–	+
	VpJHY9	August 2017	+	–	–	+	+	+	+	+	+	+	+
	VpJHY10	August 2017	+	–	–	+	+	+	+	+	–	–	–
	VpJHY11	September 2017	+	–	–	+	+	+	+	+	–	–	–
	VpJHY12	September 2017	+	–	–	+	+	+	+	+	–	–	–
	VpJHY13	July 2018	+	–	–	+	+	+	+	+	–	–	–
	VpJHY14	July 2018	+	–	–	+	+	+	+	+	–	–	–
	VpJHY15	August 2018	+	–	+	+	+	+	+	+	+	+	+
	VpJHY16	August 2018	+	–	–	+	+	+	+	+	+	+	+
	VpJHY17	August 2018	+	–	–	+	+	+	+	+	–	–	–
	VpJHY18	September 2018	+	–	–	+	+	+	+	+	+	–	+
	VpJHY19	September 2018	+	–	–	+	+	+	+	+	–	–	–
	VpJHY20	September 2018	+	–	–	+	+	+	+	+	–	–	–
<i>Macrobrachium nipponense</i>	VpJHY21	July 2016	+	–	–	+	+	+	+	+	–	–	–
	VpJHY22	August 2016	+	–	–	+	+	+	+	+	–	–	–
	VpJHY23	August 2016	+	–	–	+	+	+	+	+	–	–	–
	VpJHY24	August 2017	+	–	–	+	+	+	+	+	–	–	–
	VpJHY25	September 2017	+	–	–	+	+	+	+	+	–	–	–
	VpJHY26	August 2018	+	–	–	+	+	+	+	+	–	–	+
	VpJHY27	August 2018	+	–	–	+	+	+	+	+	–	–	–
	VpJHY28	August 2018	+	–	–	+	+	+	+	+	–	–	–
	VpJHY29	August 2018	+	–	–	+	+	+	+	+	–	–	–
	VpJHY30	August 2018	+	–	–	+	+	+	+	+	+	–	+
	VpJHY31	September 2018	+	–	–	+	+	+	+	+	–	–	–
	VpJHY32	September 2018	+	–	–	+	+	+	+	+	–	–	–
<i>Penaeus vannamei</i>	VpJHY33	July 2016	+	–	–	+	+	+	+	+	–	–	–
	VpJHY34	August 2016	+	–	–	+	+	+	+	+	–	–	–
	VpJHY35	August 2016	+	–	–	+	+	+	+	+	+	+	+

(Continued)

TABLE 2 | Continued

Source (species)	Strain no.	Sample date	tlh	Virulence-associated genes		ICEs conserved-core genes					Resistance genes		
				tdh	trh	int	attR	tral	traC	setR	strA/strB	dfrA1/dfr18	sul2
<i>Macrobrachium rosenbergii</i>	VpJHY36	August 2016	+	–	–	+	+	+	+	+	+	+	+
	VpJHY37	August 2016	+	–	–	+	+	+	+	+	–	–	–
	VpJHY38	June 2017	+	–	–	+	+	+	+	+	–	–	–
	VpJHY39	July 2017	+	–	–	+	+	+	+	+	+	+	+
	VpJHY40	July 2017	+	–	–	+	+	+	+	+	–	–	–
	VpJHY41	August 2017	+	–	–	+	+	+	+	+	+	+	+
	VpJHY42	August 2017	+	–	–	+	+	+	+	+	–	–	–
	VpJHY43	August 2017	+	–	–	+	+	+	+	+	–	–	–
	VpJHY44	August 2017	+	–	–	+	+	+	+	+	–	–	–
	VpJHY45	August 2017	+	–	–	+	+	+	+	+	–	–	–
	VpJHY46	June 2018	+	–	–	+	+	+	+	+	–	–	–
	VpJHY47	July 2018	+	–	–	+	+	+	+	+	–	–	–
	VpJHY48	August 2018	+	–	–	+	+	+	+	+	+	+	+
	VpJHY49	August 2018	+	–	–	+	+	+	+	+	–	–	–
	VpJHY50	August 2018	+	–	–	+	+	+	+	+	–	–	–
	VpJHY51	September 2018	+	–	–	+	+	+	+	+	–	–	+
	VpJHY52	August 2016	+	–	–	+	+	+	+	+	–	–	+
	VpJHY53	September 2016	+	–	–	+	+	+	+	+	–	–	–
	VpJHY54	August 2017	+	–	–	+	+	+	+	+	–	–	–
	VpJHY55	July 2018	+	–	–	+	+	+	+	+	–	–	–
	VpJHY56	August 2018	+	–	–	+	+	+	+	+	+	–	+
	VpJHY57	August 2018	+	–	–	+	+	+	+	+	–	–	+
	VpJHY58	August 2018	+	–	–	+	+	+	+	+	–	–	–
	VpJHY59	September 2018	+	–	–	+	+	+	+	+	–	–	–



that resistance to heavy metals in *V. parahaemolyticus* did not vary with shrimp species, tolerance was shown to be very prevalent in ICE-positive strains with more than eight antibiotic resistance phenotypes. These contrasting results may be a result of inappropriate, variable releases of industrial wastes to aquaculture environments (Jiang et al., 2019), because industrial pollutants enhance selection for antibiotic resistance and *vice versa*. Abundant double-resistant bacteria threaten human health if contaminated products are consumed (Silva et al., 2018).

Phylogenetic Relationships of Resistant *V. parahaemolyticus* Isolates

Genomic DNA of the eight *V. parahaemolyticus* strains was individually digested with the restriction endonuclease *NotI*,

and the resulting DNA fragments were resolved by PFGE. This analysis revealed different genomic finger prints of the strains tested (**Figure 3**). Fingerprinting analysis of the relatedness of the eight ICE-positive isolates produced 14 to 19 restriction bands that ranged from 20.5 to 1135 kb. Cluster analysis of the PFGE profiles revealed seven pulsotypes with $\geq 87\%$ similarity, which indicates isolates belonging to the same epidemic strain (Seifert et al., 2005). The isolates were assigned to four distinct clusters, with 62.5% assigned to Clusters A to C and one that was more distantly related assigned to Cluster D. Simpson's diversity index (0.9872) indicated high diversity among these isolates. Clustering analysis of the genomic fingerprints revealed eight distinguishable *NotI*-PFGE types, demonstrating that the MDR of ICEs-positive strains isolated for the prevalence analysis exhibited various genotypes. According

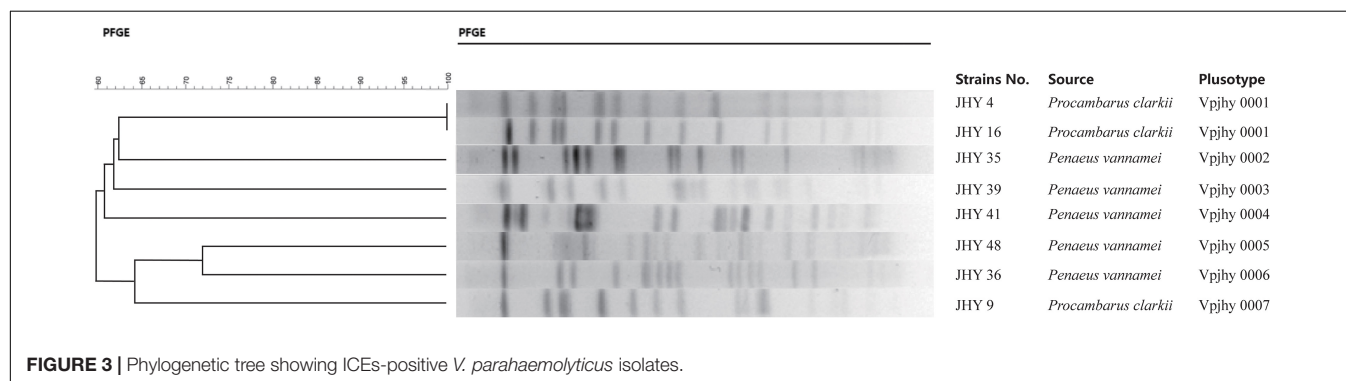
TABLE 3 | Heavy metal resistance of *V. parahaemolyticus* isolates.

Heavy metals	MIC ($\mu\text{g/mL}$)										Resistant	
	6.25	12.5	25	50	100	200	400	800	1600	3200	<i>n</i>	(%)
Ni							1	a			2	25
Cr								a	1	1	3	37.5
Cd							a	5	1	2	6	75
Cu					1	1	4	a	2		7	87.5
Pb								1	2	5	8	100
Mn									a		1	12.5
Zn							a	7	1		8	100
Hg	2	a	3	2							5	62.5

MIC, minimum inhibitory concentrations. ^aMinimal inhibition concentration of standard strain *E. coli* K12.

TABLE 4 | Susceptibility of *V. parahaemolyticus* harboring the SXT/R391 family of integrative and conjugative elements to heavy metals and antibiotics.

Sample	Heavy metal tolerance	Antimicrobial susceptibility	Source
<i>Vibrio parahaemolyticus</i> JHY4	Cr, Cd, Cu, Pb, Zn, Hg	CHL, SXT, RIF, AMP, SPT, KAN, TM, STR	<i>Procambarus clarkii</i>
<i>Vibrio parahaemolyticus</i> JHY9	Ni, Cd, Cu, Pb, Zn	CHL, SXT, RIF, AMP, SPT, KAN, TM, STR	<i>Procambarus clarkii</i>
<i>Vibrio parahaemolyticus</i> JHY16	Cr, Cd, Cu, Pb, Zn, Hg	CHL, SXT, RIF, AMP, SPT, KAN, TM, STR	<i>Procambarus clarkii</i>
<i>Vibrio parahaemolyticus</i> JHY35	Cd, Cu, Pb, Zn	CHL, SXT, RIF, AMP, SPT, KAN, TM, STR	<i>Penaeus vannamei</i>
<i>Vibrio parahaemolyticus</i> JHY36	Cu, Pb, Mn, Zn, Hg	CHL, SXT, RIF, AMP, SPT, KAN, TM, STR	<i>Penaeus vannamei</i>
<i>Vibrio parahaemolyticus</i> JHY39	Cr, Cd, Pb, Zn	CHL, SXT, RIF, AMP, SPT, KAN, TM, STR	<i>Penaeus vannamei</i>
<i>Vibrio parahaemolyticus</i> JHY41	Cd, Cu, Pb, Zn, Hg	CHL, SXT, RIF, AMP, SPT, KAN, TM, STR	<i>Penaeus vannamei</i>
<i>Vibrio parahaemolyticus</i> JHY48	Ni, Cu, Pb, Zn, Hg	CHL, SXT, RIF, AMP, SPT, KAN, TM, STR	<i>Penaeus</i>



to our previous studies, the presence or absence of ICEs has no significant relevance among these strains in terms of antimicrobial resistance (He et al., 2015a), it indicating that resistance determinants may spread among genetic lineages within the *V. parahaemolyticus* population. MGEs that carry resistance genes (Song et al., 2013) may be responsible for the high variation among the genotypes and resistance phenotypes of isolates. Therefore, we suggest future research to elucidate the

precise mechanisms of resistance determinant transmission in *V. parahaemolyticus* populations.

CONCLUSION

Bacteria secrete toxin proteins or effectors into external media or directly into eukaryotic target cells to facilitate

adaption to environmental stress conditions; this response is key during the process of infection (Park et al., 2004; Cascales, 2008; Tseng et al., 2009). Previous studies have revealed that clinical isolates of *V. parahaemolyticus* produce beta-hemolysis in Wagatsuma agar, in a process known as the Kanagawa phenomenon that is linked to TDH-secreted proteins. These proteins have been recognized as primary virulence factors and effectors (Naim et al., 2001; Ono et al., 2006; Igbinosa and Okoh, 2008). Studies from different regions of the world show that *tdh* and/or *trh* genes are found in 90–99.8% of clinical strains, whereas only 0.2–10% of environmental *V. parahaemolyticus* isolates are potentially pathogenic, based on the presence of *tdh* and/or *trh* (Letchumanan et al., 2015b; Hu and Chen, 2016; Taiwo et al., 2017; Rortana et al., 2018; Jiang et al., 2019).

Previous studies disclosed that *V. parahaemolyticus* is a very diverse species and is an opportunistic pathogen in aquatic environments that is highly successful in adapting to changing environmental conditions (Song et al., 2013; Letchumanan et al., 2015a,b). Increasing aquaculture production and industrialization has led to large amounts of antibiotics being used to prevent or treat disease outbreaks in shrimp farming; consequently, multidrug resistant (MDR) and heavy metals resistant pathogens have emerged and posed serious problems in many aquatic systems. It revealed that bacteria could acquire resistance via conjugation or transformation, allowing them to adapt to the harsh environment and to cause a wide variety of diseases (Matyar, 2012). Incidents of human food poisoning from aquaculture products pose a becoming a serious clinical issue.

In the present study, we evaluated the prevalence, antimicrobial susceptibility, heavy metal resistance and genotypes of *V. parahaemolyticus* from four species of shrimp obtained from fish markets in Jiangsu (in 2016–2018), China. The results showed that ICEs-positive isolates have been given more antibiotic resistance, and the MDR strains also showed more heavy metal resistance. Consistent with previous reports (Song et al., 2013; Hu and Chen, 2016; Kang et al., 2018), we found that AMP resistance dominated among the isolates and was present in all samples tested. Although TET, sulfonamides, and quinolones are used widely in aquaculture (Holmström et al., 2003), we did not find evidence of resistance to TET in the *V. parahaemolyticus* isolates. Nine isolates that tested positive for all resistance genes (*strA/strB*, *dfrA1/dfr18*, *sul2*) exhibited intermediate susceptibility to CN and TET, indicating potential resistance to these two antibiotics. Our results for susceptibility of the *V. parahaemolyticus* isolates to CHL contrast with previous work (He et al., 2016), where we found all ICE-positive strains were resistant to CHL, possibly as a result of the ban, since 2002, of the use of CHL and derivatives (including chloramphenicol succinate) in the breeding industry in China (China Department of Agriculture, Bulletin No. 193). We suggest that ICE-positive *V. parahaemolyticus* isolates may indicate tolerance to heavy metal agents.

Wide usage of tetracyclines, sulfonamides, and (fluoro) quinolones in aquaculture has been reported (Holmström et al., 2003). In addition, animal fecal used as fertilizer of aquaculture ponds is a common practice in integrated aquaculture-agriculture system herein. Manure and urine from field-herding cattle and goats were continuously discharged directly into ponds, which could change the bacterial community composition and bring more antimicrobials and even heavy metals in the aquaculture environment. In the *Vibrionaceae*, ICEs have been demonstrated to bestow resistance to multiple antibiotics and some complex new traits through horizontal gene transfer, which could be beneficial under certain environmental conditions (Makino et al., 2003; Balado et al., 2013). However, the present study revealed that ICEs are not the major transmission mediators of resistance to antibiotics or heavy metals. Thus, we speculate that many of the antibiotic resistance genes are intrinsic, and their expressions are activated when the environmental conditions became hostile. Furthermore, few of them are commonly transferred via conjugation or transformation. These data will aid future research in controlling of aquaculture diseases, forecasting food safety incidence and improving our understanding of *V. parahaemolyticus* prevalence and behavior in the aquaculture.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

YH, SW, XZ, and JZ participated in the design and/or discussion of the study. FS, BH, and XL carried out the major experiments. YH and SW analyzed the data. YH wrote the manuscript. SW, XZ, and JZ revised the manuscript. All authors read and approved the final version of the manuscript.

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Epidemiology Characteristics of *Streptococcus pneumoniae* From Children With Pneumonia in Shanghai: A Retrospective Study

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Background: *Streptococcus pneumoniae* is the most common pathogen causing death in children under 5 years old. This retrospective surveillance aimed to analyze serotype distribution, drug resistance, virulence factors, and molecular characteristics of pneumonia isolates from children in Shanghai, China.

Methods: A total of 287 clinical pneumococcal isolates were collected from January to December in 2018 and were divided into community-acquired pneumonia (CAP) and healthcare-associated pneumonia (HAP) two groups according to where someone contracts the infection. All isolates were serotyped by multiplex sequential PCR and antimicrobial susceptibility testing was performed using E-test or disk diffusion method. The molecular epidemiology was analyzed using multilocus sequence typing and seven housekeeping genes were sequenced to identify the sequence types (STs). In addition, we investigated the presence of virulence genes via PCR.

Results: The most common serotypes were 19F, 6A, 19A, 23F, 14, and 6B, and the coverage rates of the 7-, 10- and 13-valent pneumococcal conjugate vaccines were 58.9, 58.9, and 80.5%, respectively. More PCV13/non-PCV7 serotypes and higher rate of penicillin non-susceptible *S. pneumoniae* were seen in HAP. Molecular epidemiological typing showed a high level of diversity and five international antibiotic-resistant clones were found, including Taiwan^{19F}-14, Spain^{23F}-1, Spain^{6B}-2, Taiwan^{23F}-15 and Sweden^{15A}-25. No significant difference was observed in the presence of virulence genes among the isolates obtained from CAP and HAP. All of the *S. pneumoniae* isolates carried *lytA*, *ply*, *psaA*, *pavA*, *spxB*, *htrA*, and *clpP*, and the carriage rate of *nanA* and *piaA* were 96.2 and 99.0%. Conversely, *cps2A*, *cbpA*, and *pspA* were present in 33.8–44.3% of the isolates.

Conclusions: Serotype changes and emerging multidrug-resistant international clones were found in current study. *lytA*, *ply*, *psaA*, *pavA*, *spxB*, *htrA*, and *clpP* may be good protein vaccine candidates. Long-term high-quality surveillance should be conducted to assess impact and effectiveness brought by vaccines, and provide a foundation for prevention strategies and vaccine policies.

Keywords: *Streptococcus pneumoniae*, serotypes, antibiotic resistance, pneumococcal pneumonia, pneumococcal conjugate vaccine, virulence, children

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INTRODUCTION

Streptococcus pneumoniae is a frequent colonizer of the human nasopharynx with a colonization rate of 27–65% in children (Weiser et al., 2018), whilst the cause of both invasive pneumococcal disease (including bacteremia, meningitis, etc.) and non-invasive pneumococcal disease such as pneumonia and otitis media under the condition of the immunocompromised or microflora imbalance (GBD 2016 Lower Respiratory Infections, 2018; Weiser et al., 2018). It presents as a burden associated with high morbidity and mortality globally. As the global estimates reported, of all pneumococcal deaths in HIV-uninfected children in 2015, 81% of them died of pneumonia (Wahl et al., 2018). Centers for Disease Control and Prevention of America recommends that pneumonia can be divided into two types according to place where someone contracts the infection, community-acquired pneumonia (CAP) which is defined as when someone develops pneumonia in the community (not in a hospital) and healthcare-associated pneumonia (HAP) which is defined as when someone develops pneumonia during or following a stay in a healthcare facility¹.

In the lower respiratory infections in 195 countries in 2016, *S. pneumoniae* was estimated to be responsible for 341029 deaths of children younger than 5 years (GBD 2016 Lower Respiratory Infections, 2018). By far, lower respiratory infection incidence and mortality in children is mostly attributed to pneumococcal pneumonia. Vaccines and antibiotics are considered as effective methods against *S. pneumoniae*. Immunizing with vaccines was suggested by WHO to prevent *S. pneumoniae* infections (Pneumococcal vaccines WHO position paper, 2012). A reduction in CAP of > 40% after introduction of PCV7 has also been reported (Falup-Pecurariu, 2012). In Shanghai, pneumococcal vaccines belong to the second category of vaccines and vaccination is given only on a voluntary basis at their own expense, which maybe the cause of low vaccination rate of PCV. On the other hand, antimicrobial therapy is the common anti-infection treatment. However, with the changes in *S. pneumoniae* serotype and antibiotic resistance over time, the current treatment options are constantly being adjusted as well. The epidemiological data of pneumococcus on children with pneumonia in Shanghai is scarce at present. In this study, we aimed to analyze serotype distribution, antibiotic resistance, virulence factors and molecular characteristics of pneumonia isolates identified from children in Shanghai to provide data support for development of pneumococcal infection prevention strategies and vaccines.

MATERIALS AND METHODS

Clinical Isolates and Population

The retrospective surveillance was conducted at Shanghai Children's Hospital, which is the first specialist children's hospital in China, with about 2.5 million outpatients visiting and 44,000 hospitalized each year. A total of 287 *S. pneumoniae*

isolates were collected from patients diagnosed with pneumonia between January and December in 2018. CAP included the isolates obtained from an outpatient or collected earlier than 48 h after hospitalization, while specimens obtained more than 48 h after admission were included as HAP in this investigation (Sader et al., 2018).

Clinical and epidemiological information was systematically extracted from the medical records, including demographics of the patient, symptoms and findings at hospitalization, underlying, and other potential characteristics. The protocol for present study was approved by the Shanghai Children's Hospital Ethics Committee (Shanghai Jiao Tong University School of Medicine). The retrospective study was to obtain the genus and species of the bacteria and did not affect the patients, the Review Board consequently exempted the informed consent requirements. Only one isolate was collected from each patient. Duplicate strains and patients colonized by bacteria with no clinical symptoms were excluded from the study.

Microbiology Methods

The pneumococcal isolates analyzed in current study were collected and cultured in line with the need of clinical procedures. Specimens were collected by professional staff or doctors and transported to the department of clinical microbiology within 2 h, which were inoculated onto 5% sheep blood agar plates and incubated at 35°C, 5% CO₂ for 18–24 h. All isolates were identified by typical colony morphology, optochin assays and confirmed by the matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF MS; Bruker Daltonik GmbH, Bremen, Germany). Strains identified as *S. pneumoniae* were stored in 40% sterile glycerol broth at –80°C for subsequent analysis.

Serotyping

S. pneumoniae isolates were serotyped by multiplex sequential PCR (MP-PCR), and a primer pair targeting *cpsA* was used as a positive control in each reaction (Pai et al., 2006). Serogroup 6A/B were identified using the method described previously (Jin et al., 2009). If the serotype was not detected by the method mentioned above, the strain was classified as non-typeable. Afterwards, the coverage rates of PCV7, PCV10, and PCV13 were estimated by calculating the percentage of isolates expressed the serotypes included in the vaccines.

In vitro Antimicrobial Susceptibility Testing

Antimicrobial resistance testing of all 287 isolates were determined by E-test and Kirby-Bauer disk tests. In our study, we used E-test assay (AB Biodisk, Solna, Sweden) to measure the minimum inhibitory concentrations (MICs) to penicillin. The susceptibility to clindamycin, erythromycin, linezolid, moxifloxacin, sulfamethoxazole-trimethoprim and vancomycin was assessed using the disk diffusion method (Oxoid Ltd, Basingstoke, UK). All susceptibility tests and results interpretations were performed following the guidelines and criteria established by the Clinical and Laboratory Standard Institute (CLSI) 2018. The quality-control strain was *S. pneumoniae* ATCC 49619, which included in each set of tests

¹Prevention CfDca. *Causes of Pneumonia*. Available online at: <https://www.cdc.gov/pneumonia/causes.html>.

to ensure the reliability of the results. Isolates resistant to three or more kinds of antibiotics tested were defined as MDR *S. pneumoniae* in this study.

Multilocus Sequence Typing

To determine the STs of the isolates, multilocus sequence typing (MLST) analysis was carried out in accordance with the *S. pneumoniae* MLST protocol (Enright and Spratt, 1998). In our experiment, we used the seven housekeeping genes (*aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt*, and *ddl*), which were amplified by PCR using primers previously described (Enright and Spratt, 1998). The internal fragments amplified were sequenced on both strands by the Sanger method using the primers that were used for the initial amplification. Alleles and sequence types (STs) were confirmed by querying the pneumococcal MLST database (<http://pubmlst.org/spneumoniae/>). The STs obtained were then compared with Pneumococcal Molecular Epidemiology Network (PMEN) clones (<http://www.pneumogen.net/pmnen/>). STs that were different from any known ST were submitted for new name assignment. The relatedness between the isolates was constructed by eBURST version3.0 software. Strains were assigned to a clonal complex (CC) based on the stringent group definition of six of seven shared alleles (Feil et al., 2004).

Detection of Virulence Genes

A total of 12 genes related to virulence were detected by PCR using published primers (Ibrahim et al., 2004; Shakrin et al., 2014; Bryant et al., 2016; Kang et al., 2016), including capsular polysaccharide (*cps2A*), autolysin (*lytA*), pneumococcal surface protein A (*pspA*), choline binding protein A (*cbpA*), neuraminidase (*nanA*), ion transporters (*piaA*), pneumolysin (*ply*), pneumococcal surface adhesin A (*psaA*), pneumococcal adherence and virulence factor A (*pavA*), pyruvate oxidase (*spxB*), serine protease high-temperature requirement A (*htrA*), and caseinolytic protease (*clpP*). The PCR products were analyzed by gel electrophoresis and sequencing. The positive products were confirmed by comparing to the online database via BLAST.

Statistical Analysis

Antibiotic resistance was analyzed with the WHONET 5.6 software, while SPSS 24.0 was used for statistical analysis. Chi-square test or Fisher's exact test were used for significance comparison of categorical data, whereas *t*-test or Rank-sum test were used for comparing quantitative data. $P < 0.05$ was considered to be statistically significant.

RESULTS

Demographic and Clinical Characteristics

The total collection presented 287 *S. pneumoniae* isolates causing pneumonia, of which 243 from CAP and 44 from HAP. As was shown in **Table 1**, 90.9% of these strains (261/287) were isolated from children aged 0–5 years old and the male to female sex ratio was 1.3:1. Number of cases diagnosed in summer (during June to August) were a little lower than other seasons. The common chronic diseases in this study are congenital heart disease

(28/287) and asthma (14/287). Concurrent infection was noted in 48.8% patients. No children were vaccinated with pneumococcal conjugate vaccine (PCV) in current study. In the aggregate, discharge data showed that all patients had a favorable prognosis. CAP was the most common in the respiratory department whereas the rate of HAP in the gastroenterology dept is higher. There is no statistical difference in the sex, age, season, prognosis and other clinical and demographic characteristics between CAP and HAP.

Serotype Distribution and Vaccine Coverage

Of the 287 pneumococcal isolates, 261 isolates (90.9%) were successfully serotyped and 19F (33.4%) was the most common serotype, followed by 6A (11.8%), 19A (9.8%), 23F (8.4%), 14 (8.4%), 6B (8.0%), 34 (2.8%), 15B/C (2.4%), and 15A (2.1%). Other uncommon serotypes were detected in fewer than five strains each, which included serotype 7C (3), 11A (3), 20 (1), 4 (1), 33F (1), 9V (1), and 18 (1). The rest 26 isolates were classified as non-typeable. The serotype distribution of pneumococcal strains isolated is shown in **Figure 1**.

The overall vaccine coverage rates of PCV7, PCV10, and PCV13 serotypes were 58.9, 58.9, and 80.5%, respectively. HAP had a lower vaccine serotype coverage rate than CAP. A higher rate of PCV13/non-PCV7 serotypes was noticed in HAP. Simultaneously, serotype 4, 9V, 15B/C, 7C, 18, 20 and 33F were only observed in CAP.

Antibiotic Susceptibility

The total prevalence of penicillin non-susceptible *S. pneumoniae* (PNSP) was 31.7% including penicillin-intermediate *S. pneumoniae* (PISP, 26.8%) and penicillin-resistant *S. pneumoniae* (PRSP, 4.9%) (**Table 2**). Most strains showed high resistance to erythromycin and clindamycin (>95%). No drug-resistant strains to linezolid, moxifloxacin and vancomycin were observed in this study. In addition, resistance to sulfamethoxazole-trimethoprim was seen in 76.7% of isolates.

Approximately 74.9% (215/287) of the isolates were defined as MDR. Resistance of pneumococcus to the agents above among different serotypes was also assessed and it was found that the antibiotic resistance varied by serotype. Serotypes 19F, 19A, and 23F prevailed in PNSP isolates, and almost all 19F isolates were resistant to sulfamethoxazole-trimethoprim. PCV13 covered 85.1% (183/215) of the MDR strains, which was higher than that for PCV7 (61.4%, 132/215). Emerging serotypes (11A, 15B/C, 18, 20, 34, 7C, and 9A) accounted for 8.4% MDR. Compared with CAP, there were higher rates of PNSP (30.9 vs. 36.4%) and MDR (74.5% vs. 77.3%) in HAP.

MLST

Sixty-four STs were identified by MLST analysis among the 287 isolates. The five predominant STs were ST271 ($n = 72$, 25.1%), ST320 ($n = 30$, 10.5%), ST3173 ($n = 24$, 8.4%), ST876 ($n = 14$, 4.9%), and ST81 ($n = 13$, 4.5%), which were mainly related to serotype 19F, 19A, 6A/B, 14, and 23F, respectively. Nine clonal complexes and 39 singletons were obtained using eBURST version3.0 software analysis for the homology relationship

TABLE 1 | Clinical and demographic characteristics of children with CAP (*n* = 243) and HAP (*n* = 44) in Shanghai.

	CAP (<i>n</i> = 243)	HAP (<i>n</i> = 44)	Total (<i>n</i> = 287)	%	<i>P</i>
Sex					0.202
Male	135 (55.6)	29 (65.9)	164	57.1	
Female	108 (44.4)	15 (34.1)	123	42.9	
Age (months)					0.881
<12	49 (20.2)	11 (25.0)	60	20.9	
12–24	66 (27.2)	12 (27.3)	78	27.2	
24–60	105(43.2)	18 (40.9)	123	42.9	
>60	23 (9.5)	3 (6.8)	26	9.1	
Disease-onset season					0.992
December-February	59 (24.3)	11 (25.0)	70	24.4	
March-May	69 (28.4)	13 (29.5)	82	28.6	
June-August	49 (20.1)	8 (18.2)	57	19.9	
September-November	66 (27.2)	12 (27.3)	78	27.2	
Chronic disease					
Congenital heart disease	20 (8.2)	8 (18.2)	28	9.8	0.077
Asthma	14 (5.8)	0	14	4.9	0.211
Concurrent infection					
<i>Mycoplasma pneumoniae</i>	61 (25.1)	9 (20.5)	70	24.4	0.509
<i>Rhinovirus</i>	23 (9.5)	2 (4.5)	25	8.7	0.439
<i>Respiratory syncytial virus</i>	14 (5.8)	2 (4.5)	16	5.6	1.000
<i>Influenza virus</i>	15 (6.2)	0	15	5.2	0.185
<i>Epstein-Barr virus</i>	8 (3.3)	1 (2.3)	9	3.1	1.000
<i>Cytomegalovirus</i>	5 (2.1)	0	5	1.7	1.000
Wards of hospitalization					
Respiratory medicine	181 (74.5)	25(56.8)	206	71.7	0.017
Priority ward	42 (17.3)	9 (20.5)	51	17.8	0.613
Gastroenterology	5 (2.1)	5 (11.4)	10	3.5	0.008
ICU	5 (2.1)	2 (4.5)	7	2.4	0.650
Others*	10 (4.1)	3 (6.8)	13	4.5	0.690
Fever (days), median (IQR)	2 (1-4)	2 (1-4)	-	-	0.657
Antibiotic (days), median (IQR)	7.21 (6-8)	6.69 (5-7.5)	-	-	0.395
Hospitalization (days), median (IQR)	7 (6-8)	7 (6-9)	-	-	0.112
Cure	243 (100)	44 (100)	287	100	

*Other wards of hospitalization, including Neurology, Cardiology and Neonatology, Otolaryngology-Head and Neck Surgery.

between these STs (**Figure 2**). Among the 9 CCs, the most prevalent clonal complex CC271 (including ST271, ST236, ST320, ST1968 etc.) accounted for 42.9% (123/287) of the isolates, followed by CC3173 (10.5%, 30/287) and CC81 (4.9%, 14/287).

Comparing the isolates with the PMEN clones (at least 6 of 7 MLST alleles shared), five international antibiotic-resistant clones were found in this study, including Taiwan^{19F}-14, Spain^{23F}-1, Spain^{6B}-2, Taiwan^{23F}-15 and Sweden^{15A}-25. The isolates belonging to these international clones or their single locus variants (SLVs) made up of 40.8% of all strains. The dominating of the five international clones was Taiwan^{19F}-14. Furthermore, CC271 related to the Taiwan^{19F}-14 was mainly associated with the serotype 19 group, 88 of which were serotype 19F and 25 were 19A. Sweden^{15A}-25 was also identified in this study and this group of isolates included two STs, ST63 (*n* = 2) and SLV ST2248 (*n* = 4), with serotypes 15A and 14, respectively. What's more, one new *aroE* allele and five new STs were found in

our study. There were no significant differences in the major STs and distribution between HAP and CAP.

Presence and Distribution of Virulence Genes

The presence of virulence genes did not show any significant difference among the isolates obtained from CAP and HAP (**Table 3**). Irrespective of the source of the isolation, all isolates carried *lytA*, *ply*, *psaA*, *pavA*, *spxB*, *htrA*, and *clpP* genes. In general, most of the isolates harbored *nanA* (96.2%) and *piaA* (99.0%). Significant association was suggested between carriage rate and serotype in *cps2A*, *cbpA*, *pspA* and *nanA*. Besides, *cps2A*, *cbpA*, and *pspA* was also associated with clonal complex.

cps2A was present in 44.3% isolates and all serotypes 19A and 14 possessed it. The majority of serogroup 19 isolates carried *cbpA*, including 19F (86.5%) and 19A (89.3%). Serotypes 6A and 6B were the most dominant serotypes to carry *pspA*.

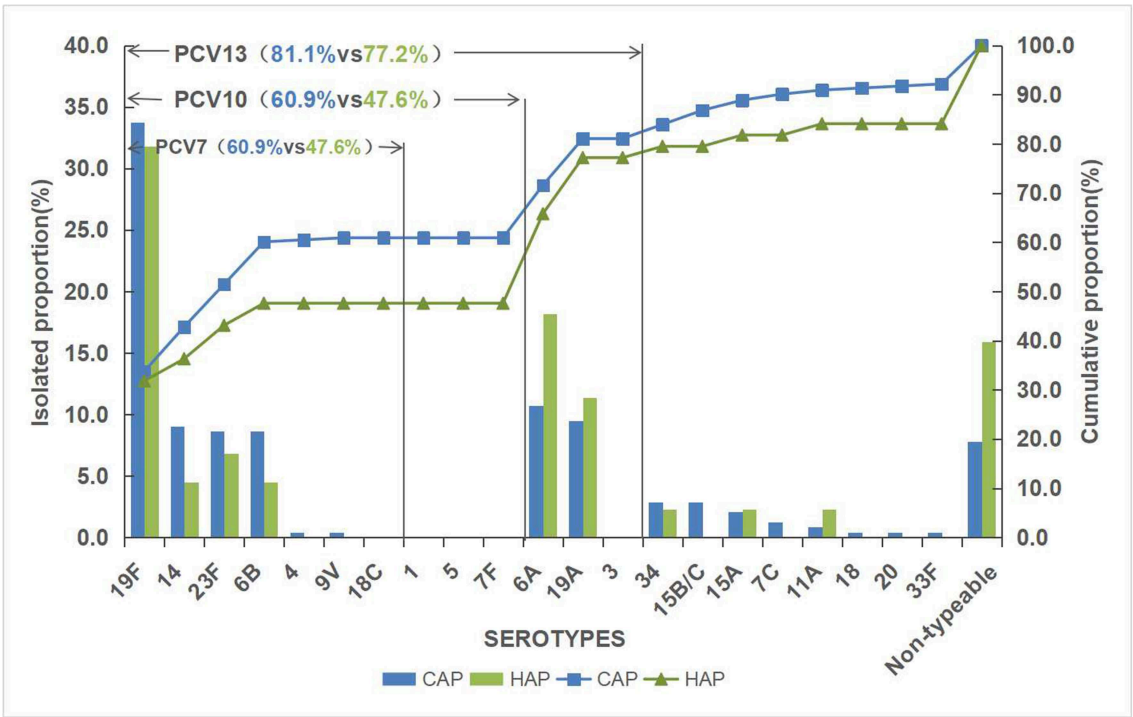


FIGURE 1 | Serotype distribution of pneumococcal strains isolated in Shanghai in 2018.

TABLE 2 | Antimicrobial resistance of pneumococcal isolates from children with CAP (n = 243) and HAP (n = 44).

Agent (%)	CAP (N = 243)			HAP (N = 44)			TOTAL (N = 287)		
	PSSP (n = 168)	PISP (n = 65)	PRSP (n = 10)	PSSP (n = 28)	PISP (n = 12)	PRSP (n = 4)	PSSP (n = 196)	PISP (n = 77)	PRSP (n = 14)
SXT	69.0	92.3	90.0	67.9	100	100	68.9	93.5	92.9
ERY	99.4	96.9	100	100	100	100	99.5	97.4	100
CLI	98.8	96.9	80.0	100	100	75.0	99	97.4	78.6
VAN	0	0	0	0	0	0	0	0	0
LZD	0	0	0	0	0	0	0	0	0
MXF	0	0	0	0	0	0	0	0	0

SXT, sulfamethoxazole-trimethoprim; ERY, erythromycin; CLI, clindamycin; VAN, vancomycin; LZD, linezolid; MXF, moxifloxacin.

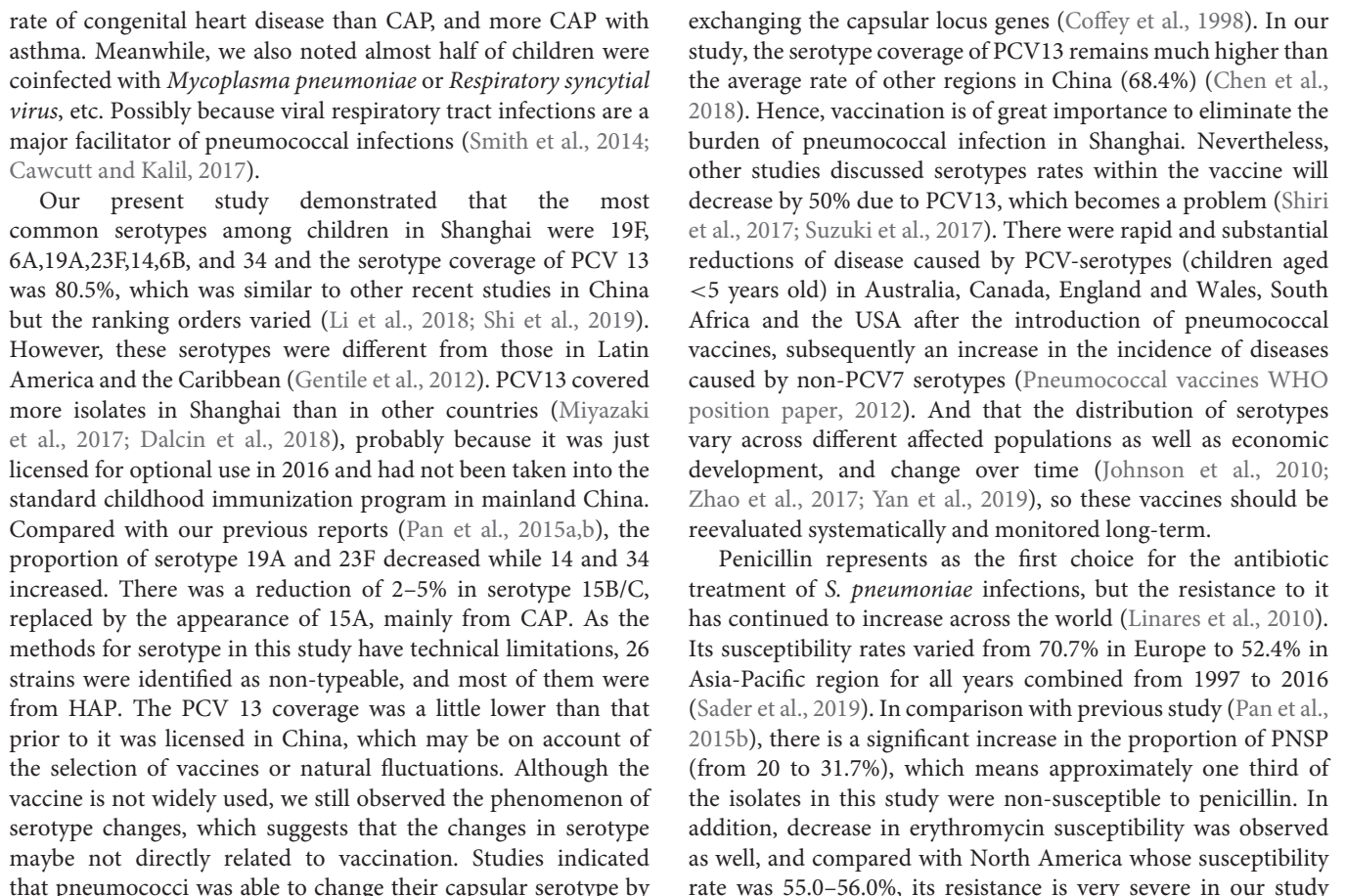
The relationship between virulence patterns and serotypes of *S. pneumoniae* isolated from CAP and HAP was listed in Table 4. Based on the studied genes, the most common virulence pattern in current study was *lytA-ply-psaA-pavA-spxB-htrA-clpP-cbpA-nanA-piaA* (27.9%), with 19F accounting for the majority and 90% were MDR, followed by pattern *lytA-ply-psaA-pavA-spxB-htrA-clpP-cps2A-nanA-piaA* (16.7%) that contains a variety of serotypes.

DISCUSSION

WHO reported that pneumonia accounts for 16% of all deaths of children under 5 years old, killing 920,136 children in 2015, with the most common cause of bacterial pneumonia being *S.*

*pneumoniae*². In this study, we identified a total of 287 pediatric patients diagnosed with pneumococcal pneumonia from January to December 2018 in Shanghai, China. Most of them (261,90.9%) were under 5 years old and diagnosed less in the summer, which was in line with the recent results from China (Cai et al., 2018). A male sexual superiority was also noticed among the study population, so as in other studies (Cai et al., 2018; Arushothy et al., 2019). In current study, chronic diseases were observed in 15.7% patients, which is an independent risk factor for pneumonia-related mortality in children (Zhang et al., 2013; Sonego et al., 2015; Nguyen et al., 2017). HAP has a higher

²World Health Organization. *Pneumonia-Key facts*. Available online from: <https://www.who.int/news-room/fact-sheets/detail/pneumonia>.



Our present study demonstrated that the most common serotypes among children in Shanghai were 19F, 6A, 19A, 23F, 14, 6B, and 34 and the serotype coverage of PCV 13 was 80.5%, which was similar to other recent studies in China but the ranking orders varied (Li et al., 2018; Shi et al., 2019). However, these serotypes were different from those in Latin America and the Caribbean (Gentile et al., 2012). PCV13 covered more isolates in Shanghai than in other countries (Miyazaki et al., 2017; Dalcin et al., 2018), probably because it was just licensed for optional use in 2016 and had not been taken into the standard childhood immunization program in mainland China. Compared with our previous reports (Pan et al., 2015a,b), the proportion of serotype 19A and 23F decreased while 14 and 34 increased. There was a reduction of 2–5% in serotype 15B/C, replaced by the appearance of 15A, mainly from CAP. As the methods for serotype in this study have technical limitations, 26 strains were identified as non-typeable, and most of them were from HAP. The PCV 13 coverage was a little lower than that prior to it was licensed in China, which may be on account of the selection of vaccines or natural fluctuations. Although the vaccine is not widely used, we still observed the phenomenon of serotype changes, which suggests that the changes in serotype maybe not directly related to vaccination. Studies indicated that pneumococci was able to change their capsular serotype by

Penicillin represents as the first choice for the antibiotic treatment of *S. pneumoniae* infections, but the resistance to it has continued to increase across the world (Linares et al., 2010). Its susceptibility rates varied from 70.7% in Europe to 52.4% in Asia-Pacific region for all years combined from 1997 to 2016 (Sader et al., 2019). In comparison with previous study (Pan et al., 2015b), there is a significant increase in the proportion of PNSP (from 20 to 31.7%), which means approximately one third of the isolates in this study were non-susceptible to penicillin. In addition, decrease in erythromycin susceptibility was observed as well, and compared with North America whose susceptibility rate was 55.0–56.0%, its resistance is very severe in our study

TABLE 3 | Virulence genes among major serotypes and clonal complexes (%).

Categories	cps2A		cbpA		pspA		nanA		piaA		lytA		ply		psaA		pavA		spxB		htrA		cipP		Total		
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	
Source																											
CAP	111	45.7	107	44	83	34.2	234	96.3	241	99.2	243	100	243	100	243	100	243	100	243	100	243	100	243	100	243	84.7	
HAP	16	36.4	20	45.5	14	31.8	42	95.5	43	97.7	44	100	44	100	44	100	44	100	44	100	44	100	44	100	44	15.3	
P = 0.252																											
P = 0.861																											
P = 0.763																											
P = 1.000																											
P = 0.394																											
Serotypes																											
19F	6	6.3	83	86.5	7	7.3	96	100	95	99	96	100	96	100	96	100	96	100	96	100	96	100	96	100	96	33.4	
6A	10	29.4	2	5.9	30	88.2	34	100	34	100	34	100	34	100	34	100	34	100	34	100	34	100	34	100	34	11.8	
6B	4	17.4	0	0	18	78.3	23	100	23	100	23	100	23	100	23	100	23	100	23	100	23	100	23	100	23	8.0	
19A	28	100	25	89.3	2	7.1	27	96.4	28	100	28	100	28	100	28	100	28	100	28	100	28	100	28	100	28	9.8	
23F	13	54.2	3	12.5	7	29.2	20	83.3	24	100	24	100	24	100	24	100	24	100	24	100	24	100	24	100	24	8.4	
14	24	100	24	100	4	16.7	24	100	23	95.8	24	100	24	100	24	100	24	100	24	100	24	100	24	100	24	8.4	
p = 0.000																											
p = 0.000																											
p = 0.000																											
p = 0.001																											
p = 0.597																											
Clonal complexes																											
CC271	35	28.5	96	78.0	10	8.1	121	98.4	121	98.4	123	100	123	100	123	100	123	100	123	100	123	100	123	100	123	42.9	
P = 0.000																											
P = 0.000																											
P = 0.169																											
P = 0.802																											
Total	127	44.3	127	44.3	97	33.8	276	96.2	284	99.0	287	100	287	100	287	100	287	100	287	100	287	100	287	100	287	100	

(Sader et al., 2019). It was reported that the susceptibility rates were lower among isolates from pediatric patients than adults for penicillin and azithromycin (Sader et al., 2019). In the Chinese consensus, antibiotics use in children is restricted due to the concern for safety and risk of adverse events. For instance, despite the widely use of fluoroquinolones for their highly effective in adults, their use in children is limited because of the side-effect and toxicity they may cause, such as destructive arthropathy and influence on the central nervous system (Patel and Goldman, 2016). The increase resistance maybe attributed to the selection pressure caused by the widespread use of β -lactam and macrolide antibiotics that were used as first-line therapy in children for their little side-effect and lower toxicity (Bradley et al., 2011). HAP with a higher rate of PNSP and proportion of MDR than CAP was noticed in this study, which suggested that the strains within the community were more susceptible and the strains obtained within hospital were more resistant. The higher resistance in HAP may be due to the higher rates of PCV13/non-PCV7 serotypes because of the significant relationship between antibiotic resistance and serotypes. Our data showed that PCV13 covered 85.1% MDR isolates, which suggested vaccine has the potential to control the spread of MDR (Maraki et al., 2010, 2018). Against all isolates vancomycin, linezolid and moxifloxacin exhibited good activity, which could be alternatives for treatment of PRSP and MDRSP infections.

The most common STs in this study was ST271,ST320,ST3173, and ST876, which was similar to other reports in other regions of China (Li et al., 2018; Shi et al., 2019; Yan et al., 2019). When comparing with the multi-center study in Shanghai in 2013 (Pan et al., 2015b), we observed that the dominating STs were basically the same, with a reduction in ST81 and increase in ST3173 and ST876. The rate of clones registered as MDR PMEN clones was essentially equal but different in content composition. Except for four common international antibiotic-resistant clones Taiwan^{19F}-14, Spain^{23F}-1, Spain^{6B}-2 and Taiwan^{23F}-15 in Shanghai, Sweden^{15A}-25 was newly observed in our study. After the introduction of PCV13, the increasing serotype 15A was noted in Norway, Canada and Japan (Steens et al., 2013; Ricketson et al., 2014; Naito et al., 2016). In our study, six isolates belongs to Sweden^{15A}-25/ST63 and its single locus variant. Most of the clones in 2018 carried the same serotypes in 2013. In addition, ST2248, a SLV of Sweden^{15A}-25/ST63, were detected in serotype 14. Five new STs were also obtained, related to serotype 34, 6A/B and 15B/C. With the popularization of vaccines, a careful monitoring should be required in the future about the Sweden^{15A}-25/ST63 known as one of the MDR PMEN clones (Hackel et al., 2013).

Dispensable genes, not required for bacterial growth, provide survival advantages to *S. pneumoniae*. We detected the prevalence of a total of 12 virulence factors of particular important. These genes play a role in adherence to host cells, evasion of host immune responses and promotion of the biofilms formation to enhance bacterial survival competitiveness (Brooks and Mias, 2018). Comparison with other investigations, the detection rate varied across the regions and isolates disease related or colonization-related (Qin et al., 2013; Fu et al., 2019). Our data indicated that there is no difference between

TABLE 4 | The relationship between virulence pattern and serotypes of *S. pneumoniae* isolated from CAP and HAP.

Virulence pattern	Isolates (No.)	Proportion (%)	CAP (No.)	HAP (No.)	Related serotypes (No.)
<i>lytA-ply-psaA-pavA-spxB-htrA-clpP-pspA</i>	1	0.3	0	1	untyped (1)
<i>lytA-ply-psaA-pavA-spxB-htrA-clpP-piaA</i>	1	0.3	1	0	Untyped (1)
<i>lytA-ply-psaA-pavA-spxB-htrA-clpP-cps2A-nanA</i>	1	0.3	1	0	14 (1)
<i>lytA-ply-psaA-pavA-spxB-htrA-clpP-cps2A-piaA</i>	1	0.3	1	0	33F (1)
<i>lytA-ply-psaA-pavA-spxB-htrA-clpP-pspA-piaA</i>	2	0.7	2	0	23F (2)
<i>lytA-ply-psaA-pavA-spxB-htrA-clpP-nanA-piaA</i>	29	10.1	22	7	19F (8),6A (3),6B (4),23F (5),15A (1),18 (1),untyped (7)
<i>lytA-ply-psaA-pavA-spxB-htrA-clpP-cbpA-nanA</i>	1	0.3	1	0	19F (1)
<i>lytA-ply-psaA-pavA-spxB-htrA-clpP-cps2A-pspA-piaA</i>	3	1.0	3	0	19A (1),untyped (2)
<i>lytA-ply-psaA-pavA-spxB-htrA-clpP-cps2A-nanA-piaA</i>	48	16.7	44	4	14 (19),23F (11),11A (1),15A (1),15B/C (4),19A (2),19F (3),20 (1),6B (1),9V (1),untyped (4)
<i>lytA-ply-psaA-pavA-spxB-htrA-clpP-cbpA-nanA-piaA</i>	80	27.9	67	13	19F (76),15A (2),6A (1),untyped (1)
<i>lytA-ply-psaA-pavA-spxB-htrA-clpP-cbpA-pspA-piaA</i>	1	0.3	1	0	23F (1)
<i>lytA-ply-psaA-pavA-spxB-htrA-clpP-pspA-nanA-piaA</i>	38	13.2	31	7	6A (19),6B (15),23F (3),19F (1),
<i>lytA-ply-psaA-pavA-spxB-htrA-clpP-cps2A-cbpA-pspA-piaA</i>	2	0.7	1	1	23F (1),untyped (1)
<i>lytA-ply-psaA-pavA-spxB-htrA-clpP-cps2A-cbpA-nanA-piaA</i>	29	10.1	23	6	19A (24),19F (1),23F (1),15B/C (1),11A (1),untyped (1)
<i>lytA-ply-psaA-pavA-spxB-htrA-clpP-cps2A-pspA-nanA-piaA</i>	36	12.5	31	5	6A (10),34 (6),14 (4),6B (3),7C (3),19F (1),15B/C (1),11A (1),4 (1),untyped (6)
<i>lytA-ply-psaA-pavA-spxB-htrA-clpP-cbpA-pspA-nanA-piaA</i>	7	2.4	7	0	19F (4), 15A (2), 6A (1)
<i>lytA-ply-psaA-pavA-spxB-htrA-clpP-cps2A-cbpA-pspA-nanA-piaA</i>	7	2.4	7	0	19F (1),19A (1),34 (2),15B/C (1),untyped (2)

CAP and HAP isolates in the presence and distribution of virulence genes. The positive rate of *cps2A*, *cbpA*, *pspA*, and *nanA* varied from different serotypes. It's worth noting that nine isolates were negative for the housekeeping gene *cpsA* similar to other reports. Studies indicated that non-encapsulated *S. pneumoniae* could also cause 3–19% of pneumococcal diseases and the current conjugate vaccines may be ineffective against them because of serotype specificity (Keller et al., 2016). The appearance of serotype replacement and non-encapsulated strains suggested a new type vaccine should be designed to prevent pneumococcal infections. Virulence genes are crucial candidate targets for the development of next-generation protein vaccines. For instance, a study reported that generate neutralizing antibodies immunization with pneumococcal neuraminidases *nanA*, *nanB*, and *nanC* was able to increase survival in mice (Janapatla et al., 2018). Our results showed that there is a high prevalence in *nanA*, *piaA*, *lytA*, *ply*, *psaA*, *pavA*, *spxB*, *htrA* and *clpP* whereas lower in *cps2A*, *cbpA*, and *pspA*. A requisite for a vaccine candidate is that the selected gene (s) is widely distributed in the target pneumococcal population (Cornick et al., 2017). The extremely high carriage rate suggests the potential to develop vaccines. However, this current study suggests that *cps2A*, *cbpA*, and *pspA* might be not suitable to be candidates for vaccines in Shanghai.

In conclusion, our study described the epidemiology characteristics of *S. pneumoniae*. from children with pneumonia in Shanghai. 19A/E, 6A/B, 23F and 14 were identified as the predominating serotypes in 2018. PCV13 serotype coverage was a little reduced than before. Compared with CAP, isolates from HAP had more PCV13/non-PCV7 serotypes, higher rate of PNSP and higher proportion of MDR. Moreover, our results revealed the type of clonal disseminations, and more

attention should be paid to the emerging Sweden^{15A}-25/ST63 with the popularization of vaccines. *lytA*, *ply*, *psaA*, *pavA*, *spxB*, *htrA* and *clpP* were observed in all isolates, which may be candidates for next generation vaccines. Finally, long-term high-quality surveillance should be conducted to assess impact and effectiveness brought by vaccines, and provide a foundation for prevention strategies and vaccine policies.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript.

AUTHOR CONTRIBUTIONS

HZ, WZ, and FP conceived and designed the experiments. WZ and YYS performed antibiotic susceptibility testing and serotyping. WZ and BW performed MLST and virulence genes detection. HZ, YS, TZ, and CW contributed strains and case data collection. WZ wrote the first draft of the manuscript, and all authors contributed to manuscript revision, read and approved the submitted version.

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Helicobacter pylori and Its Antibiotic Heteroresistance: A Neglected Issue in Published Guidelines

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“Heteroresistance” is a widely applied term that characterizes most of the multidrug-resistant microorganisms. In microbiological practice, the word “heteroresistance” indicates diverse responses to specific antibiotics by bacterial subpopulations in the same patient. These resistant subpopulations of heteroresistant strains do not respond to antibiotic therapy *in vitro* or *in vivo*. Presently, there is no standard protocol available for the treatment of infections caused by heteroresistant *Helicobacter pylori* in clinical settings, at least according to recent guidelines. Thus, there is a definite need to open a new discussion on how to recognize, how to screen, and how to eliminate those problematic strains in clinical and environmental samples. Since there is great interest in developing new strategies to improve the eradication rate of anti-*H. pylori* treatments, the presence of heteroresistant strains/clones among clinical isolates of the bacteria should be taken into account. Indeed, increased knowledge of gastroenterologists about the existence of heteroresistance phenomena is highly required. Moreover, the accurate breakpoints should be examined/determined in order to have a solid statement of heteroresistance among the *H. pylori* isolates. The primary definition of heteroresistance was about coexistence of both resistant and susceptible isolates at the similar gastric microniche at once, while we think that it can be happened subsequently as well. The new guidelines should include a personalized aspect in the standard protocol to select a precise, effective antibiotic therapy for infected patients and also address the problems of regional antibiotic susceptibility profiles.

Keywords: *Helicobacter pylori*, heteroresistance, antibiotic resistance, guidelines, antibiotic therapy

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative and transmissible microorganism that resides in the deep gastric mucosa of humans. Current estimation of people infected with this microorganism is around 4.4 billion of the world's population (Kusters et al., 2006). The infection causes serious digestive disease such as chronic gastritis, peptic ulcer disease

(PUD), and gastric cancer in 1–10% of colonized individuals (Blaser, 1990, 1992; Parsonnet et al., 1991; Atherton, 2006). It is also suspected of inducing or worsening Parkinson's disease (Dobbs et al., 2016). For reasons that are unclear, many of the colonized persons remain asymptomatic for their whole life (Malfertheiner et al., 2018b). Treating *H. pylori* infection induces the regression of mucosa-associated lymphoid tissue (MALT) lymphoma and has recently been considered as a preventive approach for the management of gastric cancer (Bayerdörffer et al., 1995; Wong et al., 2004; Atherton, 2006). The elimination of this pathogen is also reducing or eliminating Parkinson's disease symptoms (Liu et al., 2017). Many guidelines have been established that outline a number of therapeutic regimens to provide optimal treatments using conventional antibiotics and proton pump inhibitors (PPIs). However, the lack of an ideal therapy is still a major challenge in *H. pylori*-related treatment. Apart from the many antibiotics applied to fight the infection, a skyrocketing increase in antimicrobial resistance is hampering the elimination of *H. pylori* (Chiba et al., 1992; Gao et al., 2010). Although patient compliance and other environmental factors are partially involved, antibiotic resistance is mainly responsible for *H. pylori* treatment failures (Graham, 1998; Björkholm et al., 2001; De Francesco et al., 2010; Graham and Fischbach, 2010; Thung et al., 2016). Prevalence of antibiotic resistance among *H. pylori* strains varies in different geographic areas (Björkholm et al., 2001; Glupczynski et al., 2001; Megraud, 2004; Fischbach and Evans, 2007; Gao et al., 2010; Megraud et al., 2012; Khaiboullina et al., 2016). This global problem has triggered a universal drive to find a better solution. Following the rapid increase in resistance rates for conventional antibiotics used for eliminating *H. pylori*, many studies showed failures of first, second, and third lines of treatments (Wolle et al., 2002; Zullo et al., 2007; Kim et al., 2011; Oleastro et al., 2011; Saracino et al., 2012; Tonkic et al., 2012; Hsiang et al., 2013; Maleknejad et al., 2015; Kori et al., 2017; Macias-Garcia et al., 2017). The ultimate goal in therapeutic regimens against *H. pylori* is to achieve more than 90% eradication rate, which has currently proved unreachable (Zagari et al., 2018). Most recent worldwide reports together with meta-analyses indicate that the efficacy of antibiotics available to treat *H. pylori* infections has been significantly reduced (Figueiredo et al., 2005; Suzuki and Mori, 2018). In addition, emergence of multidrug-resistant *H. pylori* strains has been devastating for clinicians and microbiologists aiming to eliminate this infection (Kwon et al., 2003; Boyanova, 2009). The situation is made worse by the lack of a vaccine against *H. pylori* (Luo et al., 2018; Pan et al., 2018; Malfertheiner et al., 2018a,b). Now that antibiotic resistance has been recognized as a growing problem in the treatment of *H. pylori* infection, a much less investigated phenomenon among bacteria, namely, "heteroresistance" should be addressed. An understanding of the causes of increasing prevalence of resistant strains or, in other words, heteroresistant strains, could be pivotal in the design of effective guidelines both on national and international scales. In this paper, we discuss first the general concept of heteroresistance reported

for some *H. pylori* strains; second, we will compare this resistance to co-infection with this bacterium. Ultimately, we hope for a reformulation of the treatment options of antibiotic-resistant *H. pylori* infections.

The phenomenon of heteroresistance is based on the growth differences in bacterial subpopulations within the same strain in response to a particular antibiotic (El-Halfawy and Valvano, 2013; Wang et al., 2014; El-Halfawy and Valvano, 2015). "Heteroresistance" can be one of several terms applied to multidrug-resistant microorganisms (Alam et al., 2001; Wannet, 2002; Plipat et al., 2005; Matteo et al., 2006; Hawley et al., 2008; Goldman et al., 2014; He et al., 2017). Heteroresistant clones are able to survive in the presence of antibiotics in both *in vitro* and *in vivo* niches (Yeldandi et al., 1988; Gillespie, 2002; Ribes et al., 2010; Russo et al., 2011). Due to the lack of standard methods of characterizing heteroresistance, its detection is poor (Hofmann-Thiel et al., 2009; Huang et al., 2010; Goldman et al., 2014; He et al., 2017). The first heteroresistant Gram-negative bacterium, *Haemophilus influenzae*, was discovered in 1947 (Alexander and Leidy, 1947). Since then, not many bacteria have been listed as heteroresistant, even though this phenomenon is widely spread across many bacterial species. The Clinical and Laboratory Standards Institute (CLSI), one of several international bodies dealing with antimicrobial resistances, has published many reports determining resistant, sensitive, and intermediately resistant organisms. However, there is no established definition of heteroresistant strains (Osato et al., 2001; El-Halfawy and Valvano, 2015). Additionally, practitioners are not fully aware of the frequency and clinical activity of heteroresistant isolates. Therefore, the focus of this paper is to pinpoint the clinical impact of *H. pylori* heteroresistant strains and to highlight the urgent need for revised guidelines to manage and cure this infection.

HETERORESISTANT *H. PYLORI*

The emergence of heteroresistance in *H. pylori* resistant strains has never been discussed in published guidelines (Malfertheiner et al., 2007, 2012; Graham and Shiotani, 2008; Fock et al., 2013; Subspecialty Group of Gastroenterology, 2015; Sugano et al., 2015; Zagari et al., 2015; Chey et al., 2017; Mahachai et al., 2017; Smith et al., 2017). Matteo et al. found two *H. pylori* strains that significantly differed in antibiotic sensitivity even though they were obtained from two antral biopsies isolated from a single patient (Matteo et al., 2008). The minimum inhibitory concentration (MIC) for amoxicillin in those strains varied between 2 and 0.06 µg/ml, respectively. This finding brings a new insight about the importance of heteroresistant strains and the need for their detection prior to antibiotic prescription. The important clinical consequence of the existence of heteroresistant *H. pylori* strains is the possibility of their further propagation despite antibiotic therapy. The absence of an accurate and rigorous approach hampers the exact determination of the real prevalence of these strains in the affected individuals. Additionally, data about persistence or virulence of these heteroresistant bacteria are currently lacking (El-Halfawy and Valvano, 2013; Didelot et al., 2016; Halaby et al., 2016).

In consideration of the likely failure of treatment of heteroresistant strains, the rapid increase of gastroduodenal diseases is both predictable and expected. Consequently, the high potential of heterogeneity reported for this bacterium must be thoroughly researched (Yamaoka, 2012).

HETERORESISTANT STRAINS VERSUS CO-INFECTIONS

There is a common belief among microbiologists about the existence of two or more different *H. pylori* strains colonizing the same human stomach (Jorgensen et al., 1996; Kersulyte et al., 1999; Lai et al., 2016; Mansour et al., 2016; Raymond et al., 2016). It seems that following bacterial colonization of our stomach, *H. pylori* is able to use its remarkable genetic variability to create new variants (Jiang et al., 1996; Gottke et al., 2000; Spechler et al., 2000; Suerbaum, 2000; Gravina et al., 2016). Given the large potential of *H. pylori* in generating new genetically diverse isolates, the co-infections theory, in our opinion, needs to be updated by the incorporation of the heteroresistance concept. According to the theory of heteroresistance, human gastric mucosa can be a territory for both resistant and sensitive *H. pylori* strains exposed to specific antibiotics while the origin of isolates remains identical. During infections, approximately 5% of them are actually mixed infections caused by independent *H. pylori* strains. As such, evolved pathogen population is composed of both genetically identical and different strains (Lai et al., 2016). It is important to investigate if heteroresistant strains affect the final outcome (severe or mild diseases) of this infection or not. Additionally, the exact prevalence of co-infections and heteroresistance *in vivo* should be investigated in greater detail in future studies. Clearly, both phenotypical and genotypical analyses are required to answer this difficult question.

AN EXPLANATION FOR INCONSISTENT FINDINGS IN *IN VITRO* AND *IN VIVO* EXPERIMENTS

Currently, we are still far from understanding the exact mechanisms involved in the development of this less-recognized biologic action among the bacteria. Clinicians are well aware of inconsistencies between *in vitro* and *in vivo* susceptibility tests (Glupczynski, 1993; Best et al., 1997; Loo et al., 1997; Bereswill et al., 1999; Chatsuwan and Amyes, 1999; van der Voort et al., 2000; Gonzalez et al., 2001; Warburton-Timms and McNulty, 2001; Adeniyi et al., 2009). Unfortunately, there is no universally standard method to determine MIC for *H. pylori* species. We suggest that heteroresistance is a likely cause of this inconsistency. Our main evidence for this claim is the concept of the simultaneous presence of various *H. pylori* genotypes in the human stomach that are associated with different susceptibility profiles (Kim et al., 2003; Graham and Fischbach, 2010; Lee et al., 2014). Because independent subpopulations of *H. pylori* are growing rapidly, we detect numerous different

isolates with confusing susceptibility patterns originating from the same patient. Propagation of various *H. pylori* isolates *in vitro* can lead to conflicting results of antibiotic susceptibility testing. Thus heteroresistance is a major clinical issue, which should attract very intense attention in the upcoming years.

NEW GUIDELINE AGAINST *H. PYLORI*?

Similar to other bacterial infections, *H. pylori* management was a challenging area of the research for decades after its discovery (Malfertheiner et al., 2007, 2012; Subspecialty Group of Gastroenterology, 2015; Zagari et al., 2015; Isaeva et al., 2016; Authors, Responsible in representation of the DGVS, 2017; Chey et al., 2017; Mahachai et al., 2017; Smith et al., 2017). As far as diagnosis is concerned, there is reasonable consensus between scientists. However, there is absolutely no agreement on the optimal therapeutic intervention especially in the case of first-line treatment (Chey et al., 2007; Malfertheiner et al., 2007, 2012, 2017; Asaka et al., 2010). Overall, the mechanisms of resistance to all antibiotics used against *H. pylori* are well known (Debets-Ossenkopp et al., 1996; Burns et al., 1998; Megraud, 1998, 2003; van der Wouden et al., 2001; Keating, 2013). The current body of evidence is based on the fact that many antibiotic therapy failures are the result of genetic variations among the different *H. pylori* isolates that cause mixed infections in a host. The open question, however, is about the impact of heteroresistance on the development of severe gastroduodenal disorders in infected patients. Indeed, a long list of experiments is necessary to pinpoint the actual role of heteroresistance in the development of *H. pylori* persistent infections. Thus, updated guidelines would lead to a more effective cure if they would consider the practical approaches in dealing with heteroresistant strains. As such, this paper can be useful in the preparation for next Maastricht meetings aimed to enrich the content of current guidelines with the concept of heteroresistant *H. pylori* strains.

FUTURE PERSPECTIVES OF *H. PYLORI* TREATMENT

Antibiotic resistance among clinical *H. pylori* isolates is rapidly disseminating worldwide and we have to think deeply how to better manage it. At present, low doses of prescribed antibiotics, slow bacterial growth rate, bacterial coccoid forms, and genetic mutations in *H. pylori* are the major reasons for antibiotic resistance reported so far. Research into the presence of heteroresistance isolates of this bacterium been sorely neglected. Integration of heteroresistance phenomenon in *H. pylori* in clinical treatment decisions is essential. The primary definition of heteroresistance was about coexistence of both resistant and susceptible isolates at the similar gastric microniche at once, while we think that it can be happened subsequently as well. Currently, there are no data describing in detail the heteroresistant *H. pylori* strains, which reduces the usefulness of the current guidelines. We firmly believe that this is the time to improve our understanding of the heteroresistance phenomena and

incorporate this into uniform guidelines. Furthermore, there is a definite need to initiate discussions and learn how to recognize, manage, screen, and eliminate these infectious strains from clinics and the environment. Undoubtedly, there is an urgent necessity for new guidelines describing the heteroresistance phenomena of *H. pylori* strains.

CONCLUSION

To the best of our knowledge, this is the first paper suggest new challenge for designing the new useful guideline according to the evidence-based findings about the antibiotic resistance. Indeed, increased knowledge of gastroenterologists about the existence of heteroresistance phenomena is highly required. Moreover, the accurate breakpoints should be examined/determined in order to have a solid statement of heteroresistance among the *H. pylori* isolates.

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Understanding the Epidemiology of Multi-Drug Resistant Gram-Negative Bacilli in the Middle East Using a One Health Approach

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In the last decade, extended-spectrum cephalosporin and carbapenem resistant Gram-negative bacilli (GNB) have been extensively reported in the literature as being disseminated in humans but also in animals and the environment. These resistant organisms often cause treatment challenges due to their wide spectrum of antibiotic resistance. With the emergence of colistin resistance in animals and its subsequent detection in humans, the situation has worsened. Several studies reported the transmission of resistant organisms from animals to humans. Studies from the middle east highlight the spread of resistant organisms in hospitals and to a lesser extent in livestock and the environment. In view of the recent socio-economical conflicts that these countries are facing in addition to the constant population mobilization; we attempt in this review to highlight the gaps of the prevalence of resistance, antibiotic consumption reports, infection control measures and other risk factors contributing in particular to the spread of resistance in these countries. In hospitals, carbapenemases producers appear to be dominant. In contrast, extended spectrum beta lactamases (ESBL) and colistin resistance are becoming a serious problem in animals. This is mainly due to the continuous use of colistin in veterinary medicine even though it is now abandoned in the human sphere. In the environment, despite the small number of reports, ESBL and carbapenemases producers were both detected. This highlights the importance of the latter as a bridge between humans and animals in the transmission chain. In this review, we note that in the majority of the Middle Eastern area, little is known about the level of antibiotic consumption especially in the community and animal farms. Furthermore, some countries are currently facing issues with immigrants, poverty and poor living conditions which has been imposed by the civil war crisis. This all greatly facilitates the dissemination of resistance in all environments. In the one health concept, this work re-emphasizes the need to have global intervention measures to avoid dissemination of antibiotic resistance in humans, animals and the environment in Middle Eastern countries.

Keywords: colistin, ESBL, carbapenemases, one health, MDROs

INTRODUCTION

In the 1940s, the discovery of antibiotics was seen as one of medicine's major achievements that saved millions of lives (van Hoek et al., 2011). However, in the last decade antimicrobial resistance has significantly increased in bacteria and reduced the effectiveness of many clinically important antibiotics (Seiffert et al., 2013). Gram-negative bacilli (GNB) are among the most common causative agents of infectious diseases (Tian et al., 2012). Members of this family are ubiquitous, i.e., can be found in humans and animals' intestinal microflora, but also in the environment (Verraes et al., 2013). Among other resistant organisms, GNB are distinct in view of their complex mechanisms of resistance. These are mainly mediated via the production of extended spectrum beta lactamases (ESBL), AmpC and carbapenemases (Schill et al., 2017). These hydrolyzing enzymes confer bacterium resistance toward the most common class of antibiotics prescribed nowadays in clinical settings: beta lactams (Ruppe et al., 2015). Furthermore, resistance genes of these enzymes are often located on plasmids harboring resistance determinants to other classes of antibiotics; thus challenging therapeutic options when infectious diseases do occur (Ruppe et al., 2015). The dissemination GNB resistant to extended spectrum cephalosporins and carbapenem, necessitates the re-use of colistin (a polymyxin E antibiotic) previously abandoned due to its toxicity and side effects (Olaitan et al., 2014b). The re-introduction of colistin in recent years has also seen the emergence of resistance, further complicating the clinical situation. Colistin resistance occurs either via chromosomal mutations that mediates the alteration of the lipid A moiety of the lipopolysaccharide chain (Baron et al., 2016); or via the acquisition of an *mcr* gene (Olaitan et al., 2016a).

Previously known to be confined to the hospital settings, multi-drug resistant organisms (MDROs) are nowadays widely spread in animals and the environment (Rafei et al., 2015a). Dandachi et al. (2018a) reported the wide dissemination of ESBL producers as well as colistin resistant GNB in poultry, cattle, swine and companion animals in Mediterranean countries. For instance, several studies have shown that multi-drug resistance (MDR) can be readily transferred from one ecosystem to another via direct/indirect contact with contaminated animals and/or animal products (Huijbers et al., 2014), dust (Blaak et al., 2015), air (von Salviati et al., 2015), contaminated wastewaters (Guenther et al., 2011), and soil fertilized with animal manure (Laube et al., 2014). Humans, animals, and the environment together therefore form an interconnected system that should be carefully addressed in terms of bacterial resistance, antibiotic stewardship, and infection control measures.

In this context, Middle Eastern countries are thus of special interest. The dissemination of MDROs in this region of the world involves an interplay of over/misuse of antibiotics in humans and animals, the absence of infection control measures and most importantly the recent continuous population mobilization due to socio-economic conflicts and multiple war crises. In this review, our aim is to describe the epidemiology of extended spectrum cephalosporin, carbapenem and colistin resistant GNB in humans, animals and the environment in the Middle Eastern

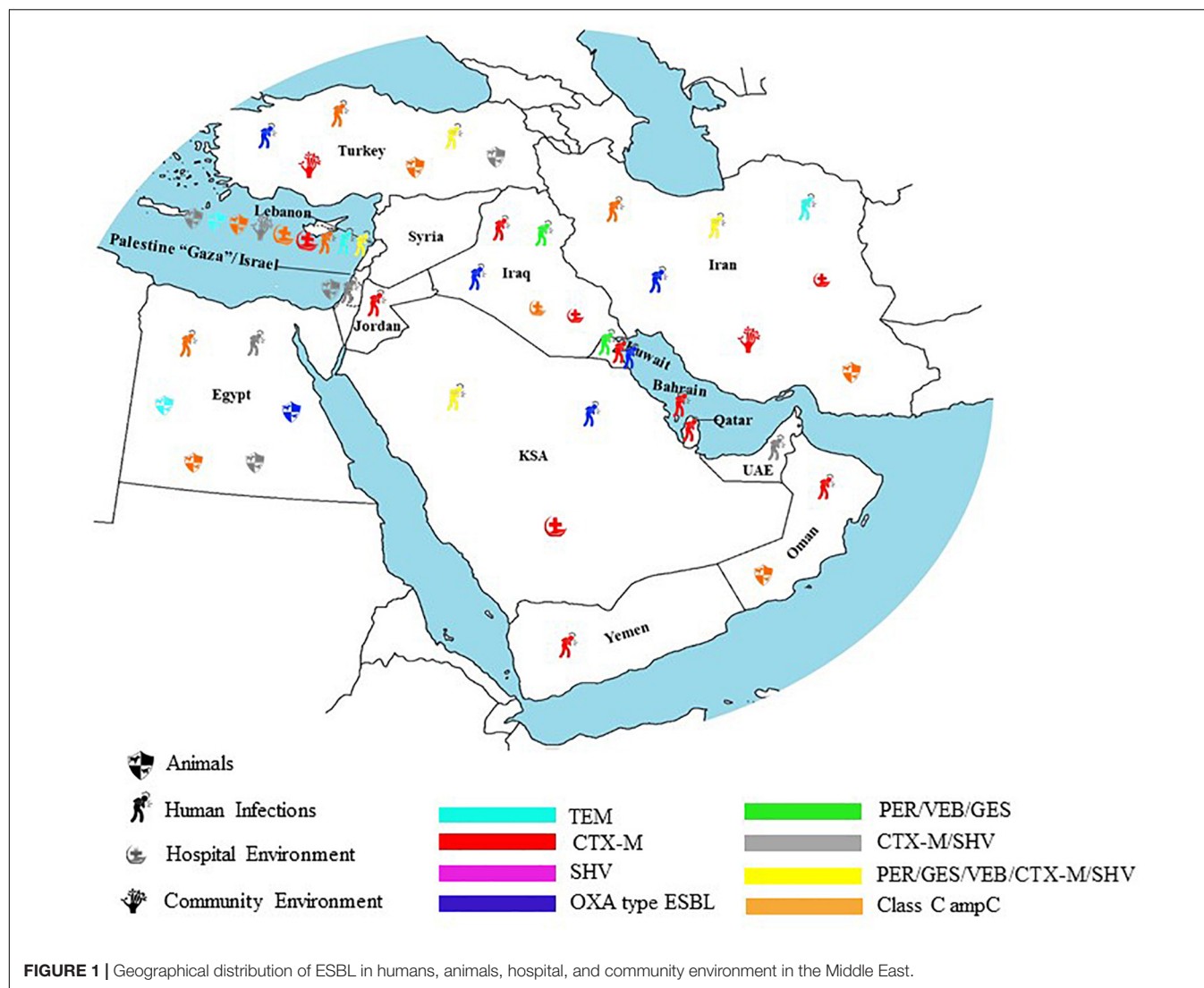
area. The Middle East includes 15 countries: Bahrain, Egypt, Iraq, Iran, Jordan, the Kingdom of Saudi Arabia (KSA), Kuwait, Lebanon, Palestine, Qatar, Sultanate Oman, Syria, Turkey, the United Arab Emirates (UAE), and Yemen. Our attempt is to highlight the gaps in bacterial resistance reports, antibiotic consumption data as well as infection control measures in this distinct area of the world.

DISTRIBUTION OF MULTI-DRUG RESISTANT ORGANISMS IN HUMANS

Infections With ESBL/AmpC Producers

Extended spectrum cephalosporins and penicillin's have been widely used in clinical settings due to their wide spectrum of activity as well as their negligible toxicity compared to other antimicrobial agents (Bush and Bradford, 2016). Their unrestricted use by physicians, in addition to their purchasing ease, without medical prescription in the community pharmacies, plays an important role in the emergence of bacteria resistant to these antimicrobial agents (Figure 1).

In Iran, studies have shown that in *Klebsiella* spp., resistance to extended spectrum beta lactams is mainly mediated via the production of CTX-M variants (CTX-M-15, CTX-M-3, CTX-M-8, CTX-M-1, and CTX-M-2) (Feizabadi et al., 2010; Peerayeh et al., 2014; Bialvaei et al., 2016; Akya et al., 2018; Dehshiri et al., 2018) and to a lesser degree via SHV (SHV-12, SHV-11, SHV-5) (Feizabadi et al., 2010; Shakib et al., 2018), TEM genes (Gholipour et al., 2014; Maleki et al., 2018) and others (VEB, PER, and GES) (Sedighi et al., 2017). pAmpC beta lactamase genes were reported by two studies in clinical isolates of *Klebsiella pneumoniae* (Mansouri et al., 2012; Kiaei et al., 2018). PFGE and ERIC-PCR analysis in these studies showed the presence of different clones in each clinical center (Akya et al., 2018; Hashemizadeh et al., 2018; Kiaei et al., 2018; Maleki et al., 2018). This is with the exception of one study where high clonal relatedness among ESBL *K. pneumoniae* strains was reported (Ghaffarian et al., 2018). Mehrgan et al. (2010) showed that intensive care unit (ICU) or medical ward stays are significantly associated with the acquisition of ESBL *Klebsiella* spp. Indeed, these resistant organisms are often found in patients who are very young and who have not yet developed full immunity, thus making them susceptible to opportunistic pathogen infections (Mehrgan et al., 2010). Similarly, to *Klebsiella* spp., CTX-M-15 followed by SHV, TEM and to a lesser extent CIT, were the most common beta lactamase genes detected in clinical strains of *Escherichia coli* (Gholipour et al., 2014; Rezai et al., 2015; Shayan and Bokaeian, 2015; Bialvaei et al., 2016). It is worth noting the detection of CTX-M, TEM, and CIT beta lactamases in diarrheagenic *E. coli* strains: Enterotoxigenic and Enteropathogenic ones (Heidary et al., 2014; Aminshahidi et al., 2017). These *E. coli* patotypes are always pathogenic when present in human intestines (Fratamico et al., 2016). Moreover, one recent study reported the isolation of CTX-M-15 extraintestinal pathogenic *E. coli* ST131 from inpatients and outpatients in Iran (Namaei et al., 2017). Statistical analysis indicated that ESBL producing ST131 *E. coli* strains were positively associated with CTX-M variants, CTX-M-15, and



TEM beta lactamases (Namaei et al., 2017). Moreover, strictly pathogenic species producing ESBL were also detected: CTX-M-1/CTX-M-15 *Salmonella* spp. and CTX-M-15/CMY-2 *Shigella* spp. (Salimian Rizi et al., 2015; Bialvaei et al., 2016; Aminshahidi et al., 2017). The high incidence of cephalosporins resistance in pathogenic bacteria in this country may be attributed in part to their inappropriate and high use in clinical settings (Amin et al., 2018); this is in addition to their extensive utilization in the Iranian community via self-medication (SM) (Zamanlou et al., 2018). Other ESBL producers that have been detected in clinical settings in Iran include CTX-M-15/TEM-169, SHV-12 producing *Enterobacter cloacae* (Peymani et al., 2014). On the other hand, in *Pseudomonas aeruginosa*, the major ESBL types were OXA-10/OXA-4, PER-1, VEB-1, and GES-1 (Mirsalehian et al., 2010; Alikhani et al., 2014; Farshadzadeh et al., 2014; Emami et al., 2015; Davodian et al., 2016; Amirkamali et al., 2017). This is followed by CTX-M, TEM-116, SHV-12, DHA and hyper-produced ADC enzymes (Bokaeian et al., 2014; Fazeli and Momtaz, 2014; Rafiee et al., 2014). Only one study in Iran revealed 13 distinct profiles

among 100 ESBL/carbapenem resistant *P. aeruginosa* isolated from burn patients via RAPD analysis. A dominant RAPD type was observed consisting of 80 isolates, thus revealing the possible existence of endemic clones circulating among patients (Neyestanaki et al., 2014).

In Turkey, ESBL production in *E. coli* and *K. pneumoniae* is mainly mediated via CTX-M-group1 (CTX-M-15 and CTX-M-1) and CTX-M-group2. Others include PER and OXA-10 in *E. coli* (Elaldi et al., 2013; Gorgec et al., 2015; Iraz et al., 2015). Furthermore, CMY-2, CIT, MOX, EBC, FOX, and ACT-1 have been detected in *E. coli* and *Klebsiella* spp., respectively (Demirbakan et al., 2008; Sari et al., 2013; Yilmaz et al., 2013). PFGE analysis showed no major clonal relationship per species in each clinical center (Durmaz et al., 2015; Gorgec et al., 2015). Multivariate analysis showed that urinary catheter insertion was a common risk factor for acquiring an infection with an ESBL quinolone resistant *E. coli* strain in inpatients and outpatients alike (Durmaz et al., 2015). Moreover, in two other studies the risk factors for the development of an ESBL

K. pneumoniae blood stream infections were high, with the duration of hospitalization being a common factor (Serefhanoglu et al., 2009). Other factors included prior antibiotic use and the use of aminoglycosides (Tanir Basaranoglu et al., 2017). Other ESBL producing Enterobacteriaceae detected in Turkey include CTX-M/TEM/SHV/qnrA aac(6')-ib *Enterobacter* spp. and VEB-1/qnrA1 *Providencia stuartii* (Nazik et al., 2009, 2011; Erdem et al., 2018). Interestingly, Agin et al. (2011) reported an outbreak of *Salmonella enterica* serovar typhimurium producing SHV-12 and CTX-M-3 ESBLs. In view of this, handwashing and disinfection procedures in addition to the establishment of an active surveillance program were initiated. These infection control measures led to the containment of the outbreak after 2 years. As for non-fermenters, PER-1 was the main ESBL type detected in *P. aeruginosa* and *Acinetobacter* spp. alike (Atilla et al., 2012; Erac et al., 2013; Keskin et al., 2014). In *Pseudomonas* spp. additional types were also detected such as OXA-10, OXA-14, and GES-1 (Aktas et al., 2012; Er et al., 2015).

In Lebanon, clinical epidemiological studies showed the predominance of CTX-M-15 and SHV-5a in *E. coli* and *K. pneumoniae* (Charrouf et al., 2014; Daoud et al., 2017). Furthermore, one report described the presence of SHV-11/CTX-M-15/acc(6')-lb-cr/qnrbl producing ST336 *K. pneumoniae* (Tokajian et al., 2015). PFGE analysis revealed clonal diversity among ESBL producing *E. coli* and *K. pneumoniae* (Daoud et al., 2017). As for the effect of antibiotic prescription and its correlation with the level of bacterial resistance, Daoud et al. (2017) found a significant association between aztreonam resistance and the use of penicillin's, and between cefuroxime, ceftazidime, cefoxitin, ciprofloxacin resistance and 3rd/4th generation cephalosporins use in *Klebsiella* spp. Moreover, one study reported the detection of four unrelated ESBL producing *Shigella sonnei* isolated from the stool samples of patients admitted for bacillary dysentery. These isolates harbored the CTX-M-15 gene on the plasmid and were flanked by ISEcp1 (Sabra et al., 2009).

In Israel, one study found low prevalence of ESBL producers in a clinical center (Chazan et al., 2009). The authors suggest that one of the reasons for this finding is the strict supervision of antibiotic prescription applied in their hospital; in addition to the limited use of ceftazidime (Chazan et al., 2009). Another study in the same country, argued that recent hospitalization, urinary tract infection (UTI) prophylaxis and *Klebsiella* spp. UTI are risk factors for the development of community acquired ESBL UTI (Dayan et al., 2013). Another has found that prior antipseudomonal therapy and empirical cephalosporin therapy are independent risk factors for UTI, caused by an ESBL producing *Proteus mirabilis* (Cohen-Nahum et al., 2010). As for the underlying genes of resistance, one study showed the presence of CTX-M-2, CTX-M-15, and CTX-M-14 in predominantly ST131 *E. coli* strains (Karfunkel et al., 2013). In this study, 93 and 51% of the isolates were co-resistant to fluoroquinolones and gentamicin, respectively. Transformation experiments suggest that aminoglycosides resistance is co-carried on the same plasmid harboring the CTX-M gene (Karfunkel et al., 2013). Other studies in Palestine have found clonal diversity among ESBL producing *E. coli* clinical strains (Adwan et al., 2014;

Tayh et al., 2016). On the other hand, in Israel, Karfunkel et al. (2013) reported the dominance of the ST131 among 41 CTX-M positive *E. coli* strains isolated for community onset bacteremia (COBSI) at Tel Aviv Sourasky Medical Center. In this center, the incidence of COBSI has increased 2.7-fold over a 7 year period. This increase appears to be correlated with the clonal expansion of ST131 *E. coli* strains carrying the blaCTX-M-14 and blaCX-M-15 genes (Karfunkel et al., 2013). ESBL production by *K. pneumoniae* in clinical settings was reported, whereby CTX-M-15, CTX-M-14a, CTX-M-3, SHV-12, SHV-5, and SHV-33 were detected (Tayh et al., 2017). In Jordan, very few studies have addressed the prevalence of ESBL producers in clinical settings. However, blaCTX-M (CTX-M-15, CTX-M-1, and CTX-M-9) was the only ESBL type detected in Enterobacteriaceae notably *E. coli* and ST131 *K. pneumoniae* strains (Hayajneh et al., 2015; Aqel et al., 2017; Nairoukh et al., 2018).

In Iraq, CTX-M-1, SHV, TEM producing *E. coli* strains were reported in recurrent UTI patients. In this report, MDR was significantly higher in ESBL *E. coli* versus non-ESBL ones (Al-Mayahie and Al Kuriashy, 2016). Similar results were obtained in a study addressing ESBL producers in pregnant/non-pregnant women with symptomatic genital tract infection. It is worth mentioning that ESBL producers co-resistant to non-beta lactam antibiotics is of special interest in this category; this is in view of the narrow choice of antibiotics that could be used in this category of patients (Al-Mayahie, 2013). Furthermore, in this country, CTX-M, SHV, TEM, and OXA ESBLs were described in clinical isolates of *Morganella morganii* with high resistance toward minocycline, trimethoprim-sulfamethoxazole and ciprofloxacin (Al-Muhanna et al., 2016). In parallel, VEB, PER, and OXA-10 were detected in high risk strains of *P. aeruginosa*: ST244, ST235, ST308, and ST654 (van Burgh et al., 2018).

In Kuwait, diverse genetic profiles of ESBL producing *E. coli* strains were detected in inpatients and outpatients alike (Dashti et al., 2014). CTX-M-15 followed by SHV-12, CMY-2, CTX-M-14, CTX-M-56, and CTX-M-2 are the most common ESBL types detected (Dashti et al., 2014; Jamal et al., 2015). In contrast to *E. coli*, one study in Kuwait reported identical PFGE profiles of *K. pneumoniae* SHV-112 positive strains isolated from blood and urine specimens of ICU patients (Dashti et al., 2010a). Another study however, reported different sequence types of *K. pneumoniae* detected in hospitalized patients: ST677, ST16, ST107, and ST485 producing CTX-M-15, SHV-11, and CTX-M-14 beta lactamases (Jamal et al., 2015).

In KSA, ST131 followed by ST38 *E. coli* strains producing ESBL appears to be predominant in clinical settings (Alghoribi et al., 2015; Alyamani et al., 2017; Yasir et al., 2018). In these, the main ESBL types detected were: CTX-M-15, CTX-M-9, CTX-M-1, CTX-M-8/25, CTX-M-2, CTX-M-14, SHV-12, and SHV-5 (Shibl et al., 2012; Al Sheikh et al., 2014; Alyamani et al., 2017; Yasir et al., 2018). Indeed, one study has shown that ESBL producers were significantly more resistant to aminoglycosides, ciprofloxacin and trimethoprim-sulfamethoxazole (Hassan and Abdalhamid, 2014). Al-Otaibi and Bukhari (2013) found that recurrent UTIs, surgical

intervention, children with vesicoureteric reflux and patients who had underlying renal transplant and renal disease are all possible risk factors for the acquisition of an ESBL UTI *E. coli* strain.

As for ESBL *K. pneumoniae*, the situation appears to be similar to their *E. coli* counterparts (Ahmad et al., 2009; Hassan and Abdalhamid, 2014; Somily et al., 2014). This is with the exception to the additional detection of other CTX-M variants such as CTX-M-3, CTX-M-82, CTX-M-57, and CTX-M-27 in *K. pneumoniae* as compared to *E. coli* strains (Al-Qahtani et al., 2014). In addition, in view of the wide diversity of ESBL *K. pneumoniae* isolates, it seems that clonal spread plays a negligible role in the dissemination of these strains (Al-Qahtani et al., 2014). Moreover, one study reported the detection of CTX-M-14 and SHV-12 in clinical isolates of *Citrobacter freundii* and *Enterobacter* spp. (Al Sheikh et al., 2014). SHV-5, CMY-2, and DHA-1 were also detected in *Enterobacter* spp. isolated from clinical settings in KSA (Abdalhamid et al., 2017a). On the other hand, VEB, GES, and OXA-10 were detected in *P. aeruginosa* clinical strains (Al-Agamy et al., 2012; Tawfik et al., 2012).

ESBL production in *Acinetobacter baumannii* on the other hand, was mediated via CTX-M and GES variants (Alyamani et al., 2015; Al-Agamy et al., 2017). Similarly, to other ESBL producing GNB in KSA, MLST analysis revealed the presence of a wide variety of sequence types in ESBL *A. baumannii* strains (Alyamani et al., 2015; Al-Agamy et al., 2017) (Table 1). Moreover, one study addressing the hajj pilgrims of Marseille, reported the detection of 2 CTX-M-2 producing *Salmonella* spp. Both strains were gentamicin and colistin resistant, in addition, they belonged to the epidemic Newport serotype ST45 (Olaitan et al., 2015). This finding calls for improved public health surveillance during the Hajj period in order to prevent the dissemination of MDROs in KSA and worldwide (Olaitan et al., 2015).

In Bahrain, CTX-M-grp1 and CTX-M-grp9 with high resistance to ciprofloxacin, nitrofurantoin, and trimethoprim-sulfamethoxazole have been described as the predominant ESBL types detected in *E. coli* and *K. pneumoniae* clinical strains (Bindayna and Murtadha, 2011; Shahid et al., 2014; Zowawi et al., 2014). In Qatar, CTX-M-group1, CTX-M-group9, TEM and SHV dominated the *E. coli* and *K. pneumoniae* clinical isolates (Sid Ahmed S.S. et al., 2016; Eltai et al., 2018b). In United Arab Emirates, CTX-M-15 and SHV-258 were detected in *K. pneumoniae* isolated from inpatients (Alfaresi et al., 2011). In parallel, CTX-M-15, CTX-M-3, and CTX-M-14 producing ST131 *E. coli* strains were also reported (Peirano et al., 2014). This same *E. coli* sequence type was isolated recently from the urine sample of a 76-year-old male patient. This isolate harbored the blaCTX-M-27 gene carried on an IncFII-FIA-FIB plasmid along with aminoglycosides (aadA5, strA, and strB), sulfonamide (sul1 and sul2), TET [tet(A)], macrolides (mphA), and trimethoprim (dfrA17), resistance determinants (Mutti et al., 2018). Ranjan Dash et al. (2018) found that age, gender, recurrent UTI and catheterization

are significant factors for developing an ESBL UTI in United Arab Emirates.

In the Sultanate of Oman, the main risk factors for ESBL infections in children was suggested to include being female, severe illness, prolonged hospital stays and previous exposure to antimicrobials (Al Muharrmi et al., 2008). As for the ESBL types detected, only one study showed the presence of CTX-M-15 producing a clinical *E. coli* strain (Zowawi et al., 2014).

Last, but not least in the gulf region, in Yemen, CTX-M-15, SHV-11, SHV-76, and SHV-184 were detected in clonally diverse *K. pneumoniae* clinical isolates (Gharout-Sait et al., 2014). On the other hand, CTX-M-15 was observed in ST131 *E. coli* strains (Alsharapy et al., 2018). As it becomes evident, ST131 is highly associated with ESBL production in the Middle Eastern region as well as other countries across the world: Israel (Karfunkel et al., 2013), KSA (Alghoribi et al., 2015; Alyamani et al., 2017; Yasir et al., 2018), Iran (Moghanni et al., 2018), Bulgaria (Markovska et al., 2012), Ecuador (Zurita et al., 2019), and Spain (Merino et al., 2016).

In Egypt, ESBL producers are widely spread in hospitals. One recent study showed a significant association between 3rd generation cephalosporins and resistance fluoroquinolones, gentamicin and tetracycline in hospital acquired infections (Galal et al., 2018). CTX-M-1, CTX-M-9, CTX-M-15, CTX-M-14, and SHV-12 were reported in *E. coli* strains isolated from different clinical origins (Hassan et al., 2012; Abdelaziz et al., 2013a; Abdallah et al., 2015b; Helmy and Kashef, 2017).

Additionally, TEM and SHV variants were also reported by El-Badawy et al. (2017), who found that among 61 clinical isolates of *E. coli* producing ESBL, SHV-11, and TEM-214 were predominant followed by others such as SHV-48, TEM-206, TEM-57, TEM-135, TEM-207, TEM-34, and TEM-176. This study was the first to report the detection of GES in *E. coli* strains isolated from Egyptian patients. A total of 92.30% ESBL *E. coli* isolates belonged to the ST131 clone and 45.83% of them belonged to the O25b serotype (El-Badawy et al., 2017). The association of *E. coli* ST131 with high antimicrobial resistance and virulence was previously reported in the literature (Can et al., 2015). On the other hand, CTX-M-15, CTX-M-14, SHV-11, and SHV-12 were detected in ESBL positive *K. pneumoniae* strains (Abdelaziz et al., 2013a; Abdallah et al., 2015b). Considerable resistance against aminoglycosides, fluoroquinolones and trimethoprim-sulfamethoxazole was observed in these isolates (Abdallah et al., 2017). Other ESBL producers detected in Egyptian hospitals include CTX-M-14/CTX-M-15 *Enterobacter* spp. (Abdallah et al., 2015b; Galal et al., 2018), CTX-M-15/SHV *P. mirabilis*, CTX-M-15/SHV *C. freundii*, CTX-M-14 *Serratia marcescens* (Helmy and Kashef, 2017; Galal et al., 2018) and CTX-M *Salmonella* spp. (Abdallah et al., 2017). CTX-M-14, CTX-M-15, and SHV ESBL types were detected in *P. aeruginosa* and *A. baumannii* (Abdelkader et al., 2017; Alkasaby and El Sayed Zaki, 2017; Helmy and Kashef, 2017). Furthermore, as for AmpC production, CMY variants (CMY-2, CMY-42, CMY-102), DHA-1, EBC, FOX, and MOX were detected

TABLE 1 | Sequence and plasmid types associated with ESBL genes in humans, animals, and environment in the Middle East.

Country	ESBL gene	Reservoir	Species	Sequence type/phylogroup	Plasmid type
Iran	CTX-M	Humans	<i>E. coli</i>		
			<i>K. pneumoniae</i>		
			<i>E. cloacae</i>		
			<i>Salmonella</i> spp.		
			<i>Shigella</i> spp.		
	TEM	Hospital environment	<i>E. coli</i>		
		Community environment	<i>A. baumannii</i>		
		Humans	<i>E. coli</i>		
			<i>K. pneumoniae</i>		
			<i>E. cloacae</i>		
			<i>A. baumannii</i>		
			<i>P. aeruginosa</i>		
		Hospital environment	<i>E. coli</i>		
			<i>P. aeruginosa</i>		
			<i>A. baumannii</i>		
	SHV	Community environment	<i>E. coli</i>		
			<i>K. pneumoniae</i>		
			<i>E. cloacae</i>		
			<i>P. aeruginosa</i>		
		Humans	<i>E. coli</i>		
			<i>K. pneumoniae</i>		
			<i>E. cloacae</i>		
			<i>P. aeruginosa</i>		
	OXA	Animals	<i>E. coli</i>		
			<i>E. coli</i>		
		Hospital environment	<i>P. aeruginosa</i>		
			<i>E. coli</i>		
		Humans	<i>P. aeruginosa</i>		
			<i>E. coli</i>		
	GES	Hospital environment	<i>E. coli</i>		
			<i>K. pneumoniae</i>		
	VEB	Humans	<i>P. aeruginosa</i>		
			<i>E. coli</i>		
	PER	Humans	<i>K. pneumoniae</i>		
			<i>P. aeruginosa</i>		
			<i>K. pneumoniae</i>		
			<i>A. baumannii</i>		
Turkey	CTX-M	Humans/community environment	<i>P. aeruginosa</i>		
			<i>E. coli</i>	–	
			<i>E. coli</i>	A, D, B1, B2	
		Animals	<i>K. pneumoniae</i>		
		Humans	<i>Enterobacter</i> spp.		
		Humans	<i>Enterobacter</i> spp.		
	TEM	Humans/animals	<i>E. coli</i>		
			<i>K. pneumoniae</i>		
		Humans	<i>Enterobacter</i> spp.		
			<i>E. coli</i>		
	SHV	Humans	<i>Enterobacter</i> spp.		
			<i>S. paratyphi</i>		
			<i>E. coli</i>	A, D, B1, B2	
		Animals	<i>E. coli</i>		
			<i>E. coli</i>		
		Community environment	<i>E. coli</i>		
	OXA	Humans	<i>P. aeruginosa</i>		
			<i>E. coli</i>		
		Community environment	<i>P. aeruginosa</i>		
			<i>E. coli</i>		
	GES	Humans	<i>P. aeruginosa</i>		
			<i>K. pneumoniae</i>		
	VEB	Humans	<i>E. coli</i>		
			<i>P. aeruginosa</i>		
	PER	Humans	<i>A. baumannii</i>		
			<i>E. coli</i>		

(Continued)

TABLE 1 | Continued

Country	ESBL gene	Reservoir	Species	Sequence type/phylogroup	Plasmid type
Lebanon	CTX-M	Humans	<i>E. coli</i>		
			<i>K. pneumoniae</i>		
			<i>Shigella</i> spp.		
		Animals	<i>E. coli</i>	ST10, ST617, ST58, ST69, ST1303, ST156, ST5470, ST354, ST155, ST3224	
		Community environment	<i>E. coli</i>	ST328, ST405, ST34, ST48, ST131, ST120, ST2067, ST10, ST38, ST410	IncFII
				ST38, ST1431, ST46	IncFIA
				ST212	IncY
				ST617, ST4144, ST6470, ST6222, ST90, ST38, ST4608, ST6894, ST127	
			<i>K. pneumoniae</i>	ST22, ST336, ST15, ST16	IncFIIk
	TEM	Humans	<i>C. freundii</i>		IncY
			<i>C. braakii</i>		IncY
			<i>E. coli</i>		
		Animals	<i>K. pneumoniae</i>		
			<i>E. coli</i>		
		Hospital environment	<i>K. pneumoniae</i>		
			<i>E. coli</i>		
			<i>K. pneumoniae</i>		
	SHV	Humans	<i>E. coli</i>		
			<i>K. pneumoniae</i>		
		Animals	<i>E. coli</i>		
			<i>K. pneumoniae</i>		
		Community environment	<i>E. coli</i>	ST46	IncFII
Palestine/Israel	CTX-M	Humans		ST617, ST6470, ST90, ST4608, ST6480, ST4144	
		Animals	<i>E. coli</i>		
	TEM	Humans	<i>K. pneumoniae</i>		
			<i>E. coli</i>		
			<i>K. pneumoniae</i>		
		Animals	<i>E. coli</i>		
Jordan	CTX-M	Humans	<i>E. coli</i>	ST131	
			<i>K. pneumoniae</i>	A2, C	
Iraq	CTX-M	Humans	<i>E. coli</i>		
			<i>M. morganii</i>		
			<i>M. morganii</i>		
		Animals	<i>E. coli</i>		
	TEM	Humans	<i>M. morganii</i>		
			<i>E. coli</i>		
			<i>M. morganii</i>		
		Animals	<i>P. aeruginosa</i>	ST244, ST308	
			<i>P. aeruginosa</i>	ST235	
Bahrain	CTX-M	Humans	<i>P. aeruginosa</i>		
			<i>E. coli</i>		
			<i>K. pneumoniae</i>		
	TEM	Humans	<i>E. coli</i>		
			<i>K. pneumoniae</i>		
			<i>E. coli</i>		

(Continued)

TABLE 1 | Continued

Country	ESBL gene	Reservoir	Species	Sequence type/phylogroup	Plasmid type
Qatar	CTX-M	Humans	<i>E. coli</i> <i>K. pneumoniae</i>		
	TEM	Humans	<i>E. coli</i> <i>K. pneumoniae</i>		
	SHV	Humans	<i>E. coli</i>		
United Arab Emirates	CTX-M	Humans	<i>E. coli</i>	ST131 ST131	IncFII-FIA-FIB
	SHV	Humans	<i>K. pneumoniae</i>		
Oman	CTX-M	Humans	<i>E. coli</i>		
Yemen	CTX-M	Humans	<i>E. coli</i> <i>K. pneumoniae</i>	ST131 ST1399, ST340, ST405, ST147, ST29	
	SHV	Humans	<i>K. pneumoniae</i>	ST1399, ST340, ST405, ST147	
Kuwait	CTX-M	Humans	<i>E. coli</i> <i>K. pneumoniae</i>	ST131, ST405, ST38	
	SHV	Humans	<i>E. coli</i>	ST131	
	SHV	Humans	<i>K. pneumoniae</i>		
KSA	CTX-M	Humans	<i>E. coli</i>	ST131, ST493, ST73, ST2346, ST1193, ST92, ST421, ST636, ST410, ST3268, ST120, ST602, ST224, ST1196, ST2852, ST58, ST1011, ST6438, ST167, ST8162, ST4981, ST1284, ST648, ST117, ST457, ST394, ST69, ST38, ST10	
			<i>K. pneumoniae</i>		
			<i>C. freundii</i>		
			<i>Enterobacter</i> spp.		
			<i>A. baumannii</i>	ST195	
			<i>E. cloacae</i>		
	TEM	Community environment	<i>E. coli</i>	ST493, ST73, ST131, ST2346, ST1193, ST92, ST421, ST636, ST410, ST4040, ST46, ST38, ST3268, ST405	
				ST648, ST117, ST394, ST69, ST8162, ST4981, ST1284	
			<i>A. baumannii</i>	ST195	
		Animals	<i>E. coli</i>		
	SHV	Humans	<i>E. coli</i> <i>K. pneumoniae</i> <i>C. freundii</i> <i>Enterobacter</i> spp.		
		Animals	<i>E. coli</i>		
	OXA	Humans	<i>E. coli</i> <i>P. aeruginosa</i>		
	GES	Humans	<i>A. baumannii</i>	ST1, ST2, ST15, ST17, ST113, ST114, ST115, ST116	
			<i>P. aeruginosa</i>		
Egypt	VEB	Humans	<i>P. aeruginosa</i>		
	CTX-M	Humans	<i>E. coli</i>	ST405, ST68, ST131, ST648; Phylogroup A, B1, B2, D	
			<i>K. pneumoniae</i>		
			<i>Enterobacter</i> spp.		
			<i>Salmonella</i> spp.		
			<i>P. mirabilis</i>		
			<i>C. freundii</i>		
			<i>S. marcescens</i>		
			<i>A. baumannii</i>		
			<i>P. aeruginosa</i>		

(Continued)

TABLE 1 | Continued

Country	ESBL gene	Reservoir	Species	Sequence type/phylogroup	Plasmid type
		Animals	<i>E. coli</i>	ST131 D	IncFII
	TEM	Humans	<i>K. pneumoniae</i> <i>A. baumannii</i>		
		Animals	<i>E. coli</i> <i>Salmonella</i> spp.	B1, C, D	
	SHV	Humans	<i>E. coli</i> <i>A. baumannii</i> <i>P. aeruginosa</i>	ST68, Phylogroups A and D	
		Animals	<i>E. coli</i>	D	
	OXA	Animals	<i>E. coli</i>	C, B2	

References are cited in the main text.

in clinical isolates of Enterobacteriaceae such as *E. coli*, *K. pneumoniae*, and *P. mirabilis* (Abdelaziz et al., 2013a; Helmy and Wasfi, 2014; Wassef et al., 2015).

Infections With Carbapenemase Producers

In Iran, *K. pneumoniae* is the most common carbapenemase producing Enterobacteriaceae clinical isolate. Carbapenem resistance in these is often mediated by NDM-1 and OXA-48 production followed by KPC (KPC-2), VIM (VIM-1 and VIM-4), and IMP (Rastegar Lari et al., 2013; Azimi et al., 2014; Nobari et al., 2014; Fazeli et al., 2015; Rajabnia et al., 2015; Firoozeh et al., 2016, 2017; Sedighi et al., 2017; Shahcheraghi et al., 2017; Armin et al., 2018; Ghotaslou et al., 2018; Hosseinzadeh et al., 2018; Moghadampour et al., 2018a). The majority of the studies reported no clonal relatedness among isolated carbapenem resistant *K. pneumoniae* in each center (Shahcheraghi et al., 2013; Jafari et al., 2018; Kiaei et al., 2018). This is with the exception of three centers where an identical genotype was observed for VIM-1 producers (Nobari et al., 2014), for NDM-1 producers the strains were distributed into two major clonal complexes including ST13 and ST392 (Shoja et al., 2018) and among NDM-1 and/or OXA-48 positive ones the predominant cluster/pulstotype was associated to ST11 and ST893 (Solgi et al., 2018). In this latter study, OXA-48 and NDM-1 genes were located on IncL/M and IncFII plasmids, respectively. These transferable plasmids are known as potent contributors to the dissemination of resistance genes including NDM-1, OXA-48, and CTX-M-15 among enterobacterial species (Solgi et al., 2018). OXA-48 and NDM (NDM-1, NDM-7) were dominant in carbapenem resistant *E. coli* strains (Hojabri et al., 2017; Solgi et al., 2017b). In one study, isolated strains belonged to the ST131 (Hojabri et al., 2017) whereas in the other one, carbapenemase producing *E. coli* strains were distributed into 18 different sequence types with the predominance of ST648 and ST167 (Solgi et al., 2017b). Interestingly, in one of the aforementioned studies, OXA-48 was found on the same transferable plasmid type IncL/M that was previously detected in *K. pneumoniae* (Solgi et al., 2017b) (Table 2). This finding emphasizes the role of the IncL/M incompatibility group in the horizontal

gene transfer of the OXA-48 gene among Enterobacteriaceae. In contrast, in the same study, NDM-1 was detected on an IncA/C plasmid type. In this study, ST648 and ST167 were dominant in NDM-1 and/or OXA-48 producing *E. coli* strains (Solgi et al., 2017b). Concerning *Salmonella*, two VIM positive strains were reported in Iran (Shahcheraghi et al., 2017). In *A. baumannii*, OXA-23 were dominant in all studies addressing carbapenem resistant *Acinetobacter* spp. in clinical settings (Azizi et al., 2015; Zanganeh and Eftekhari, 2015; Shoja et al., 2016, 2017; Mohajeri et al., 2017; Sarikhani et al., 2017; Zafari et al., 2017; Rezaei et al., 2018; Shirmohammadlou et al., 2018). Other carbapenem resistance genes included OXA-24, OXA-58, IMP, VIM, KPC, GIM, SIM, and SPM (Peymani et al., 2011; Azimi et al., 2015; Bagheri Josheghani et al., 2015; Aghamiri et al., 2016; Maspi et al., 2016; Moghadam et al., 2016; Khorvash et al., 2017; Armin et al., 2018; Soltani et al., 2018). Isolated strains of carbapenem resistant *A. baumannii* are genetically diverse with the predominance of International clone I and II (Peymani et al., 2011; Savari et al., 2017; Mahdian et al., 2015; Sarhaddi et al., 2017). The rapid evolution of bacterial resistance in *Acinetobacter* spp. could be attributed to its genome plasticity that allows the acquisition and loss of mobile genetic elements (plasmids, transposons) that modifies the organism's genomic structure (Savari et al., 2017). As for non *baumannii* species, only one study reported the detection of OXA-23 and SPM producing *Acinetobacter nosocomialis* in patients with blood infections (Pourabbas et al., 2016). In *P. aeruginosa*, MBLs were the most common carbapenemases including: IMP (IMP-1 and IMP-55) and VIM variants (VIM-1, VIM-2) (Abiri et al., 2015; Lari et al., 2015; Mirbagheri et al., 2015; Moosavian and Rahimzadeh, 2015; Azizi et al., 2016; Saffari et al., 2016; Kazeminezhad et al., 2017; Dogonchi et al., 2018; Pournajaf et al., 2018; Rostami et al., 2018). Only two studies reported the detection of OXA-23 and SPM-1 in Iranian clinical isolates of *P. aeruginosa* (Ostad Asadolah-Malayeri et al., 2016; Azimi et al., 2018). Akhi et al. (2018) found that the main risk factor for acquiring an MBL infection is non-intensive wards hospitalization. Whether the dissemination of carbapenem resistant *P. aeruginosa* in Iran is polyclonal or not, cannot be assumed. This is because the genetic relatedness was investigated in only two studies; in one of these different

TABLE 2 | Sequence and plasmid types associated with carbapenemase genes in humans, animals, and environment in the Middle East.

Country	Carbapenemase gene	Reservoir	Species	Sequence type/phylogroup	Plasmid type	
Iran	Class D oxacillinases	Humans	<i>E. coli</i>	ST131, ST167, ST1431, ST5005, ST889, ST10, ST3737, ST410, ST5114, ST5164, ST315, ST6350, ST648, ST167, ST410, ST431, ST3134	IncL/M	
				ST178, ST6826	IncA/C	
			Hospital environment	<i>K. pneumoniae</i>	A, B	
				<i>A. baumannii</i>	ST2	
				<i>P. aeruginosa</i>		
		<i>K. pneumoniae</i>				
		<i>A. baumannii</i>				
		KPC	Humans	<i>K. pneumoniae</i>	B	
			NDM	Humans	<i>E. coli</i>	C,D, ST167, ST131
					ST3044, ST167, ST178, ST6826	IncA/C
		ST315			IncL/M	
	<i>K. pneumoniae</i>	C, D				
		ST11, ST893		IncFII		
	VIM	Community environment		<i>Enterobacter</i> spp.		
				<i>A. baumannii</i>		
				<i>P. aeruginosa</i>		
			<i>P. aeruginosa</i>			
			<i>K. pneumoniae</i>			
		Hospital environment	<i>K. pneumoniae</i>			
			Humans	<i>K. pneumoniae</i>	ST13, ST392	
				<i>Salmonella</i> spp.		
				<i>A. baumannii</i>		
	<i>P. aeruginosa</i>					
IMP	Community environment	<i>P. aeruginosa</i>				
		<i>K. pneumoniae</i>				
	Humans	<i>A. baumannii</i>				
		<i>P. aeruginosa</i>				
		<i>P. aeruginosa</i>				
Turkey	Class D oxacillinases	Community environment	<i>P. aeruginosa</i>			
			<i>K. pneumoniae</i>			
			Hospital environment	<i>E. coli</i>		
				<i>K. pneumoniae</i>	ST11, ST258, ST16	
				<i>K. oxytoca</i>		
		<i>E. cloacae</i>				
		<i>E. aerogenes</i>				
		Humans	<i>P. mirabilis</i>			
			<i>P. stuartii</i>			
			<i>P. rettgeri</i>			
	<i>C. freundii</i>					
	<i>S. marcescens</i>					
	NDM	Humans	<i>M. morgani</i>			
			<i>R. planticola</i>			
			<i>A. baumannii</i>	ST2, ST15, ST157, ST10, ST158		
			<i>P. aeruginosa</i>			
			<i>E. coli</i>			
		Humans	<i>K. pneumoniae</i>			
			<i>E. cloacae</i>			
			<i>P. rettgeri</i>			
VIM			Humans	<i>E. coli</i>		
				<i>K. pneumoniae</i>		
	<i>E. cloacae</i>					
	<i>P. aeruginosa</i>					
	<i>P. aeruginosa</i>					

(Continued)

TABLE 2 | Continued

	IMP	Humans	<i>K. pneumoniae</i> <i>E. cloacae</i> <i>P. aeruginosa</i>		
Palestine/Israel	Class D oxacillinases	Humans	<i>P. mirabilis</i>		Incl/M
		Humans	<i>E. coli</i>	ST512	
	KPC		<i>K. pneumoniae</i>	ST340, ST277, ST376	
				ST327, ST512, ST258	pKpQIL
Lebanon	NDM	Hospital and community environment	Enterobacteriaceae		
		Humans	<i>A. baumannii</i>	ST103, ST2, ST183	
	VIM	Hospital and community environment	Enterobacteriaceae		
		Humans	<i>P. aeruginosa</i>	ST1562, ST1563, ST1564	
	Class D oxacillinases	Humans	<i>E. coli</i>	ST405	Incl/M
			<i>K. pneumoniae</i>		Incl/M
			<i>E. cloacae</i>		Incl/M
			<i>S. marcescens</i>		Incl/M
			<i>M. morgani</i>		Incl/M
			<i>R. ornithinolytica</i>		
			<i>A. baumannii</i>	ST2, ST25, ST1, ST570, ST85, ST600	
			<i>A. pittii</i>		
		Animals	<i>E. coli</i>	ST38	
			<i>A. baumannii</i>	ST2, ST491, ST493, ST20, ST492	
		Community environment	<i>E. coli</i>	ST38	InclH2
				–	Incl
			<i>K. pneumoniae</i>	ST16	Incl
			<i>A. baumannii</i>		
	NDM	Humans	<i>K. pneumoniae</i>	ST14, ST25	
Syria	VIM	Hospital environment	<i>E. cloacae</i>		
		Humans	<i>P. aeruginosa</i>	ST233, ST699, ST654	
	IMP	Animals	<i>P. aeruginosa</i>	ST1759, ST1760, ST1761, ST1762	
		Humans	<i>P. aeruginosa</i>	ST446, ST654	
	Class D oxacillinases	Humans	<i>E. coli</i>	ST38	
			<i>K. pneumoniae</i>	ST101, ST1633	
	NDM	Humans	<i>E. coli</i>		
			<i>K. pneumoniae</i>	ST278	
			<i>E. cloacae</i>		
			<i>P. rettgeri</i>		
			<i>C. braakii</i>		
			<i>A. baumannii</i>	ST85	
Jordan	Class D oxacillinase	Humans	<i>K. pneumoniae</i>	A1, A3, B	Incl/M
		Hospital environment	<i>A. baumannii</i>		
	NDM	Humans	<i>E. coli</i>		
			<i>K. pneumoniae</i>	A2, C	InclFIB, InclFI
Iraq	VIM	Humans	<i>E. cloacae</i>		
			<i>E. cloacae</i>		
	Class D oxacillinases	Humans	<i>A. baumannii</i>	ST2, ST136, ST194, ST623, ST792, ST793	
			<i>K. pneumoniae</i>		
	NDM	Humans	<i>P. aeruginosa</i>		
Kuwait	IMP	Humans	<i>K. pneumoniae</i>		
			<i>P. aeruginosa</i>		
	Class D oxacillinases	Humans	<i>E. coli</i>	ST405, ST38	
			<i>K. pneumoniae</i>	ST677, ST16, ST107, ST485	
			<i>E. cloacae</i>		
			<i>M. morgani</i>		

(Continued)

TABLE 2 | Continued

KSA	NDM	Humans	<i>A. baumannii</i>	ST158	pK50a
				ST195, ST208, ST229, ST436, ST450, ST452, ST499	
			<i>E. coli</i>	ST448	IncX3
			<i>K. pneumoniae</i>		
			<i>E. cloacae</i>		
	VIM	Humans	<i>P. stuartii</i>		
			<i>M. morganii</i>		
			<i>E. coli</i>		IncA/C
			<i>K. pneumoniae</i>		IncA/C
			<i>K. oxytoca</i>		
	IMP	Humans	<i>E. cloacae</i>		
		Humans	<i>P. aeruginosa</i>		
	Class D oxacillinases	Humans	<i>A. baumannii</i>		
			<i>E. coli</i>		
			<i>K. pneumoniae</i>	ST29, ST709, ST37, ST111, ST974, ST29, ST199, ST48, ST348, ST152, ST340, ST17, ST298, ST16, ST11, ST353	
			<i>E. cloacae</i>		
			<i>E. aerogenes</i>		
			<i>A. baumannii</i>	ST2, ST19, ST20, ST25, ST236, ST1096, ST945, ST208, ST450, ST452, ST499, ST195, ST229, ST436, ST152, ST348, ST199, ST1045	
	NDM	Humans	<i>K. pneumoniae</i>		
			<i>E. cloacae</i>		
			<i>E. aerogenes</i>		
			<i>A. baumannii</i>		
			<i>E. coli</i>		
Bahrain	VIM	Community environment Humans	<i>K. pneumoniae</i>		
			<i>K. oxytoca</i>		
			<i>E. cloacae</i>		
			<i>A. baumannii</i>	ST1, ST2, ST195, ST196, ST487, ST489, ST20	
			<i>A. baumannii</i>	ST1, ST2, ST195, ST196, ST487, ST489, ST20	
	IMP	Humans	<i>A. baumannii</i>	ST208, ST452	
			<i>A. baumannii</i>		
			<i>P. aeruginosa</i>		
			<i>E. coli</i>		
			<i>K. pneumoniae</i>		
Qatar	Class D oxacillinases	Humans	<i>A. baumannii</i>	ST229	
			<i>K. pneumoniae</i>		
			<i>P. aeruginosa</i>		
			<i>E. coli</i>		
			<i>K. pneumoniae</i>		
	NDM	Humans	<i>A. baumannii</i>		
			<i>K. pneumoniae</i>		
			<i>P. aeruginosa</i>		
			<i>E. coli</i>		
			<i>K. pneumoniae</i>	ST14, ST15, ST23, ST48	
United Arab Emirates	VIM	Humans	<i>Citrobacter</i> spp.		
			<i>S. marcescens</i>		
			<i>K. pneumoniae</i>	ST14	IncX3
			<i>E. coli</i>		
			<i>K. pneumoniae</i>	ST14	HI1b
	Class D oxacillinases	Humans		ST147	IncFII
			<i>E. cloacae</i>		
			<i>K. pneumoniae</i>		
			<i>E. coli</i>	ST648	IncL/M
				ST138	
Oman	Class D oxacillinases	Humans	<i>K. pneumoniae</i>	ST753, ST754	IncL/M

(Continued)

TABLE 2 | Continued

Yemen	NDM	Humans	<i>A. baumannii</i>	ST195	
			<i>E. coli</i>	ST2527	IncF
				ST4107	IncX3
			<i>K. pneumoniae</i>	ST15, ST147	IncH1B
				ST372	IncFII
				ST11	IncL/M, IncH1B
	VIM	Humans	<i>K. pneumoniae</i>		
	Class D Oxacillinase	Humans	<i>A. baumannii</i>	ST2	
	NDM	Humans	<i>K. pneumoniae</i>	ST1399, ST147, ST29, ST405, ST340	
			<i>E. cloacae</i>		
Egypt	Class D oxacillinases	Humans	<i>E. coli</i>	ST101, ST648	
			<i>K. pneumoniae</i>		
			<i>Salmonella</i> spp.		
			<i>S. marcescens</i>		
			<i>A. baumannii</i>	ST331, ST410, ST208, ST108, ST411, ST409, ST236	
	KPC	Animals	<i>P. aeruginosa</i>		
		Humans	<i>K. pneumoniae</i>		
			<i>K. pneumoniae</i>		
			<i>E. cloacae</i>		
			<i>A. baumannii</i>		
	NDM	Animals	<i>P. aeruginosa</i>		
		Humans	<i>K. pneumoniae</i>		
			<i>E. coli</i>	ST5018	IncI1-ly
			<i>K. pneumoniae</i>	ST147, ST11, ST17	IncR
			<i>E. cloacae</i>		
	VIM		<i>S. marcescens</i>		
			<i>A. baumannii</i>	ST103	
		Animals	<i>P. aeruginosa</i>	ST233	
		Humans	<i>K. pneumoniae</i>		
			<i>E. coli</i>	A, B	
			<i>K. pneumoniae</i>		
			<i>S. maltophilia</i>		
			<i>A. baumannii</i>		
			<i>P. aeruginosa</i>	ST233, ST198, ST303, ST629, ST507, ST406, ST274, ST990, ST683, ST884, ST738	

References are cited in the main text.

genotypes (Akhi et al., 2018) were detected whereas in the other, the strains were distributed into three distinct genotypes (Azimi et al., 2018).

In Turkey, *K. pneumoniae* were the most common GNB resistant to carbapenems (Us et al., 2010). Resistance to these agents is mainly mediated via the production of OXA-48 carbapenemases (Nazik et al., 2012, 2014; Iraz et al., 2015; Baran and Aksu, 2016; Haciseyitoglu et al., 2017; Kutlu et al., 2018). Some studies found a clonal relationship between isolated OXA-48 strains in the clinical center investigated (Castanheira et al., 2014; Karabay et al., 2016; Haciseyitoglu et al., 2017) while others did not (Kilic et al., 2011; Nazik et al., 2012; Iraz et al., 2015). Interestingly, in one of the studies, post the detection of a clonal spread of OXA-48 *K. pneumoniae* in a tertiary care center, infection control measures including hand washing, high level surface disinfection, screening of colonization before admission

were strictly followed (Ece et al., 2018). Later on, this resulted in a significant decrease of the rate of carbapenem resistant *K. pneumoniae* (Ece et al., 2018). Other types of carbapenemases were also detected in Turkish isolates of *K. pneumoniae*: NDM, VIM and IMP (Balkan et al., 2014; Candan and Aksoz, 2015; Cakar et al., 2016; Guven Gokmen et al., 2016). In *E. coli* strains, OXA-48, NDM, and VIM were detected (Gulmez et al., 2008; Carrer et al., 2010; Kilic et al., 2011; Nazik et al., 2012; Baron et al., 2016; Cakar et al., 2016; Kuskucu et al., 2016; Kutlu et al., 2018). Other carbapenemases producing Enterobacteriaceae include: OXA-48 and MBL *Enterobacter* species (Carrer et al., 2010; Baron et al., 2016; Haciseyitoglu et al., 2017), OXA-48 *C. freundii*, *S. marcescens*, *P. mirabilis*, *M. morgani*, *Raoultella planticola*, *P. stuartii*, and *Providencia rettgeri* (Carrer et al., 2010; Baron et al., 2016). Otlu et al. (2018) reported the detection of two genetically indistinguishable OXA-48/NDM-1 producing

P. rettgeri isolates. These strains were isolated from two different patients about 7 months apart in the same unit. These data show that Turkey is an endemic area of OXA-48 producers, thus warranting the urgent implementation of infection control measures as well as antibiotic stewardship programs (ASP). In non-fermenters, OXA-23, OXA-24, and OXA-58 were detected in *A. baumannii* with no evidence of clonal dissemination (Kulah et al., 2010; Ciftci et al., 2013; Metan et al., 2013; Castanheira et al., 2014; Cicek et al., 2014; Aksoy et al., 2015; Ahmed S.S. et al., 2016; Direkel et al., 2016). In two studies, co-resistance to colistin was detected (Ergin et al., 2013; Keskin et al., 2014); this fact is threatening and should be taken into real consideration since colistin is currently a last resort therapeutic agent against carbapenem resistant organisms (Olaitan et al., 2014b). On the other hand, MBL (VIM-1, VIM-2, VIM-38, IMP-1, and IMP-9) (Iraz et al., 2014; Yilmaz et al., 2014; Er et al., 2015; Malkocoglu et al., 2017) and OXA-23/OXA-58 were detected in clonally diverse *P. aeruginosa* (Tasbent and Ozdemir, 2015).

In Syria, the epidemiology of MDR is unknown due to the civil war crisis. However, injured Syrian refugees are considered a source of MDROs in the country they are residing in (Peretz et al., 2014b), Reinheimer et al. (2016). Indeed, recent studies showed the introduction of ST85 NDM-1 positive *A. baumannii* into Lebanese clinical settings from wounded Syrian refugees (Rafei et al., 2014b, 2015b). Subsequently, NDM-1 positive *Acinetobacter* spp. were isolated from Lebanese patients (Rafei et al., 2015b). Similarly, NDM/OXA-48 *K. pneumoniae* and *E. coli* and NDM producing *E. cloacae*, *P. rettgeri*, and *Citrobacter braakii* were isolated from Syrian refugees in North Palestine (Lerner et al., 2016). In both reports, the origin of the detected isolates could not be determined; the infection might have been acquired on the battlefield from environmental sources, during the patients stay in Syrian hospitals or during evacuation from Syria to another territory (Rafei et al., 2014b). Instead, what is sure is that screening of refugees arriving from countries with unknown epidemiology of carbapenem resistance, upon hospital admission, is a must and is crucial in order to contain the dissemination of these highly resistant MDROs (Lerner et al., 2016).

In Lebanon, in early 2012, OXA-48 and OXA-48/NDM-1 positive *E. coli* and *K. pneumoniae*, respectively, were isolated from the blood and urine cultures of Iraqi patients (El-Herte et al., 2012). Indeed, the most common carbapenemases detected in Enterobacteriaceae isolated from Lebanese hospitals are class D oxacillinases; these include OXA-48/OXA-232 *E. coli*, ST14 NDM-1 *K. pneumoniae*, OXA-48, OXA-162, OXA-232 *K. pneumoniae*, OXA-48/OXA-232 *E. cloacae* and OXA-48 producing *S. marcescens*, *M. morgani*, and *Raoultella ornithinolytica* (Hammoudi et al., 2015b; Al-Bayssari et al., 2016; Tokajian et al., 2016; Hammoudi Halat et al., 2017; Alousi et al., 2018). In two studies, the OXA-48 gene was located on the same plasmid IncL/M in *E. coli* and *K. pneumoniae* alike (Hammoudi Halat et al., 2017; Alousi et al., 2018). This emphasizes the crucial role that mobile genetic elements play in the spread of resistance determinants between different species. On the other hand, within the same species, no clonal relatedness was observed among carbapenem resistant

K. pneumoniae strains (Baroud et al., 2013). NDM-1 producing *K. pneumoniae* belonging to ST14 was reported by Alousi et al. (2018). Furthermore, an NDM-1 ST15 *K. pneumoniae* strain was isolated from the urine sample of an old Syrian refugee in Lebanon (Salloum et al., 2017). ST15 is heavily reported in hospitals worldwide such as Nepal (Stoesser et al., 2014), Vietnam (Tada et al., 2017), Thailand (Netikul et al., 2014), and China (Hu et al., 2013). The successful dissemination of ST15 *K. pneumoniae* could be attributed to its ability to acquire several resistance genes with no fitness cost (Toth et al., 2014). On the other hand, bacterial resistance in *A. baumannii* have largely evolved in Lebanon since its first detection by Matar et al. (1992). OXA-58 were at first the most common carbapenemase detected in clinical isolates of *A. baumannii* (Giannouli et al., 2009; Di Popolo et al., 2011), thereafter, OXA-23 and OXA-24 dominated (Rafei et al., 2014a; Hammoudi et al., 2015a,b; Hammoudi Halat et al., 2017). The dissemination of carbapenem resistant *A. baumannii* in Lebanese hospitals appears to be mainly mediated via the international clone II (Al Atrouni et al., 2016; Dahdouh et al., 2016; Hajjar Soudeihia et al., 2018). However, horizontal gene transfer has also played a major role. This is illustrated in a study conducted by Kanj et al. (2018) who found that the prevalence of OXA-23 positive *A. baumannii* have significantly increased between 2007, 2008, and 2013. Molecular analysis revealed only a 22% genomic relatedness among isolated strains. This emphasizes the role of horizontal gene transfer in the dissemination of resistance determinants among *A. baumannii* in Lebanese hospitals. Only one study reported the detection of non *baumannii* *Acinetobacter* species: *Acinetobacter pittii* producing NDM-1 and OXA-72 carbapenemases; these strains were isolated from the urine culture of a 4-month-old child and from a febrile gastroenteritis infected patient, respectively (Al Atrouni et al., 2016a). As for *P. aeruginosa* isolated from Lebanese hospitals, only MBLs were detected: IMP-1, IMP-2, IMP-15, and VIM-2 (Al Bayssari et al., 2014; Hammoudi et al., 2015b; Hammoudi Halat et al., 2017).

In Israel, KPC-3 and to a lesser extent KPC-2 producing *K. pneumoniae* appear to be endemic (Navon-Venezia et al., 2009; Leavitt et al., 2010a,b; Warburg et al., 2012; Castanheira et al., 2014). PFGE analysis showed that isolated KPC-3 *K. pneumoniae* belonged to the same genetic clone (Navon-Venezia et al., 2009). On the other hand, MLST analysis in three other studies showed the predominance of ST258 (Leavitt et al., 2010a; Warburg et al., 2012; Castanheira et al., 2014). The fact that KPC was also detected in other enterobacterial species such as *E. coli* (KPC-2) (Goren et al., 2010a,b), *Enterobacter* spp. (Lazarovitch et al., 2015), and *C. freundii* (Castanheira et al., 2014) suggests a possible monoclonal spread of KPC in *K. pneumoniae* and its subsequent successful horizontal gene transfer to other species. Other carbapenemase producing GNB detected in Palestine involve: OXA-48 located in the IncL/M plasmid in *P. mirabilis* (Chen et al., 2015), VIM-2/VIM-4 *P. aeruginosa* in Palestine (Sjolander et al., 2014) and NDM-2/OXA-23/OXA-24 *A. baumannii* in both Israel and Palestine (Castanheira et al., 2014; Sjolander et al., 2014).

In Jordan, NDM-1 and NDM-1/VIM-4 were detected in *E. coli* and *E. cloacae* clinical isolates, respectively (Aqel et al., 2018).

Furthermore, two studies reported the detection of NDM and OXA-48 in genetically diverse *K. pneumoniae* strains isolated from clinical specimens (Aqel et al., 2017, 2018). In one study, NDM-1 and OXA-48 were located on FII(K)/FIB and IncL/M, respectively (Aqel et al., 2018). The interesting finding in the second study is that distinct NDM-1 positive *K. pneumoniae* were isolated from a Yemeni patient and a native Jordanian without a history of travel, hospitalized at the same time period. In the same report, distinct OXA-48 *K. pneumoniae* was isolated from a Yemeni and also a native Jordanian treated in the same ward with specimens 12 days apart (Aqel et al., 2017). Altogether, these data highlight the importance of horizontal gene transfer and the absence of effective infection control measures in the dissemination of carbapenem resistance genes in Jordanian hospitals.

In Iraq, the most common carbapenemase producers are the non-fermenters including OXA-23/OXA-24 *A. baumannii* (Kusradze et al., 2011; Ganjo et al., 2016) and NDM (NDM-1, NDM-2), IMP and SPM *P. aeruginosa* (Al-Charrakh et al., 2016; Ismail and Mahmoud, 2018). According to Al-Charrakh et al. (2016), the high resistance of carbapenem resistant *P. aeruginosa* to non-beta lactams such as ciprofloxacin and gentamicin, can be attributed to the over-use of these antimicrobial agents in Iraqi clinical practices. In Enterobacteriaceae, only NDM-1 and SPM *K. pneumoniae* strains were detected (Hussein, 2018).

In Kuwait, VIM-4, NDM (NDM-1, NDM-7), and OXA-48 carbapenemases were detected in clinical isolates of clonally unrelated *E. coli* and *K. pneumoniae* strains (Jamal et al., 2013, 2015, 2016; Pal et al., 2017). In one study, the blaVIM gene was located on the same plasmid type IncA/C both in *E. coli* and *K. pneumoniae* (Sonnevend et al., 2017b). Moreover, several reports described the detection of NDM-1 *P. stuartii*, OXA-48, NDM-1 *M. morgani* and VIM (VIM-4), NDM-1, and OXA-48 *E. cloacae* in Kuwaiti hospitals (Jamal et al., 2013, 2015, 2016; Sonnevend et al., 2015a, 2017b). In *A. baumannii*, OXA-23 were mainly detected followed by IMP-1, VIM (VIM-1 and VIM-2) (Jamal et al., 2009; Al-Sweih et al., 2012; Zowawi et al., 2015; Wibberg et al., 2018). On the other hand, only one study reported the detection of VIM positive *P. aeruginosa* clinical strains (Zowawi et al., 2018). The *P. aeruginosa* strains were distributed into 14 sequence type clusters with some of them being recognized as highly disseminated international clones such as ST111, ST235, ST357, and ST654 (Zowawi et al., 2018). In fact, according to one report, it has been suggested that the dissemination of carbapenem resistance in the clinical settings of Kuwait appears to be promoted by immigration, in-sufficient infection control measures, environmental spread, and antibiotic misuse (Jamal et al., 2016).

In KSA, OXA-48, and MBL (NDM-1, VIM-4, and VIM-29) are the most common carbapenem resistance genes detected in Enterobacteriaceae (Memish et al., 2015; Sonnevend et al., 2015b; Algowaihi et al., 2016; Alotaibi et al., 2017; Al-Zahrani and Alsiri, 2018; Zaman et al., 2018). These isolates included mainly *K. pneumoniae* and others (*E. coli*, *E. cloacae*, and *Enterobacter aerogenes*) (Al-Agamy et al., 2013; Shibl et al., 2013; Uz Zaman et al., 2014; Memish et al., 2015; Abdalhamid et al., 2017b). In fact, several studies reported a clonal

relatedness among carbapenemase producing *K. pneumoniae* in each clinical center (Balkhy et al., 2012; Uz Zaman et al., 2014; Abdalhamid et al., 2017b). In one study, the clonal relatedness of carbapenem resistant *K. pneumoniae* was 93.2% (Abdalhamid et al., 2017b) whereas in other studies, MLST analysis revealed the predominance of certain sequence types such as ST29, ST199, and ST152 (Uz Zaman et al., 2014; Zaman et al., 2018). Furthermore, in one of the reports, the IncL/M plasmid type was predominant in OXA-48 *Klebsiella* spp. (Zaman et al., 2018). Indeed, one explanation for the MBL and OXA-48 predominance in the clinical isolates of Enterobacteriaceae in KSA is the big number of migrant workers and visitors coming from endemic areas such as India, Pakistan and Turkey (Al-Zahrani and Alsiri, 2018). Moreover, one study found that most of the patients infected with a carbapenem resistant *K. pneumoniae* had prolonged hospital stays, indwelling devices, surgical procedures, carbapenem usage and infection/carriage with MDROs (Balkhy et al., 2012). On the other hand, class D oxacillinase (OXA-23, OXA-24, and OXA-58) predominate in *A. baumannii* followed by NDM, VIM, and IMP (Alsultan et al., 2013; Elabd et al., 2015; Aly et al., 2016; Al-Agamy et al., 2017; Alhaddad et al., 2018). Clonal diversity revealed by different sequence types as well as PFGE patterns among isolated strains was reported in all the studies (Aly et al., 2014; Lopes et al., 2015; Zowawi et al., 2015; El-Mahdy et al., 2017). In one study, Aljindan et al. (2015) found that carbapenem resistant *A. baumannii* were more resistant to gentamicin, amikacin, ciprofloxacin, and tigecycline compared to the susceptible ones. In *P. aeruginosa* with high clonal diversity, VIM, IMP, VIM-1, VIM-2, VIM-4, VIM-11, VIM-28 were detected (Al-Agamy et al., 2012, 2016; Tawfik et al., 2012).

In Bahrain, VIM and class D oxacillinases (OXA-23, OXA-58, OXA-72, and OXA-40) were detected in genetically variant *P. aeruginosa* and *A. baumannii*, respectively (Mugnier et al., 2009; Zowawi et al., 2015, 2018). In Qatar, OXA-48 *E. coli*, NDM/OXA-48 *K. pneumoniae*, OXA-23 *A. baumannii*, and VIM *P. aeruginosa* were reported in clinical settings (Zowawi et al., 2014, 2015, 2018; Rolain et al., 2016).

In United Arab Emirates, NDM (NDM-1 and NDM-5), OXA-48 and to a lesser degree KPC, are the predominant carbapenemases detected in clinical isolates of *K. pneumoniae* (Dash et al., 2014; Sonnevend et al., 2015a, 2017a). MLST analysis revealed different sequence types with the most common being ST11, ST14, and ST147 (Sonnevend et al., 2013, 2015a, 2017a; Moubareck et al., 2018). ST147 is of special interest since in their study, Sonnevend et al. (2017a) reported a multi-hospital occurrence of a pan-resistant ST147 *K. pneumoniae* isolated from four patients over a 1 year period. The strains had highly similar genotypes and PFGE patterns. Furthermore, with more deep genetic analysis, extensive similarities (backbone and resistance islands) were found between these strains and the ST147 *K. pneumoniae* strains isolated in South Korea. Interestingly, one of the Korean isolates was from a patient transferred from the United Arab Emirates. This reveals the huge capacity of the ST147 *K. pneumoniae* clone in maintaining itself over a long period of time in addition to its ability to be transmitted internationally (Sonnevend et al., 2017a). Similarly, NDM and OXA-48 were also found in other GNB in the Emirati hospitals

including *E. coli*, *E. cloacae*, *Citrobacter* spp., *S. marcescens*, and *A. baumannii* (Ghazawi et al., 2012; Sonnevend et al., 2013, 2015b). In two studies, the NDM gene was located on an IncX3 plasmid (Sonnevend et al., 2013; Pal et al., 2017; Moubareck et al., 2018). According to Sonnevend et al. (2013), the Middle East is the second region where IncX3 plasmids with very similar structures that carry the blaNDM-1 were detected; found in different species, this emphasizes the role of this plasmid type on the inter-generic dissemination of this MBL gene.

In Oman, carbapenem resistance in Enterobacteriaceae (*E. coli* and *K. pneumoniae*) is mediated via the production of NDM (NDM-1 and NDM-7) and OXA-48 carbapenemases (Dortet et al., 2012; Zowawi et al., 2014). Reported sequence types for *K. pneumoniae* include ST14, ST340, ST11, and ST147 (Poirel et al., 2011a; Potron et al., 2011; Sonnevend et al., 2015a). Furthermore, as reported in United Arab Emirates, NDM-7 in *E. coli* was located on the epidemiologically important IncX3 plasmid (Pal et al., 2017). On the other hand, OXA-23 was detected in *A. baumannii* whereas in *P. aeruginosa* VIM and IMP were found (Zowawi et al., 2015, 2018). In the gulf, Zowawi et al. (2015) found that several clusters of indistinguishable OXA-23 *A. baumannii* strains are circulating. These include ST208 and ST195 that belong to the clonal complex 92, which is internationally disseminated (Chen et al., 2017; Rieber et al., 2017).

In Yemen, OXA-23 producing ST2 *A. baumannii* were isolated from clinical settings (Bakour et al., 2014); this is in addition to clonally un-related NDM-1 *K. pneumoniae* (ST1399, ST147, ST29, ST405, and ST340) and *E. cloacae* strains (Gharout-Sait et al., 2014).

In Egyptian hospitals, KPC, VIM (VIM-1, VIM-2, and VIM-29), NDM (NDM-1 and NDM-5), and OXA-48 are the predominant carbapenemases detected in Enterobacteriaceae (Abdelaziz et al., 2013b; Metwally et al., 2013; Hamdy Mohammed el et al., 2016; Abdallah et al., 2017; Barwa and Shaaban, 2017; Khalifa et al., 2017; Khalil et al., 2017; Abdulall et al., 2018; Kamel et al., 2018). Molecular analysis revealed that no clonal relationship was observed among carbapenem resistant *E. coli* and *K. pneumoniae* strains (Abdelaziz et al., 2013b; Khalifa et al., 2017; Khalil et al., 2017; Abdulall et al., 2018). The polyclonal spread of carbapenem resistant *K. pneumoniae* in Egypt is further documented in a study conducted in Italy. In this study, two NDM producing *K. pneumoniae* were isolated from unrelated patients with recent hospitalization in an Egyptian hospital (Principe et al., 2017). Isolated strains belonged to different sequence types. ST15 which was previously reported in Africa (Poirel et al., 2011b) and other Middle Eastern countries such as Lebanon (Salloum et al., 2017); and ST11 which is the sequence type to which the first NDM-1 *K. pneumoniae* strain isolated from Egypt belonged to (Abdelaziz et al., 2013b; Gamal et al., 2016). Polyclonal and horizontal gene transfer via mobile genetic elements appears to play an important role in the spread of carbapenemase producers in Egyptian clinical settings. However, more genetic analyses (MLST, plasmid typing) are needed to confirm this assumption. Other carbapenemase producers detected in Egyptian clinical settings include: VIM, KPC, NDM *E. cloacae*, OXA-48 *M. morgani* and *Salmonella*,

OXA-48, NDM-1 *S. marcescens* and VIM *Stenotrophomonas maltophilia* (Hamdy Mohammed el et al., 2016; Khalifa et al., 2017; Abdulall et al., 2018; Kamel et al., 2018). In non-fermenters, carbapenem resistance *A. baumannii* was mediated mainly via OXA-23, OXA-24, OXA-58 followed by NDM (NDM-1 and NDM-2), VIM (VIM-1 and VIM-2), IMP, SIM, and GIM (Kaase et al., 2011; Fouad et al., 2013; El-Ageery and Al-Hazmi, 2014; Lopes et al., 2015; Hamdy Mohammed el et al., 2016; Alkasaby and El Sayed Zaki, 2017; Ghaith et al., 2017; Abdulall et al., 2018; Abdulzahra et al., 2018; Kamel et al., 2018; Ramadan R.A. et al., 2018). High genetic diversity was observed among isolated strains (Al-Hassan et al., 2013; Ghaith et al., 2017; El Bannah et al., 2018). As for associated risk factors, one study showed that the empirical intake of carbapenem 1 month ago is significantly associated with the development of a carbapenem resistance caused infection (ElMahallawy et al., 2018). On the other hand, VIM (VIM-2, VIM-28, and VIM-1-like), NDM (NDM-1), IMP, and OXA-48 genes were reported in *P. aeruginosa* (El-Mahdy, 2014; Zafer et al., 2014, 2015; Khalifa et al., 2017). The majority of isolated strains were genetically diverse with different sequence types including ST233, ST303, ST198, ST629, and ST507 (Zafer et al., 2014, 2015; Khalifa et al., 2017).

Infections With Colistin Resistant Gram-Negative Bacilli

In Egypt, the first *mcr-1* producing *E. coli* isolated from a clinical setting occurred in 2016. This strain co-produced the CTX-M-15 and had a sequence type of ST1011 which was previously detected in an avian *E. coli* strain within this same country. This finding could be a direct manifestation of a zoonotic transmission of *mcr-1* from animals to humans (Elnahriry et al., 2016). Another study conducted on carbapenem resistant *A. baumannii* revealed substitutional mutations in the *pmrA/B* genes and subsequent colistin resistance. *A. baumannii* is considered an opportunistic pathogen and is usually treated with colistin if found to be carbapenem resistant. This association of colistin resistance with resistance to other antimicrobials is thus especially worrisome (Abdulzahra et al., 2018).

In Lebanon, Okdah et al. (2017) reported the detection of colistin resistance in three unrelated *K. pneumoniae* strains (ST268, ST2296, and ST348) with mutations in the *mgrB*, *phoQ*, *pmrA/B* genes in a hospital in Beirut. In Israel, one study reported the case of an Israeli patient with prior colistin administration during hospitalization and subsequent isolation of colistin resistant *K. pneumoniae* from his stool, supporting the theory of colistin resistance emergence as a result of antibiotic overuse in hospitals (Olaitan et al., 2014a). Lalaoui et al. (2019), reported the detection of colistin resistance in NDM-1 and KPC-3 harboring *K. pneumoniae* strains isolated from a medical center in Jerusalem. Resistance to colistin in these isolates was mediated by inactivation of the *mgrB* gene via an IS5-like insertion sequence (Lalaoui et al., 2019). Similarly, to nearby countries, colistin resistant *K. pneumoniae* strains in Israel are genetically diverse with different sequence types including ST16, ST76, ST258, and ST512 (Lalaoui et al., 2019). Indeed, this

country lacks quantitative investigation of the dosages and/or duration of colistin administration that significantly increase the risk of development of colistin resistance in a strain or a patient (Drozdinsky et al., 2018).

In Jordan, Nazer et al. (2015) conducted a study where they focused on critically ill cancer patients with carbapenem resistant *A. baumannii* and the adverse effects of colistin as choice of treatment. In the latter, despite 66% of the patients being cleared of their respiratory infections with colistin resistant *A. baumannii*, nephrotoxicity and even mortality were significantly associated with this therapeutic regimen. This warrants quantitative studies that are not necessarily targeted at determining doses and frequency that lead to emergence of *A. baumannii* colistin resistant strains, but are rather targeted at finding treatments for different types of infections in different populations (critically ill cancer patients for example) with minimal side effects and optimal outcomes (Nazer et al., 2015).

In the region of the Arabian Peninsula, colistin resistance is a public health challenge that is worth addressing. In the United Arab Emirates for instance, *K. pneumoniae* strains were isolated from different hospitals in different emirates. ST147 *K. pneumoniae* was isolated from a hospital in Abu Dhabi as well as from two different hospitals in Um al Quwain (Sonnevend et al., 2017a). This strain was not only carbapenem resistant through the blaOXA-181 gene but was also colistin resistant through an insertion in its mgrB gene. Interestingly enough, the insertion into the mgrB gene which resulted in colistin resistance was in fact the functional blaOXA-181 gene (Sonnevend et al., 2017a). Those findings imply that not only is there a spread of this ST over a large geographic area, but also that this strain is one of many that have developed resistance to both carbapenems and colistin and therefore has the potential to cause epidemics (Sonnevend et al., 2017a). Moreover, a study conducted in Dubai, on clinical isolates from hospitals with the broadest medical and surgical exposure in the country to assess resistance to carbapenems as well as to colistin, found that 31.4% of the carbapenem resistant *K. pneumoniae* strains isolated were also colistin resistant (Moubareck et al., 2018). The mechanism of colistin resistance was not identified but was confirmed not to be the *mcr-1* plasmid mediated gene. While 40% of the *K. pneumoniae* isolates that were both colistin and carbapenem resistant were sporadic cases, 31.4% were associated with the *K. pneumoniae* ST14 clone, which is locally prevalent. Along with the fact that Dubai is a major economical, touristic, and medical city in the region, the above information showcases the potential of Dubai playing an important role in the spread of colistin resistance from a One Health Concept perspective (Moubareck et al., 2018). Indeed, in the United Arab Emirates, only one ST131 *E. coli* strain harboring the *mcr-1* gene was reported (Sonnevend et al., 2016).

In Qatar, a colistin resistant clinical *E. coli* strain positive for the *mcr-1* gene was recently reported. This isolate belongs to ST95, known to cause meningitis in humans as well as severe avian infections. It is worth mentioning that this strain had an ISAp11 element in the same plasmid carrying the *mcr-1* gene and the pap2-like phosphatase gene (Forde et al., 2018).

The PAP2-like phosphatase can potentially contribute to colistin resistance by modifying the lipid A of the GNB outer membranes. The extent to which this gene contributes to colistin resistance in bacteria remains unknown but is worth investigating (Forde et al., 2018). Similarly, in Bahrain, the clinical colistin resistant *E. coli* strains (ST648 and ST224) were associated with the *mcr-1* gene being on an IncI2 plasmid type (Sonnevend et al., 2016).

Additionally, in Oman, a clinical isolate of *E. coli* carrying *mcr-1* was isolated in 2016. This strain belongs to ST10 and also harbors a plasmid of the IncI2 type. The detection of colistin resistance in ST10 *E. coli* is worrisome given that this clonal group has been known to mediate the spread of ESBL and quinolone resistance genes globally (Mohsin et al., 2018). In Kuwait, the development of colistin resistance in *Acinetobacter* spp. was evaluated in 2011. Of a total of 250 strains collected from eight governmental hospitals, 12% were found to be resistant to colistin. Compared to 0% in 2009, this significant increase prevalence could be attributed to the sudden increase in colistin prescription due to the global emergence of MDR infections (Al-Sweih et al., 2011).

In the KSA, a study by Mirnejad et al. (2018), focused on resistance to polymyxin B rather than colistin (polymyxin E). Those two antibiotics however, cover the same spectrum of organisms and can be used interchangeably as they have very similar mechanisms of action (Mirnejad et al., 2018). It was found that 13.2% of *A. baumannii* strains collected were resistant to polymyxin B (Memish et al., 2012). Another study found that the rate of resistance to colistin among *A. baumannii* in the KSA increased from 2.6 to 4.7% over the course of 2 years between 2010 and 2011 (Baadani et al., 2013). The danger that accompanies the appearance of colistin resistant strains in this country was embodied in a study in which two out of seven patients involved died due to colistin resistance. In that study, there was a history of colistin use reported in all patients except for one, suggesting that sporadic emergence rather than horizontal transmission of resistance might have played a more important role in the rise of colistin resistance in the isolated strains (Garbati et al., 2013). Moreover, sporadic cases of *mcr-1* in hospitals in the KSA has previously been reported (Sonnevend et al., 2016). However, most of the studies conducted demonstrated chromosomal mutations (mgrB and phoP) responsible for colistin resistance (Uz Zaman et al., 2018). In all of these studies, no clonal relatedness was observed among isolated colistin GNB strains (Sonnevend et al., 2016; Uz Zaman et al., 2018). The polyclonal spread of colistin resistance questions the level of colistin use in hospitals of the Arabian peninsula.

While addressing the topic of colistin resistance and the One Health Concept in the KSA, it is very important to mention the yearly Muslim pilgrimage, Hajj, that takes place in the city of Mecca. Plasmid mediated *mcr-1* carrying strains of predominantly genetically diverse *E. coli* strains and to a lesser extent *K. pneumoniae* have previously been isolated from patients during the Hajj. Pilgrims arrive from different countries, different occupations, and therefore with different sources of colistin resistance acquisition. These sources might be from the environment, food, animals, or from other humans (Leangapichart et al., 2016b).

In Turkey, colistin resistance has raised great concern as it has been associated with poor prognosis (Yilmaz et al., 2016). A study done by Cizmeci et al. (2017), found that six out of eight patients with *K. pneumoniae* that are resistant to both carbapenems and colistin ended up dying when all treatment options failed. Carbapenem resistant isolates positive for the NDM-1 gene have been found to have a higher rate of concomitant colistin resistance than isolates positive for the OXA-48 gene (Cizmeci et al., 2017). Furthermore, not only is the potential for colistin resistant infections to be fatal worrisome in Turkey, their potential to cause epidemics is also worrisome; the isolation of identical colistin resistant strains circulating in the country over short periods of time validate those concerns (Metan et al., 2017).

In Iran, one study reported the isolation of two colistin resistant *P. aeruginosa* from university teaching hospitals. The two isolates presented with different sequence types and more importantly were isolated from patients with no history of colistin consumption. The mechanisms of colistin resistance in both isolates was the overexpression of MexB and MexY genes which code for MexAB-OprM and MexXY-OprM efflux pumps. Despite colistin not being a specific substrate for those efflux pumps, the over expression of the MexAB-OprM and MexXY-OprM efflux was suspected to have played a role in the development of colistin resistance (Goli et al., 2016). This theory is partly supported by the fact that the over expression of MexAB-OprM and MexXY-OprM efflux pumps has already been linked to resistance in *P. aeruginosa* in multiple antimicrobial agents such as aminoglycosides (Hocquet et al., 2003). One interesting study done by Bahador et al. (2018) found that resistance to colistin in *A. baumannii* isolates is linked to the increase in virulence factors such as biofilm formation in burn patients. This renders the treatment of such MDR more challenging, as both resistance to colistin and virulence factors must be tackled at once (Bahador et al., 2018). Furthermore, two studies reported the isolation of colistin resistant *A. baumannii* and *K. pneumoniae* with mutations in the *pmrB* and *mgrB* genes, respectively (Haeili et al., 2017, 2018) (Table 3).

Carriage of ESBL/Carbapenemase Producers

The main concern of MDROs intestinal carriage is the acquisition of MDRO caused infections with limited therapeutic options (Magwenzi et al., 2017). In addition, as the carriage can last from months to years, the asymptomatic colonization of MDROs constitute a potent reservoir for transmission and dissemination (Decker et al., 2018).

In Iran, one study reported an 18.3% rectal carriage rate of ESBL *K. pneumoniae* among ICU patients and outpatients. The main mechanism of resistance was the production of CTX-M-15 detected in 86.3% of isolated strains (Aghamohammad et al., 2018). MLST analysis revealed that isolates of *K. pneumoniae* belonged to 16 different STs with a predominance of ST15, ST147, and ST16 (Aghamohammad et al., 2018). ST15 *K. pneumoniae* is widely associated worldwide with the production of CTX-M-15 (Lee et al., 2011; Rodrigues et al., 2014; Caneiras

et al., 2019). On the other hand, carbapenem resistant Enterobacteriaceae (CRE) colonization in Iranian inpatients was associated with 3rd generation cephalosporins, meropenem, colistin, and vancomycin exposure. This is in addition to ICU hospitalization, urinary catheter, mechanical ventilation, recent surgery, patient transfers from another hospital/unit and being male (Solgi et al., 2017a). Isolated CRE included, NDM and OXA-48 producing *K. pneumoniae*, *E. coli*, *E. cloacae*, and *P. mirabilis* (Solgi et al., 2017a).

In Turkey, the rectal carriage of CRE (OXA-48, NDM-1, and IMP *K. pneumoniae*) as well as carbapenem resistant non-fermenters (CR-NF) was reported in several studies (Alp et al., 2013; Karaaslan et al., 2016). In one study, only carbapenem intake was associated with OXA-48/IMP producing *K. pneumoniae* infections (Zarakolu et al., 2016). An interesting clinical experience was the one reported by Poirel et al. (2014) when an outbreak of carbapenem resistance was suspected with the first isolation of two similar carbapenem resistant *E. cloacae* from two patients' residing in the neonatal ICU (NICU). Accordingly, nasal and rectal screening was performed for all NICU patients. In addition, contact isolation precautions were implemented as well as an intensive infection control program was performed for all staff personnel. Subsequently, after 1 month, no infection/colonization with CRE was observed (Poirel et al., 2014). This emphasizes the significance of the microbiology laboratory and infection control unit's cooperation in preventing the dissemination of CRE (Poirel et al., 2014). A more recent study, also conducted in a Turkish NICU, showed that ages less than 1 year, carbapenem administration, presence of underlying diseases, urinary catheterization, and nasogastric tube placement were independent risk factors for CRE colonization. In this study, CRE included OXA-48, IMP, NDM *K. pneumoniae*, *E. coli*, and *E. cloacae* (Karaaslan et al., 2016). On the other hand, CR-NF carriage (NDM, IMP-1, OXA-23, OXA-24, and OXA-58 producing *A. baumannii*) was correlated to an ICU stay, ampicillin carbapenem use, mean daily antibiotic use, presence of underlying diseases, surgical intervention and nasogastric tube placement (Karaaslan et al., 2016).

In Lebanon, two studies addressed the rectal carriage of ESBL producing Enterobacteriaceae in nursing home residents in Beirut and Tripoli in the north (Jallad et al., 2015). In Beirut, constipation and antibiotic intake were independent risk factors for ESBL carriage (Jallad et al., 2015); whereas in Tripoli, only antibiotic administration was found (Dandachi et al., 2016). Nursing homes are community facilities where MDROs can easily emerge and spread due to the uncontrolled or unprofessional prescription of antibiotics and inadequate environmental decontamination, waste disposal, and hygiene practices (Dandachi et al., 2016). Another study conducted in healthy infants showed that CTX-M-15, CTX-M-9, and CTX-M-2 positive Enterobacteriaceae are prevalent in the Lebanese community (Hijazi et al., 2016). Hospital birth, cesarean delivery, being formula-fed and being male are important risk factors for ESBL colonization in this category. In this report, proper hygiene was associated with a colonization rate decrease (Hijazi et al., 2016). On the other hand, Christophy et al. (2017) reported a high prevalence of carbapenem resistance

TABLE 3 | Mechanisms of colistin resistance described in GNB in the Middle East.

Country	Origin	Species	Sequence type	Mechanism of colistin resistance	References
Iran	Clinical samples	<i>A. baumannii</i>		pmrB*	Haeili et al., 2018
		<i>A. baumannii</i>		pmrA/B*	Sepahvand et al., 2016
		<i>K. pneumoniae</i>		mgrB*	Haeili et al., 2017
		<i>P. aeruginosa</i>		MexAB-OprM/MexXY-OprM**	Goli et al., 2016
Lebanon	Clinical samples	<i>K. pneumoniae</i>	ST268	mgrB*	Okdah et al., 2017
		<i>K. pneumoniae</i>	ST2296	mgrB*, PhoQ*	
		<i>K. pneumoniae</i>	ST348	pmrA/B*	
	Poultry	<i>E. coli</i>	ST515	<i>mcr-1</i>	Dandachi et al., 2018c
	Swine	<i>E. coli</i>		<i>mcr-1</i>	Dandachi et al., 2018b
Palestine	Clinical samples	<i>K. pneumoniae</i>		mgrB*	Olaitan et al., 2014a
		<i>K. pneumoniae</i>	ST512, ST76	mgrB*	Lalaoui et al., 2019
Bahrain	Clinical samples	<i>E. coli</i>	ST648, ST224	<i>mcr-1</i> on IncI2	Sonnevend et al., 2016
Qatar	Clinical samples	<i>E. coli</i>	ST95	<i>mcr-1</i> on IncHI2	Forde et al., 2018
United Arab Emirates	Clinical samples	<i>K. pneumoniae</i>	ST147	mgrB*	Sonnevend et al., 2017a
		<i>E. coli</i>	ST131	<i>mcr-1</i> on IncI2	Sonnevend et al., 2016
Oman	Clinical samples	<i>E. coli</i>	ST10	<i>mcr-1</i> on IncI2	Mohsin et al., 2018
KSA	Clinical samples	<i>K. pneumoniae</i>	ST974, ST37, ST709, ST348, ST37	PhoP*	Uz Zaman et al., 2018
		<i>K. pneumoniae</i>	ST14, ST15, ST16, ST22, ST48, ST101, ST152, ST307	mgrB*	
		<i>K. pneumoniae</i>	ST15	mgrB*, phoP*	
		<i>E. coli</i>	ST68	<i>mcr-1</i> on IncHI2	
		<i>E. coli</i>		<i>mcr-1</i>	
Egypt	Clinical samples	<i>A. baumannii</i>		pmrCAB*	Sonnevend et al., 2016
	Animal	<i>E. coli</i>	ST10	<i>mcr-1</i>	Abdulzahra et al., 2018
		<i>E. coli</i> O157		<i>mcr-1</i>	Khalifa et al., 2016
		<i>E. coli</i> O158		<i>mcr-1</i>	Lima Barbieri et al., 2017

*Mutations. **Efflux pump over-expression.

fecal carriage in cancer patients undergoing chemotherapy. The carbapenem resistant strains included mainly OXA-48/CTX-M *E. coli*, OXA-48 *E. cloacae* and VIM *Pseudomonas stutzeri*.

In Israel, a study conducted at a rehabilitation center revealed the patient's rectal carriage of CTX-M-27, CTX-M-15, CTX-M-14, CTX-M-39, CTX-M-55, SHV-5, SHV-12, and CMY-4 and CMY-2 producing *E. coli* strains of diverse sequence types including ST131 (Izdebski et al., 2013). In parallel, among patients admitted to a teaching hospital in one study, 8% were carriers of ESBL. The risk factors for this colonization were female sex and recent antibiotic intake. On the other hand, 21% of admitted patients acquired ESBL carriage. The latter was significantly associated with being older than 65 years and having an extended spectrum beta lactam antibiotic intake (Friedmann et al., 2009). Additionally, in clinical settings, one study raised concern about the real efficiency of antibiotic prophylaxis post-bowel surgery on ESBL carriage and subsequent infection. This is because in this report, immunosuppressive therapy and antibiotic use in the previous 3 months were independent risk factors for ESBL rectal carriage in this patients' category (Pfeffer et al., 2016). Moreover, in this country, the carriage of carbapenem resistance was significantly higher to ESBL positive Enterobacteriaceae in view of the number of reports. In one study, the carriage of CR-KP was significantly associated with a prolonged hospital stay, room sharing with a previously known carrier and residency

in a high carrier ward (Ben-David et al., 2011). In fact, in their study, Wiener-Well et al. (2010), found that during the surveillance of CR-KP carriage in hospitalized patients, isolated strains had identical PFGE patterns showing a clonal origin. The authors argued that strict isolation of carriers might help reduce the transmission of the CR-GNB from one patient to another (Wiener-Well et al., 2010). In another study, the CR-KP carriage was dependent on recent surgery and a sequential organ failure assessment (SOFA) score (Debby et al., 2012). Other described risk factors for CR-KP intestinal carriage include diaper use, length of hospital stay and vancomycin use (Wiener-Well et al., 2010). Adler et al. (2015) reported an increase in the rectal carriage of KPC producing *K. pneumoniae* (represented by ST258) in a post-acute care hospital (PACH) from 65% in 2008 to 80% in 2013. The acquisition source of more than 50% of the carriers was the PACH itself (Adler et al., 2015). Moreover, one report showed that the duration of CRE carriage can last for 3 months, 6 months and even up to 1 year. The carriage duration was affected mainly by repeated hospitalization and the isolation of a clinical and not surveillance positive culture (Zimmerman et al., 2013). One explanation for this finding is that recurrent hospitalization often represents re-infection and flags more severely ill people who need more time to eradicate CRE (Zimmerman et al., 2013). Furthermore, CRE infected patients might have larger loads of CRE compared to those who are only

colonized with; contributing subsequently to longer periods of continuous carriage (Zimmerman et al., 2013). Interestingly, one study assessed the risk factors responsible for the development of CRE infection after CRE colonization. These latter included ICU admission, antibiotic intake (especially fluoroquinolones and metronidazole), diabetes mellitus and central venous catheter insertion (Schechner et al., 2013). The identification of these factors are important in order to predict CRE infections and direct accordingly antibiotic empirical therapy (Schechner et al., 2013). Other carbapenem resistant species detected in Palestinian colonizers include VIM (VIM-1, VIM-35) producing *Aeromonas* species and NDM-1/OXA-10 positive *P. rettgeri* (Adler et al., 2014; Olaitan et al., 2016b). In Jordan, one study reported the rectal carriage of CTX-M-15, CTX-M-2, and CTX-M-1 *E. coli* in infants less than 1 year of age to the Pediatric unit in a hospital in Amman (Badran et al., 2016).

In the Gulf region, Dashti et al. (2010b) reported the detection of a single ESBL producing *E. coli* clone in blood cultures of neonates and health care workers' (HCW) hand in a Kuwaiti hospital. This highlight the important role that the health care personnel can play as vectors and reservoirs from which bacterial resistance can spread. This is especially true when non-adherence to proper sanitation and hand hygiene occur. In Qatar, only one study reported the fecal carriage of MDR ESBL *E. coli* in food handlers (Eltai et al., 2018c). This finding is of public health concern, since MDROs can be silently transmitted to the general community via contaminated food, contributing thus further to the dissemination of bacterial resistance (Eltai et al., 2018c).

In KSA, only two studies reported the rectal carriage of MDROs in the clinical settings. These latter included ICU patients carrying of highly diverse OXA-23 *A. baumannii*, CTX-M-15 *K. pneumoniae*, and NDM, VIM producing *P. aeruginosa* (Aljindan et al., 2015; Abdalhamid et al., 2016). Indeed, the rectal colonization of MDROs was mostly addressed in the Hajj period. One study reported in 2013 a significant CTX-M intestinal carriage in pilgrims with the rate of the latter increasing from 10.08% before Hajj to 32.56% post Hajj (Leangapichart et al., 2016a). In the same context, the same author reported similar findings in 2014. The acquisition rate of ESBL producers did not significantly differed between the 2 years (Leangapichart et al., 2017). Indeed, Leangapichart et al. also found that there was a difference not only at the level of intestinal colonization rate but also at the level of the bacterial diversity detected. For instance, *A. baumannii* strains were isolated from 26 rectal specimens and 16 pharyngeal one's post Hajj while none was detected in the samples collected prior to Hajj travel (Leangapichart et al., 2016c). It is worth mentioning the detection of one *A. baumannii* strain that is carbapenem resistant and produced the OXA-72 carbapenemase post Hajj. Likewise, an *E. coli* positive for blaNDM-5, blaCTX-M-15 was also detected after Hajj travel (Leangapichart et al., 2016c). These data emphasize the role of this season as a mediator of bacterial resistance dissemination in the KSA and worldwide. More effort is warranted for the improvement of the public health conditions during this period of the year. Moreover, recent Hajj travel should be taken into consideration when pilgrim patients are admitted to hospitals in their hometown in order to control for the introduction of new

MDROs to clinical settings. However, more studies are needed in order to characterize "recent Hajj travel" as a risk factor for MDROs fecal carriage.

In Egypt, fecal carriage of ESBL producing GNB was detected in hospitalized patients. These included CTX-M (CTX-M-15, CTX-M-14, CTX-M-2, and CTX-M-grp9) SHV and TEM ESBL type (Khalaf et al., 2009; Fam et al., 2015). Fouda et al. (2016) found that ESBL carriage was associated with increased mortality in ICU admitted patients. In the same context, two studies reported the intestinal carriage of ESBL and AmpC beta lactamases in HCW in two hospitals (Abdel Rahman et al., 2010; Bassyouni et al., 2015). As already mentioned, HCW constitute a potent reservoir of bacterial resistance when infection control measures and proper hand hygiene are lacking in a clinical center (Bassyouni et al., 2015). Furthermore, NDM-1 positive ST267 *A. baumannii* were isolated from hospitalized patients during a rectal screening surveillance in this same country (Krahn et al., 2016).

DISTRIBUTION OF MULTI-DRUG RESISTANT ORGANISMS IN ANIMALS

ESBL/AmpC Producers

In the Middle East, studies from Egypt reported the detection of TEM, SHV, CTX-M-9, CTX-M-15, and OXA-7 producing *E. coli* strains in broiler farms. The plasmid mediated AmpC beta lactamase genes blaCMY-2 and blaDHA-1 were also observed (Moawad et al., 2018). Furthermore, studies done on poultry hatcheries revealed similar results where blaTEM, followed by blaSHV, blaMOX-like, blaCIT-like, and blaFOX were the most common beta lactamase genes detected (Osman et al., 2018). BlaCTX-M-15 has also been reported in Egyptian poultry with other β -lactamase-encoding genes such as blaTEM-104, blaCMY-2, and blaOXA-30 in *E. coli* strains including the sequence type ST131 (Ahmed et al., 2013; Abdallah et al., 2015a; Ramadan H.H. et al., 2018). Multidrug-resistant *E. coli* O25b:H4 ST131 has been reported to be spread worldwide in humans, companion animals and livestock (Ahmed et al., 2013). Another study on chickens in Egypt, reported other ESBL types including blaTEM-57, blaSHV-12, blaCTX-M-14 (El-Shazly et al., 2017). As for pathogenic bacteria, two studies reported the detection of TEM ESBL type in *Salmonella* species isolated from chicken meat as well as from pigeons (Ahmed H.A. et al., 2016; Abdeen et al., 2018). Indeed, one of the main contributors to this high prevalence of ESBL/AmpC producers in the Egyptian poultry sector is the misuse of antibiotics. According to El-Shazly et al. (2017), many farmers in Egypt tend to use cefotaxime injections (a 3rd generation cephalosporin banned in poultry) to treat diseases in chicken (such as colibacillosis) after the failure of other antimicrobial treatments like fluoroquinolone and aminoglycosides. In addition, due to the low cost of antibiotics, many veterinarians still over-use antibiotics such as tetracycline, quinolone and beta lactams to treat and prevent zoonotic diseases and growth promotion (Braun et al., 2016; Hakim et al., 2017). Moreover, in pets, blaTEM along with blaSHV, blaPSE-1 and blaCTX-M were detected in *E. coli* strains

isolated from dogs in the same country (Aly et al., 2012). In cattle, TEM, SHV (SHV-11, SHV-27), and CTX-M-15 were detected in *E. coli* and *K. pneumoniae* strains (Hammad and Shimamoto, 2011; Braun et al., 2016). Another report on dairy calves, reported the detection of blaCMY-2 and blaSHV-12 genes in *Salmonella* spp. including *S. enterica* serovars *enteritidis* and *S. typhimurium* (Ahmed et al., 2009). Other studies in the Egyptian dairy products revealed the presence of CTX-M-variants (CTX-M-15, CTX-M-104, CTX-M-3), TEM-52, SHV-12, and CMY-2 producing *E. coli* strains (Ahmed and Shimamoto, 2015; Ombarak et al., 2018). Other ESBL (including OXA-10 and SHV-28) and AmpC producing GNB detected in the Egyptian bovine sector include *Klebsiella oxytoca* and *C. freundii* (Ahmed and Shimamoto, 2011).

In Palestine, blaCTX-M (including CTX-M-1, CTX-M-9) and SHV-12 were the only ESBL types detected in *E. coli* strains isolated in Chicken (Qabajah et al., 2014). Similarly, these ESBL types were detected in cattle in Israel (Adler et al., 2016). In Lebanon, a recent nationwide study conducted in chicken farms, found a considerable number of ESBL and AmpC producing GNB. These included mainly blaCTX-M, blaTEM and blaCMY genes (Dandachi et al., 2018). On the other hand, Diab et al. (2016) reported the dissemination of CTX-M-15 producing *E. coli* in Lebanese cattle. One more recent study conducted by Dandachi et al. (2018b) found that CTX-M followed by CMY are the most common beta lactamases detected in *E. coli* strains isolated from Swine farms. Both in cattle and poultry, MLST analysis revealed high variety of sequence types in isolated *E. coli* strains with some of them previously described in the literature as being common to animals as well as to humans (ST10, ST617, ST58, ST69, ST155, and ST156) (Diab et al., 2016; Dandachi et al., 2018). This emphasizes the role of livestock in the dissemination of MDROs in the one health concept.

In Turkey, CTX-M-15 was detected in *E. coli* strains belonging to the B1 phylogenetic group isolated from cattle with bovine mastitis (Pehlivanoglu et al., 2016). Furthermore, one study targeting MDROs in dogs reported the detection of CTX-M-15, blaCMY-2, blaCTX-M-3, blaCTX-M-1, and blaSHV-12 in *E. coli* isolates with A1 and D2 being the most common phylogenetic groups identified. In this report, ST131/B2 *E. coli* positive for CTX-M-15 were detected. This clone is a human pandemic one that can possibly be transmitted to humans via direct or indirect contact with companion animals (Aslantas and Yilmaz, 2017).

In the gulf and specifically in the KSA, blaSHV and blaTEM were reported in *E. coli* strains isolated from poultry (Altalhi et al., 2010; Abo-Amer et al., 2018). Furthermore, ESBL and AmpC producers were detected in the Qatari chickens and green turtles in Oman, respectively (Al-Bahry et al., 2012; Eltai et al., 2018a). In Iran, *E. coli* strains producing blaSHV were isolated from raw milk and dairy products across the country (Ranjbar et al., 2018). Furthermore, this same gene was detected in Uropathogenic *E. coli* strains isolated from dogs (Yousefi and Torkan, 2017).

Carbapenem and Colistin Resistance

Unlike ESBL and AmpC producers, carbapenemase producing GNB are not widely spread in animals of the Middle East (Figure 2). Al Bayssari et al. (2015b) reported the isolation

of ST38 *E. coli* positive for the blaOXA-48 from fowl in Lebanon. Furthermore, they detected VIM-2 carbapenemase in *P. aeruginosa* and blaOXA-23/blaOXA-58 genes *A. baumannii* strains isolated from cattle, swine and fowl (Al Bayssari et al., 2015a). Furthermore, Rafei et al. (2015a) reported the isolation of OXA-143 *A. baumannii* and OXA-24 *A. pittii* from a horse and a rabbit oral cavity, respectively. In Egypt, carbapenem resistant *K. pneumoniae* (CR-KP) have been isolated from broilers, drinking water and workers in chicken farms (Abdallah et al., 2015a). The genes responsible for resistance were blaKPC, blaOXA-48, and blaNDM. In cattle, OXA-48 and OXA-181 producing *E. coli* were detected (Braun et al., 2016).

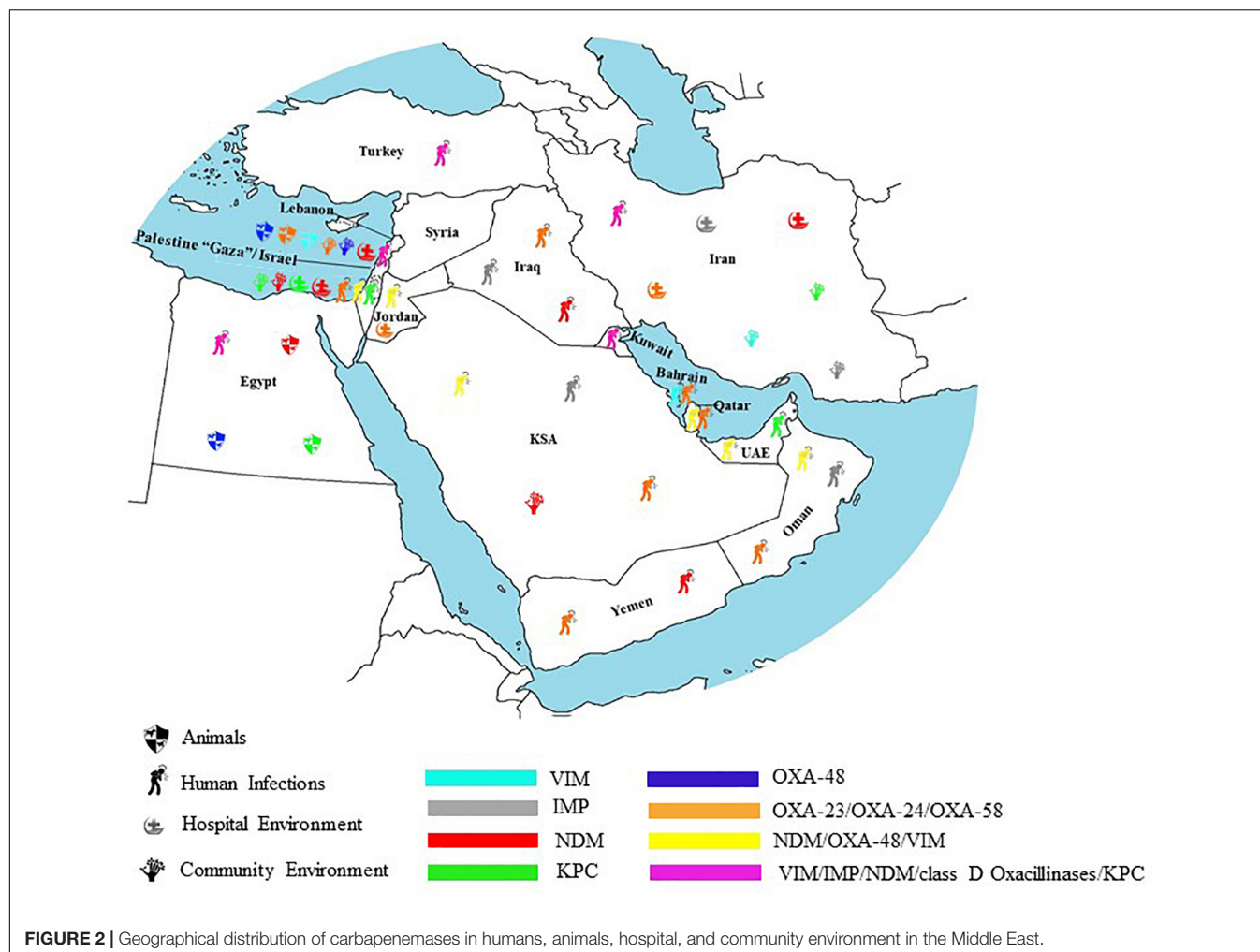
As for colistin resistance (Figure 3), in Egypt, it is known that colistin is used in animal husbandry in farms, calves, poultry, and rabbits (Lima Barbieri et al., 2017). In poultry for example, colistin has been used for colibacillosis. Colistin resistant avian isolates of *E. coli* that have been found in Egyptian farms imply that the overuse of colistin in the farming industry can indeed have participated in the emergence of colistin resistance in Egypt (Lima Barbieri et al., 2017). Indeed, in samples collected from both poultry and cattle, the *mcr-1* gene was detected (Khalifa et al., 2016; Lima Barbieri et al., 2017). In cattle, *mcr-1* was harbored by an ST10 *E. coli* strain (Khalifa et al., 2016). In the light of the One Health concept, those resistant strains can potentially enter the human food chain and result in treatment challenging infections that pose a serious threat to the Egyptian population. This is especially relevant in a country like Egypt which is known to struggle with infectious diseases and poor control of antibiotic use (Khalifa et al., 2016). On the other hand, Lebanon is considered one of the more recent countries in which colistin resistance has emerged. Dandachi et al. (2018c) reported the first detection of ESBL/*mcr-1* ST515 *E. coli* strain isolated from chicken in the South of Lebanon. *mcr-1* *E. coli* strains were also detected in Lebanese pigs (Dandachi et al., 2018b).

DISTRIBUTION OF MULTI-DRUG RESISTANT ORGANISMS IN THE ENVIRONMENT

Community Environment

In Iran, a study addressing MDROs in surface water resources found a high prevalence of CTX-M, TEM, SHV, and OXA genes among isolated *E. coli* strains (Ranjbar and Sami, 2017). In another study conducted in community water filtering systems, *P. aeruginosa* producing blaVIM-1, blaNDM, and blaIMP-1 were detected (Mombini et al., 2019). In Turkey, ESBL producing *E. coli* strains were detected in samples collected from Orontes River. The most common ESBL type was CTX-M-15 with the majority co-harboring the sulfonamide resistance gene *sul* (Kurekci et al., 2017).

In Lebanon, Diab et al. (2018) targeted the issue of MDROs contamination in three water sources: estuaries, wells and spring water (Diab et al., 2018). It was found that in estuaries, 17 ESBL and four carbapenemase producing GNB were isolated.



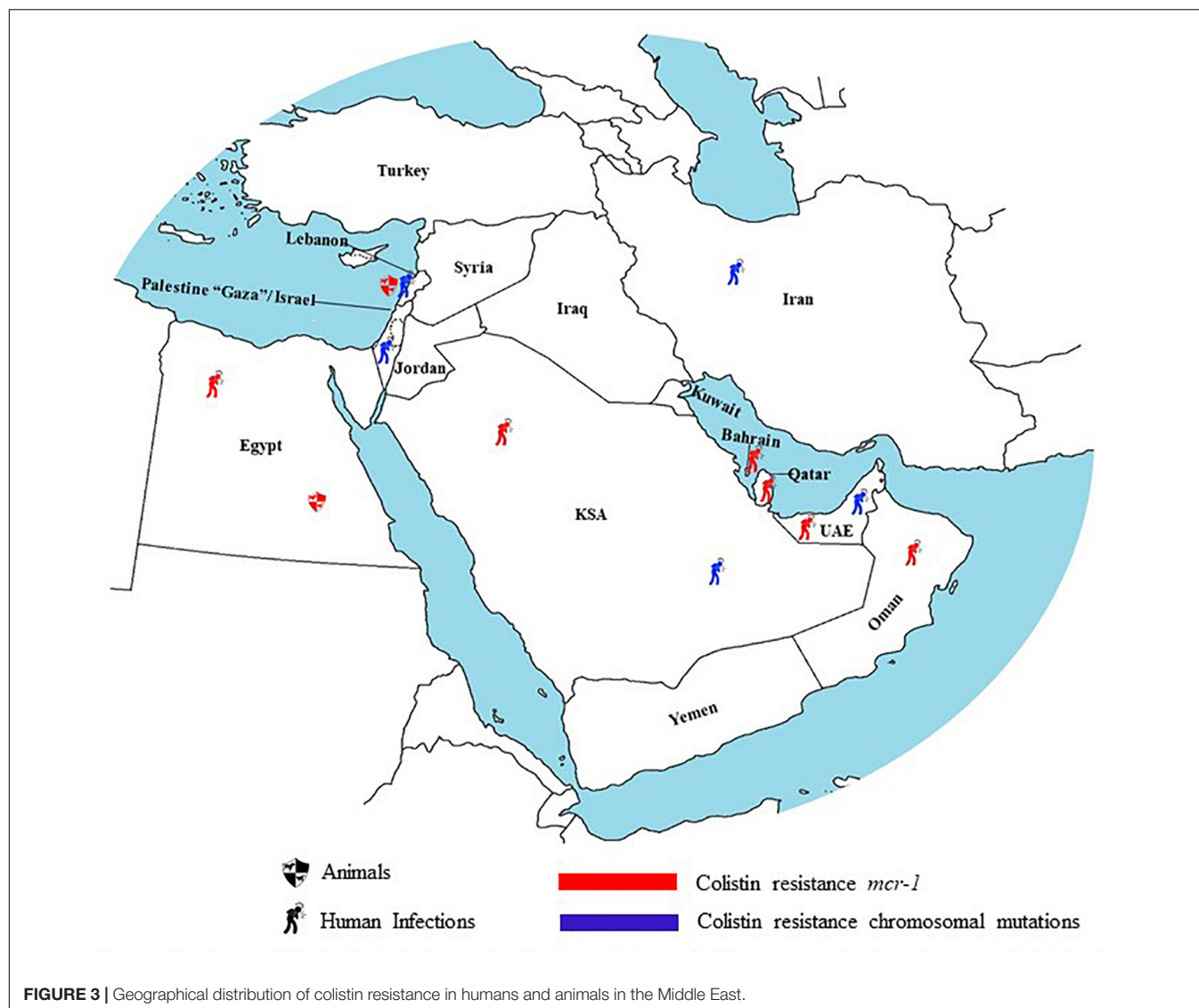
The most prevalent ESBL gene was the bla-CTX-M-15 followed by CTX-M-55, CTX-M-14, and SHV-12. IncF type plasmid was the most common plasmid type detected among ESBL. In parallel, carbapenem resistance was mediated by the presence of bla-OXA-48 carried by the IncL plasmid and the bla-OXA-244 carried by the IncHI2 plasmid. On the other hand, only ESBL producers were detected in wells and spring water. These included the blaCTX-M-15 gene located on an IncF plasmid (Diab et al., 2018). In another study, ESBL producing *E. coli* were addressed in a refugee camp and from river effluents (Tokajian et al., 2018). The prevalence of ESBL was similar in both groups (53.11% versus 49.1%). However, the latter presented with different phylogroups and sequence types; in addition, refugee camp isolated strains had higher resistance rates toward aminoglycosides, fluoroquinolones and trimethoprim-sulfamethoxazole (Tokajian et al., 2018). In this study, the most common ESBL types detected in both sources were CTX-M-15, CTX-M-27, CTX-M-14, and CTX-M-9 (Tokajian et al., 2018). Furthermore, it is worth noting the detection of OXA-72 producing *Acinetobacter calcoaceticus* in vegetables in Lebanon. The presence of MDROs in vegetables could be the result of direct animal contamination or indirect environmental

contamination with soil or irrigation water (Al Atrouni et al., 2015, 2016b). Altogether, due to the lack of proper water treatment systems in Lebanon, water sources have become major environmental reservoirs for MDROs. In Israel, one study reported the detection of blaKPC and blaNDM-1 producing Enterobacteriaceae (mainly *K. pneumoniae* and *E. cloacae*) in sewage systems (Meir-Gruber et al., 2016).

In the KSA, blaNDM-1 ST101 *E. coli* was isolated from wastewater (Mantilla-Calderon et al., 2016). In addition, Alghoribi et al. (2015) reported the detection of CTX-M producing *E. cloacae* from a community sewage.

Hospital Environment

In Iran, TEM and SHV producing *P. aeruginosa* were detected in hospital environmental samples (Gholami et al., 2017). Another study, in which the samples were taken from an ICU setting, revealed that among the *Klebsiella* species isolated, the majority were carbapenem resistant *K. pneumoniae*. The ICU's contaminated areas are a great source for the spread of MDROs in their surroundings (Moghadampour et al., 2018b). In isolated *K. pneumoniae* strains, the highest resistance rate was observed for β -lactam antibiotics and the lowest resistance



rate was toward tigecycline. In this study, blaOXA-48 was the most prevalent carbapenemase detected, followed by blaNDM (Moghadampour et al., 2018b). Furthermore, various hospitals have been suffering from antibiotic resistant *A. baumannii*. In one study, several isolates were taken from different parts of the hospital with the greatest number of *A. baumannii* coming from the ICU. These strains carried the OXA-23 and OXA-24 genes (Shamsizadeh et al., 2017). The prevalence of CRKP has been another problem in Iran, especially on hospital equipment where in one study 34 CRKP were isolated (Moghadampour et al., 2018b). Aliramezani et al. (2016) also reported the detection of carbapenem resistant GNB (OXA-23 *A. baumannii*) in instruments that are frequently used for the care of patients such as dressing sets, suction tubes, hand-washing sinks and faucets. The occurrence of MDROs in the aforementioned surfaces raise the chance of acquisition and HGT of resistance genes among patients, health care personnel and visitors, creating thus a significant source of hospital acquired infections (Aliramezani

et al., 2016). Moreover, other studies in Iran reported the isolation of carbapenemase producing *A. baumannii* (OXA-23, OXA-24, and OXA-58) from hospital environmental samples (Kulah et al., 2010; Salehi et al., 2018). Of special interest is the detection of OXA-23 and CTX-M-32 genes in air samples collected from operating theaters, ICUs, surgery and internal medicine wards (Mirhoseini et al., 2016). This study unveiled another source of the environmental route of MDROs transmission. Not only is area disinfection warranted but the application of efficient working ventilation and air quality monitoring systems is also highly needed (Mirhoseini et al., 2016).

In Turkey, only one study conducted in hospitals reported the detection of ESBL *K. pneumoniae* in broiler distilled water in a NICU (Hosbul et al., 2012). In Israel, a study conducted inside a general hospital with emphasis on the bacteria present on wheelchairs, found that *P. aeruginosa* followed by *A. baumannii* were the most common MDROs detected and exhibited resistance to all antibiotics tested, especially in the samples taken

from wheelchairs in the surgery department (Peretz et al., 2014a). Interestingly, one study assessed the prevalence of environmental contamination of CRE in the vicinity of 34 carriers. Among these 26 were spreaders with a group of six being responsible for 79% of the environmental CRE detected. Statistical analysis revealed that fecal continence was the sole independent factor associated with CRE non-spread. On the other hand, high rectal colonization with these MDROs in addition to being admitted with a respiratory disease were the only independent risk factors for CRE shedding (Lerner et al., 2015). Therefore, imminent protocols must be set to minimize contamination and spread of infections in all hospital settings.

In Lebanon, the most discussed source of MDROs contamination are of a human source whether directly or indirectly through hospital sewage. In addition to that, water is considered a pivotal driver of contamination because it acts as a reservoir for MDROs and receives them from multiple sources. Furthermore, Daoud et al. (2018) found that isolates of *E. coli* from the hospital wastewater produced CTX-M ESBL at a rate of 81.5% in one hospital and 94.4% in another. SHV beta-lactamases were produced by 55.6 and 44.4% of the isolates in each hospital, respectively. In the same context, Daoud et al. (2017), addressed the MDROs in two hospital sewage treatment plants. In this latter, ESBL and AmpC producers including *E. coli*, *E. cloacae*, *Klebsiella* spp., and *Serratia odorifera* were isolated. The most common ESBL types found were CTX-M followed by SHV, TEM, and OXA. Furthermore, only one CRE was detected (Daoud et al., 2017).

In Iraq, only one study revealed the detection of ESBL *Klebsiella* spp. and *E. coli* producing CTX-M-15, AmpC beta lactamases and SHV ESBL types, respectively, in hospital environmental samples (Huang et al., 2012). Interestingly, Obeidat et al. (2014) reported the isolation of carbapenem resistant *A. baumannii* (OXA-23 and OXA-24 producing) from a hospital environment as well as from patients respiratory tracts; the high similarity of MDR patterns suggest the persistence of these MDRO in the environment is responsible for their high colonization rates detected in the respiratory tracts of ICU patients.

In the gulf, one study in the KSA found ESBL producing GNB in hospital sewage. The sewage tank might play a significant role in the dissemination of MDROs, especially if it enters the sea and beach recreational activity areas, subsequently affecting the community population (Alghoribi et al., 2015).

ANTIBIOTIC USE IN THE MIDDLE EAST

Clinical Setting and Community

Nowadays, antibiotics are among the most common drugs prescribed worldwide. Between 2000 and 2010, antibiotic consumption increased by 20 billion standard units (Auta et al., 2019). The growing use of antibiotics through prescriptions or non-prescriptions is linked to the spread of MDROs, therefore causing a global public health concern (Auta et al., 2019).

In the Middle East, studies have shown that SM is highly prevalent. In Iran, SM ranges from 35.4 to 83%,

32 to 42% in Lebanon, 32 to 62% in Jordan, 98% in Palestine, 85% in Syria. In the Gulf, SM rates were as high as 89.2% in the United Arab Emirates, 48% in the KSA and 60% in Yemen (Khalifeh et al., 2017). This is unlike European countries where “over-the-counter” access to antibiotics is strictly regulated, thus resulting in SM prevalence rates ranging from 1 to 4% only (Alhomoud et al., 2017). The major types of antibiotics sold over-the-counter in the Middle East include penicillin, macrolides, cephalosporins, fluoroquinolones, and tetracycline (Alhomoud et al., 2017). The main reason behind the common practice of SM in Middle Eastern countries is the lack of strict policies controlling the sale of antibiotics without a prescription from pharmacies. This is in addition to the low economic status and lack of health care insurance that push individuals to retrieve medications from pharmacists to avoid consultations costs (Alhomoud et al., 2017).

Besides SM, inappropriate use of antibiotics in hospitals is another reason behind the dissemination of MDROs in the Middle East. Indeed, ASP, although implemented in some hospitals in several countries such as Lebanon, Jordan, Palestine, the KSA, the United Arab Emirates, Bahrain, Qatar, and Oman; these are still in their infancy (Nasr et al., 2017). Barriers for the implementation of ASP in the Middle East are divided mainly into two levels: individual and hospital barriers. Individually speaking, physicians often lack up-to-date knowledge for appropriate antibiotic use and resistance, reluctance for antibiotic prescription other than the usual and fear of patient complications especially in very sick patients are other individual barriers (Alghamdi et al., 2018). On the other hand, lack of expertise, unavailability of some antibiotics, lack of education/training for appropriate usage of antibiotics and antimicrobial resistance as well as a lack of financial, administrative and management support are all barriers against the implementation of ASP at the hospital level (Alghamdi et al., 2018).

Further research assessing the knowledge, attitude and practices of antibiotic prescription among expatriates is crucial for the adoption of successful programs, in order to promote the rational use of antimicrobial agents in the Middle East (Alhomoud et al., 2017). Furthermore, hospital leadership is paramount to ensure policies’ enforcement, in collaboration with physicians and other stakeholders (Alghamdi et al., 2018). On the other hand, as for SM, enforcing regulatory measures that restrict antibiotic access to “prescribed-only,” developing national resistance as well as antibiotic consumption surveillance systems can all help in reducing the rates of SM (Alhomoud et al., 2017). This is definitely in addition to public awareness campaigns addressing the proper use of antibiotics in addition to the dangers of their inappropriate use and over-intake (Alhomoud et al., 2017).

Animals and Environment

Unfortunately, it is evident that MDROs are nowadays disseminated in animals and the environment as it has been reported worldwide (Rizzo et al., 2013; Alonso et al., 2017; Dandachi et al., 2018a). Similar to humans, among other factors,

the un-regulated use of antibiotics in veterinary medicine is the main cause for MDRO dissemination (Guerra et al., 2014). Besides treatment, in animals, antibiotics are also given as growth promoters and for prophylaxis. As growth promoters, this practice is no longer applied in the European Union, but it persists in North America and other countries (Economou and Gousia, 2015). MDROs in animals can be transmitted to humans via direct/indirect contact or via the surrounding environment (Pomba et al., 2017). Despite their importance in the transmission chain, surveillance studies addressing MDROs in these two ecosystems in the Middle East are scarce. As shown in **Figures 1–3**, epidemiological studies describing the dissemination of MDR in animals and the environment were conducted in only six out of the 15 countries. The level of antibiotic consumption in livestock is unknown and thus policies to control the misuse and overuse of antimicrobial agents in veterinary medicine are not yet in place. Furthermore, the role of the environment in the transmission route is also unknown in this region of the world. In the environment, resistant bacteria can spread either due to the shedding of MDROs from human/animal waste or via the antibiotic selective pressure created by antimicrobial release in livestock and human waste streams (Dar et al., 2016). In the one health concept “the health of people is connected to the health of animals and the environment” (Centers for Disease Control and Prevention [CDC], 2018). Researchers in Middle Eastern countries are therefore recruited to conduct studies to fill the gaps of epidemiological distribution of MDROs as well as antibiotic consumption in ecosystems other than humans. Furthermore, the implementation of an integrated human-animal surveillance system where samples are obtained from both humans, livestock and the environment and then processed with a synchronized monitoring system can assist these speculations (Manyi-Loh et al., 2018). The first worldwide system integrating humans and

animals was the “DANMAP” (Danish Integrated Antimicrobial Monitoring and Resistance Program) which addresses the problem of MDR in livestock, food of animal origin and people (Dar et al., 2016).

CONCLUSION

This review shows the extensive dissemination of ESBL and carbapenemase producing GNB in Middle Eastern hospitals. The prevalence of these MDROs is less well documented in animals and the environment. However, studies reported that ESBL is common in livestock whereas carbapenemases are scarce. In the environment, to some extent both groups (ESBL and carbapenemases) were reported equally. This emphasizes that the environment plays a double route in the transmission of resistant organisms from humans to animals and vice versa. In some countries especially in the gulf, nothing is known about the spread of MDROs in animals nor the environment; therefore, a clear conclusion cannot be drawn. One major mediator of MDROs spread in the Middle East is the recent population mobilization due to the socio-economic crisis and the Syrian war. This conflict promotes the introduction of resistance genes not previously reported in those countries. The emergence of colistin resistance is another major issue. In most of the epidemiological studies, colistin susceptibility is assessed by the Kirby-Bauer technique. This method is unreliable and might underestimate the real prevalence of colistin resistance in all ecological niches.

AUTHOR CONTRIBUTIONS

ID, AC, JH, and JM wrote the manuscript. ZD corrected the manuscript. All authors approved and revised the final version of the manuscript.

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Ceftazidime/avibactam Improves the Antibacterial Efficacy of Polymyxin B Against Polymyxin B Heteroresistant KPC-2-Producing *Klebsiella pneumoniae* and Hinders Emergence of Resistant Subpopulation *in vitro*

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Due to the increasing multidrug resistance and limited antibiotics, polymyxin B revived as the last resort for the treatment of carbapenemase-producing *Klebsiella pneumoniae* (CRKP). Unfortunately, the heteroresistance hampers polymyxin B monotherapy treatment via the amplification of resistant subpopulation. Reliable polymyxin B based combinations are demanded. Ceftazidime/avibactam has been regarded as a new salvage therapy against CRKP. The occurrence of heteroresistance was confirmed by population analysis profiling (PAP). Our study demonstrated that polymyxin B and ceftazidime/avibactam combinations improved the *in vitro* antimicrobial activity of polymyxin B and delayed or suppressed the regrowth of resistant subpopulation by time-kill studies. Ceftazidime/avibactam at around MIC values ($0.5-1 \times \text{MIC}$) plus clinically achievable concentrations of polymyxin B ($0.5-2 \text{ mg/L}$) resulted in sustained killing against polymyxin B-heteroresistant isolates. Active PmrAB and PhoPQ systems and a *pmrA* mutation (G53R) in resistant subpopulation might associate with heteroresistance, but further investigation was required. Our findings suggested that the heteroresistance represented barriers to polymyxin B efficacy, and the combination of polymyxin B with ceftazidime/avibactam could be potentially valuable for the treatment of heteroresistant CRKP. Further, *in vivo* studies need to be performed to evaluate the efficacy of this combination against heteroresistant strains.

Keywords: ceftazidime/avibactam, polymyxin B, heteroresistance, KPC-2-producing *Klebsiella pneumoniae*, bacterial killing activity

INTRODUCTION

The global spread of carbapenemase-producing *Klebsiella pneumoniae* (CRKP) posed a severe challenge to public health, especially KPC-producing *K. pneumoniae* (KPC-Kp) (Willyard, 2017; Decraene et al., 2018; Gu et al., 2018). To date, available options for CRKP were limited. Therefore, polymyxin B (PMB) revived as one of the last-resort options for CRKP (Falagas and Michalopoulos, 2006; Landman et al., 2008). However, there are some challenges when clinicians use polymyxin B, such as its toxicities, unreliable plasma concentrations, and several issues with polymyxin B susceptibility testing (Ezadi et al., 2019). Additionally, bacteria employed several strategies to survive to polymyxins, including LPS modifications by activation of the two-component systems (TCSs), particularly modifications of lipid A, the efflux pumps, and plasmid-mediated resistance (Ma et al., 2018; Meletis and Skoura, 2018).

Significantly, the heteroresistance raised a diagnostic and therapeutic dilemma for clinicians, which the resistant subpopulations in heteroresistant strains were undetectable and could affect the clinical outcome (El-Halfawy and Valvano, 2015; Band and Weiss, 2019). El-Halfawy and Valvano (2015) recommended defining heteroresistance as subpopulations of an isogenic strain exhibit widely various susceptibilities to a particular antimicrobial agent, i.e., when the lowest concentration exhibiting maximum inhibition is eightfold higher than the highest non-inhibitory concentration in terms of population analysis profiling (PAP). However, this method is too laborious and complex to apply to clinical detection. Increasing studies have demonstrated that conventional susceptibility tests could misclassify heteroresistant strains as susceptible and might lead to clinical treatment failure (Band et al., 2018; Turlej-Rogacka et al., 2018; Ezadi et al., 2019). But little work has been done to evaluate the efficacy of available antibiotics against heteroresistant strains. Ceftazidime/avibactam, a β -lactam/ β -lactamase inhibitor combination, has been proposed as a new salvage therapy for severe KPC-Kp infections (Barber et al., 2018; Manning et al., 2018; Tumbarello et al., 2019). The objective of this study was to evaluate the *in vitro* effect of ceftazidime/avibactam in combination with polymyxin B against polymyxin B heteroresistance *Klebsiella pneumoniae*.

MATERIALS AND METHODS

Bacterial Strains and Characterization

Seventeen non-duplicate clinical isolates were obtained from two tertiary hospitals in Guangzhou from 2013 to 2014, as shown in **Supplementary Table S1**. All isolates were stored at -80°C and subcultured onto blood agar plate before each experiment. All isolates were reconfirmed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF). Carbapenemase genes, ESBLs genes, outer member protein genes, and *mcr-1* gene were amplified by primers described previously and then sequenced by Sanger sequencing (Pagani et al., 2003; Poirel et al., 2011; Liu et al., 2016).

Antimicrobials and Antimicrobial Susceptibility Testing

Polymyxin B (Sigma-Aldrich, United States), ceftazidime hydrate (Sigma-Aldrich, United States) and avibactam (MedChem Express, United States) were freshly prepared for each experiment and filter sterilized using a $0.22\ \mu\text{m}$ filter. Avibactam was tested at a fixed concentration of 4 mg/L (Clinical and Laboratory Standards Institute [CLSI], 2017). Mueller-Hinton broth (Oxoid, United Kingdom) supplemented with calcium and magnesium (25.0 mg/liter Ca^{2+} and 12.5 mg/liter Mg^{2+}) (CAMHB) and Mueller-Hinton II agar (Oxoid, United Kingdom) were used for susceptibility testing and all *in vitro* models. The breakpoints for polymyxin B, ceftazidime and ceftazidime/avibactam were defined by CLSI-M100-S26. Quality control was monitored with *Escherichia coli* strains ATCC 25922 and *Klebsiella pneumoniae* strain ATCC 700603.

Polymyxins Population Analysis Profiles (PAPs)

Population analysis profilings were performed to investigate the presence of polymyxin B heteroresistance in duplicate (Nicoloff et al., 2019). Fifty-microliter of dilutions of an overnight culture ($\sim 10^8$ CFU/ml) were plated on Mueller-Hinton agar plates containing polymyxin B at the following concentrations: 0.5, 1, 2, 4, 8, 16, and 32 mg/L. After overnight incubation at 37°C , colonies were counted. Agar plate preparation followed CLSI M7-09 documents for MIC determination by agar dilution assays. The detection limit of PMB resistant subpopulations was 20 CFU/ml.

To Measure the Stability of Resistant Subpopulation

After overnight growth of B1, D1, and D4 (without polymyxin B), fifty-microliter aliquots of the overnight culture were added into tubes with 16 mg/L polymyxin B. After serially diluted, suspension was plated on M-H agar plates with and without 16 $\mu\text{g}/\text{ml}$ polymyxin B to count CFU of total, and resistant subpopulation at desired time points (day 1). A subculture (1:100) was grown overnight in CAMHB without 16 $\mu\text{g}/\text{ml}$ polymyxin B, serially diluted, and plated on M-H agar with or without 16 $\mu\text{g}/\text{ml}$ polymyxin B to count CFU of parental, and resistant cells (day 2). Repeated this process in CAMHB broth without antibiotics (day 3 and 4).

Genes Expression Analysis

The polymyxin B-resistant subpopulations were collected from the last step. Cultures of parental strains and resistant subpopulations were grown in CAMHB medium without polymyxin B at 37°C with shaking to an OD600 of 0.5. The mRNA of strains was extracted by Trizol method. By the process of RT-PCR using the PrimeScriptTM RT reagent Kit with gDNA Eraser (TAKARA, China), the cDNAs were got. Then the *phoP*, *phoQ*, *mgrB*, *pmrA*, *pmrB*, *pmrC*, and *acrB* gene expression were detected through quantitative real-time PCR (qRT-PCR) using the SYBR[®] Premix Ex TaqTM II (Tli RNaseH Plus) kit (TAKARA, China), as previously described (Jayol et al., 2015). Each experiment was performed in triplicate. The expression of

target genes was normalized relative to the RNA polymerase beta subunit gene *rpoB*. Threshold cycle (Ct) numbers were confirmed by the qRT-PCR system software, and data was analyzed in accordance with the $2^{-\Delta\Delta C_t}$ method. The expression levels of the target genes were compared with those of *K. pneumoniae* ATCC 700603 (polymyxin B susceptible strain, expression = 1).

Whole-Genome Sequencing and SNPs Analysis

The whole genome sequencing of twenty-four carbapenem-resistant isolates was performed with a NextSeq 500 platform (Illumina Inc., San Diego, CA, United States). Briefly, the genomic DNA was extracted using a MiniBEST Bacteria Genomic DNA Extraction Kit (Takara, Dalian, China). To prepare the DNA library for sequencing, a QIAseq FX DNA Library Kits (Qiagen Inc., Valencia, CA, United States) was used following the manufacturer's recommendations. The quality and quantity of the libraries were assessed with LabChip GX (Perkin Elmer; Waltham, MA, United States) and Qubit dsDNA HS Assay Kit (Life Technologies, United States). All barcoded libraries were pooled together in equimolar amounts and each pool was sequenced on NextSeq 500 in PE-150 bp mode. Later, sequencing raw reads were processed for library adapter removal and filtering using FASTQ preprocessor Fastp v0.12.5 (Chen et al., 2018) and *de novo* assembly with SPAdes v3.13.0 (Bankevich et al., 2012). Genomic repeats were removed from the analyses by filtering out reads that mapped to multiple positions in *K. pneumoniae subsp. pneumoniae* HS11286 (NCBI accession number: NC_016845). Single nucleotide polymorphisms (SNPs) and insertions and deletions (indels) generated by Snippy.

Synergy Testing Using the Checkerboard Assay and Time-Kill Assay

Time-kill studies were performed using a 5-ml time kill assay with an initial inoculum of $\sim 10^6$ CFU/ml suspended in CAMHB. Each experiment was performed in duplicate. Ceftazidime-avibactam concentrations of 0.25 $\times$, 0.5 $\times$, 1 $\times$, 2 $\times$, 4 $\times$, and 8 $\times$ MIC and polymyxin B concentrations of 0.5, 1, 2, and 6 mg/L were evaluated as monotherapy. Meanwhile, avibactam was added to a final concentration of 4 mg/L. A 3-by-3 concentration matrix of ceftazidime-avibactam 0.25 $\times$, 0.5 $\times$, and 1 $\times$ MIC in combination with PMB (0.5, 1, and 2 mg/L) was evaluated. All concentrations evaluated were clinically achievable, and supratherapeutic concentrations were also selected to evaluate potential advantages of intensive dosing (Avedissian et al., 2019; Tumbarello et al., 2019). Samples were incubated with shaking (37°C, 200 rpm), and were obtained at 0, 4, 8, 12, and 24 h for quantification of bacteria. The change in log₁₀ CFU per milliliter at time t (CFU_t) compared to the baseline value (0 h) (CFU₀) was the index of pharmacodynamic effect. A > 3 log₁₀ CFU/ml reduction from baseline was considered as the bactericidal activity. Synergy was considered as a >2 log₁₀ CFU/ml reduction and additivity as a >1 to <2 log₁₀ reductions in CFU/ml caused by the combination of PMB and ceftazidime-avibactam compared to the most effective single antibiotic in the combination. Subsequently, the effects of combinations

against heteroresistant strains were evaluated using microbroth checkerboard method. Given that we could not get clear MIC results of polymyxin B due to the presence of skip-wells, the concentrations of polymyxin B in the combinations were selected as these used in time-kill assay. And ceftazidime-avibactam in the combinations was two dilutions above and four dilutions below the MIC. The fractional inhibitory concentration index (FICI) was calculated using the following equation: FICI = FICA + FICB, where FICA = MIC of drug A in a combination/MIC of drug A alone, and FICB = MIC of drug B in a combination/MIC of drug B alone. The FICI results were interpreted as synergistic (≤ 0.5), additive (> 0.5 to ≤ 1), or indifferent (> 1).

Statistical Analysis

Statistical analyses were performed using Prism 7 (GraphPad Software). The median was used to describe the average fold increase in heteroresistant strains. The two-tailed student's *t*-test was used to analyze the significance of relative gene expression level between parental strain and resistant subpopulation.

Accession Number

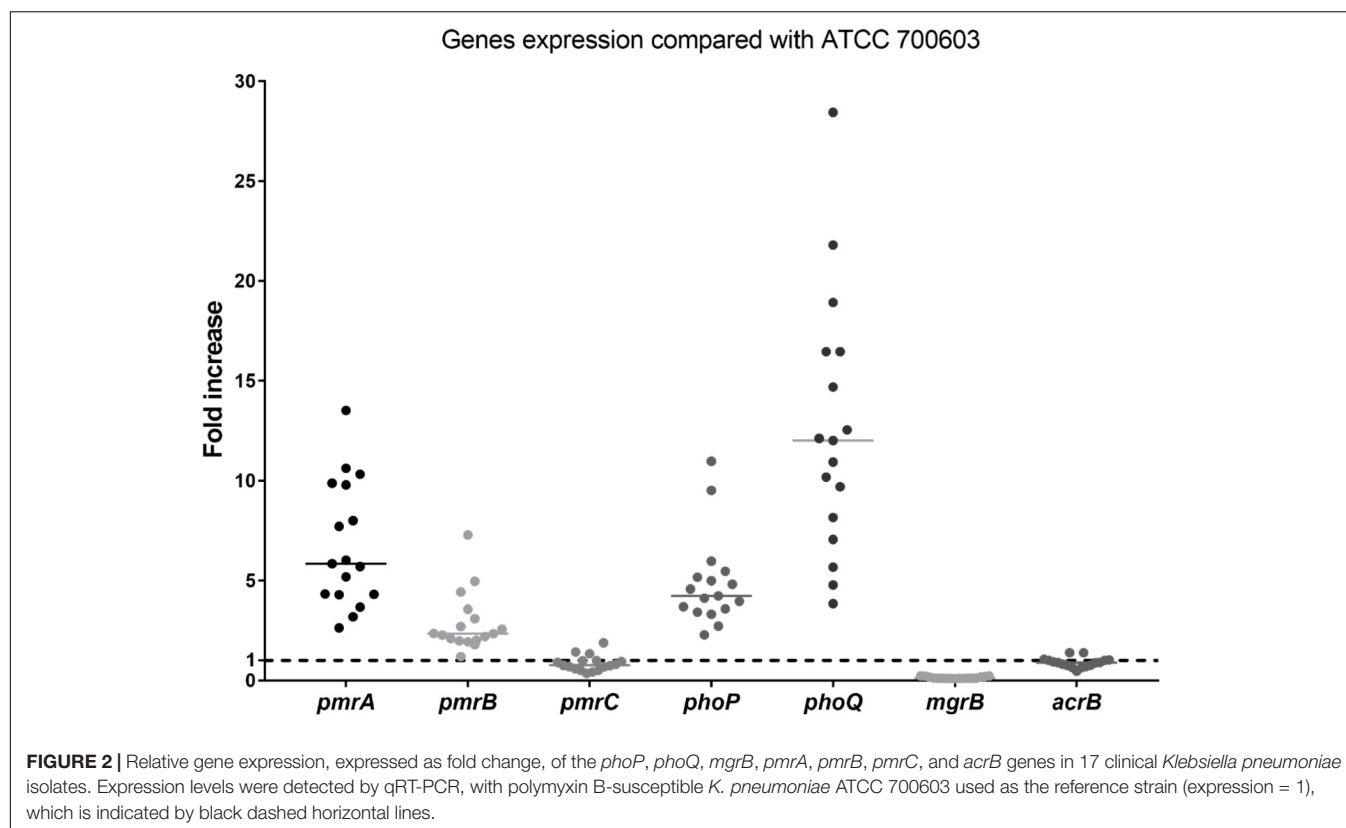
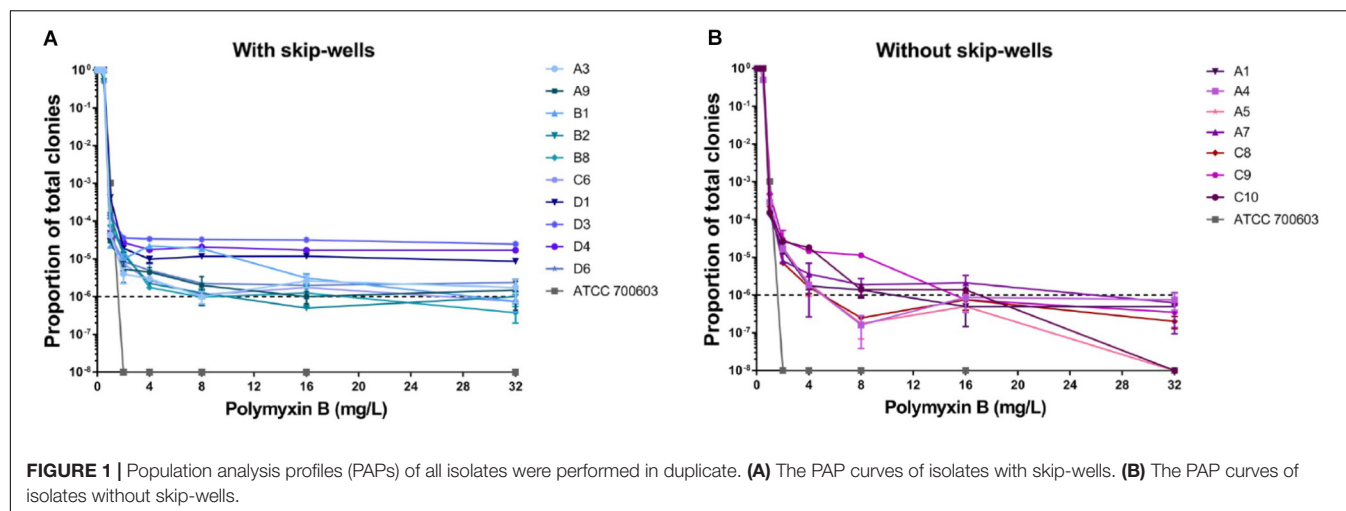
Sequence data from this study were deposited in NCBI's short read archive (SRA) under project accession number PRJNA504930.

RESULTS

Antimicrobial Susceptibility Testing and Heteroresistance Identification

In our study, all isolates belonged to ST 11 and harbored KPC-2, TEM, SHV, and CTX-M (Supplementary Table S1). All isolates remained sensitive to ceftazidime/avibactam (Supplementary Table S1). Seven isolates showed sensitive to polymyxin B with clear wells (range 0.06125–0.125 mg/L), but the presence skip-wells was observed in the other ten isolates (Supplementary Table S1). The results of PAPs indicated all isolates exhibited heteroresistant to polymyxin B. Most of our isolates (except C10 and A5) harbored minor resistant subpopulations able to withstand at least 32 mg/L polymyxin B (Figure 1). In contrast, susceptible strain (ATCC 700603) was entirely killed by 2 mg/L polymyxin B. Compared with the isolates without skip-wells, the frequencies of resistant subpopulation among isolates with skip-wells were higher (above 10^{-6}) and remained constant with polymyxin B concentrations increasing.

No isolates harbored the *mcr* gene. The differences in expression of polymyxin B resistant genes between heteroresistant strains and reference strain (polymyxin B-susceptible *K. pneumoniae* ATCC 700603) were observed (Figure 2). The median fold changes in the expression of *phoP* and *phoQ* genes were 4.18 and 11.47, respectively (Figure 2). The increasing fold changes were also observed in *pmrA* and *pmrB* genes (5.78-fold and 2.31, respectively, Figure 2). As the negative regulator of PhoPQ, the fold change of *mgrB* decreased (0.12-fold) in all polymyxin B-heteroresistant strains. There is no significant overexpression of *pmrC* (0.79-fold) and *acrB* (0.90-fold) among polymyxin B-heteroresistant strains (Figure 2).



The Resistant Subpopulation Can Survive Under Polymyxin B Pressure and Exist Stably Without Antibiotic

The stability of resistant subpopulations from three strains (B1, D1, and D4) was tested. As shown in Figures 3A–C, the resistant subpopulation could withstand and expand robustly under polymyxin B pressure, while most susceptible cells were killed over the first 2 h. After withdrawing polymyxin B, the resistant subpopulation still maintained a high level of polymyxin B resistance for 24 and 72 h, which suggested the resistant

subpopulations can exist stably. Therefore, we speculated that this phenotypic change might be constant and correlated with genetic changes.

Overexpressed PhoPQ or PmrAB System and Genetic Alternations in the Resistant Subpopulation

To determine the genetic alterations behind the stable heteroresistance phenotype, whole-genome sequencing (WGS) and quantitative PCR (qPCR) were performed on the paired

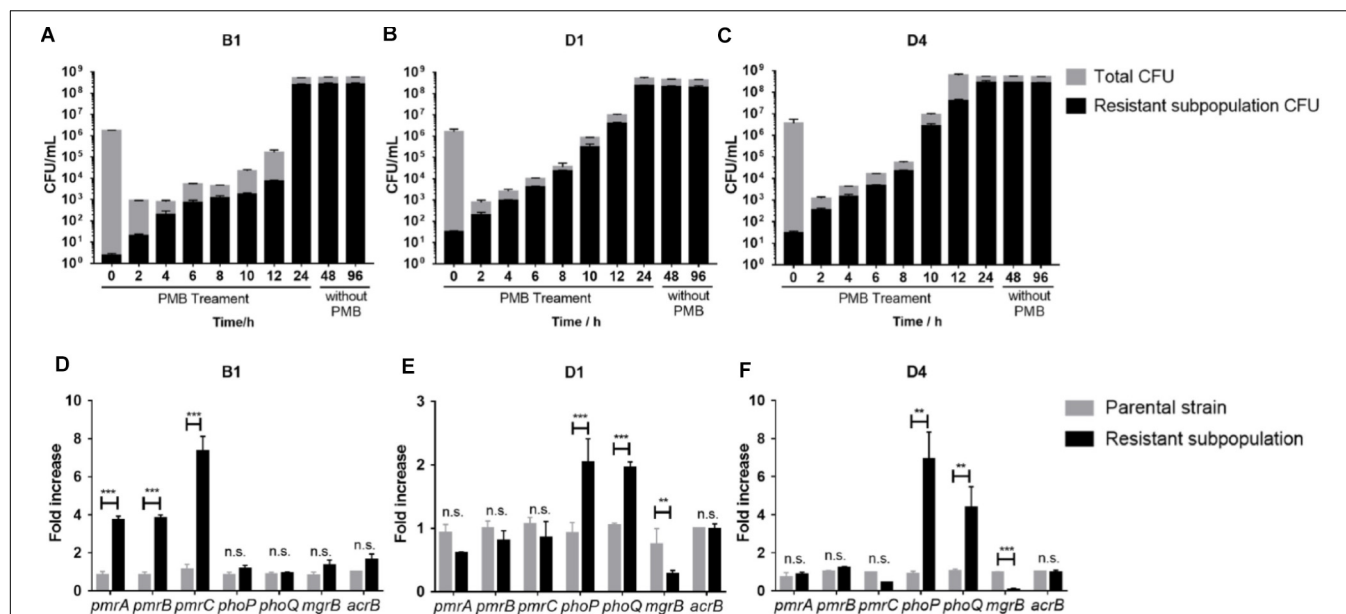


FIGURE 3 | (A–C) Polymyxin B-resistant subpopulation CFU and total CFU of B1, D1, and D4 during 24 h treatment with 16 mg/L polymyxin B in liquid culture and subculture without polymyxin B for further 24 and 72 h ($n = 3$). **(D–F)** qRT-PCR analysis of the *phoP*, *phoQ*, *mgrB*, *pmrA*, *pmrB*, *pmrC*, and *acrB* gene expression in resistant and susceptible subpopulations of B1, D1, and D4. Relative abundance was calculated by normalizing the expression of each gene to the average expression of the housekeeping gene, *rpoB* ($n = 4$). Statistically significant: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. n.s., not significantly different (unpaired t-test).

parental strain and its resistant population. Differences in the transcript levels of *pmrCAB* operon between parental strain and a resistant population of B1 were observed (Figure 3D). The expression of *phoP* and *phoQ* increased in resistant populations compared to their expression in parental cells (Figures 3E,F). As the negative regulator of PhoPQ signaling, the expression of *mgrB* was lower in resistant populations (Figures 3E,F). There was no difference in the expression of *acrB* between parental strains and resistant subpopulations. We identified a mutation in *pmrA* that led to a missense variant (G53R) in polymyxin B-resistant cells of B1. There were some mutations in other genes (Supplementary Table S2), but it is unclear whether they might contribute to polymyxin B heteroresistance.

Ceftazidime/avibactam Combinations Achieved Sustained Killing and Resistance Suppression

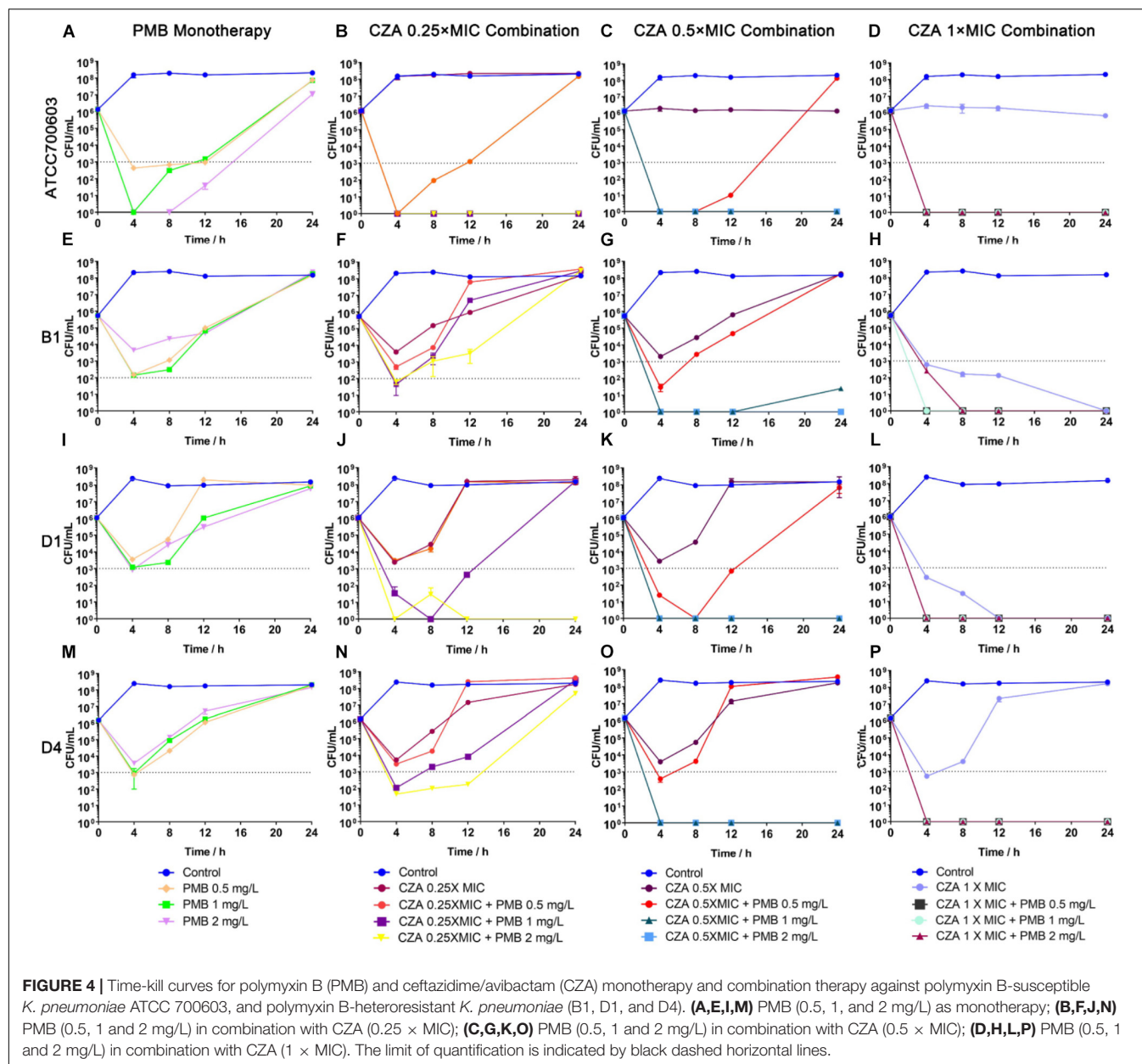
Polymyxin B displayed a stronger and more sustained initial killing ($\geq 3 \log_{10}$ by 12 h) (Figure 4A and Supplementary Table S3) against susceptible isolate ATCC 700603, while all polymyxin B treatments only led to a $\geq 2 \log_{10}$ reduction against heteroresistant isolates by 4 h and followed with bacterial re-growth (Figures 4E,I,M and Supplementary Table S3). This similar regrowth was observed even using supratherapeutic concentration against heteroresistant strains (6 mg/L) (Figure 5 and Supplementary Table S3). Ceftazidime/avibactam monotherapy showed concentration dependence. The higher concentrations ($2\times$, $4\times$, and $8\times$ MIC) displayed sustained bactericidal activity against all isolates over 24 h (Figure 5). In contrast, the bactericidal activity of ceftazidime/avibactam at

lower concentrations ($0.25\times$, $0.5\times$, and $1\times$ MIC) varied and displayed a weaker effect.

The addition of ceftazidime/avibactam improved the efficacy of polymyxin B. Combining ceftazidime/avibactam at $0.25 \times$ MIC with polymyxin B at 2 mg/L increased initial killing compared to that of monotherapy, but following regrowth was observed in B1 and D4 (Figures 4B,E,J,N). The addition of ceftazidime/avibactam at $0.25 \times$ MIC with polymyxin B (1 mg/L) did not hamper the regrowth of heteroresistant isolates (Figures 4F,I,J,N). The killing effect of combination sustained for 24 h when combining ceftazidime/avibactam at $0.5 \times$ MIC with polymyxin B (1 and 2 mg/L) (Figures 4C,G,K,O and Table 1). Colonies were undetectable over 24 h in all isolates when polymyxin B at all concentrations in combination with ceftazidime/avibactam at $1 \times$ MIC, showing a rapid and durable bactericidal activity (Figures 4D,H,L,P), and synergy was observed (Table 1). To confirm the effect of combination, microbroth checkerboard assay was performed. The FICI values against heteroresistant strains can be seen in Table 1. Similarly, the synergistic effect of combination ($FICI \leq 0.5$) was observed in B1 and D4, and the additive effect ($FICI > 0.5$ to ≤ 1) was observed in D1. Obviously, ceftazidime/avibactam strengthened the effect of polymyxin B and prevented the regrowth of polymyxin B-resistant cells. Additionally, combinations decreased the dose of each drug.

DISCUSSION

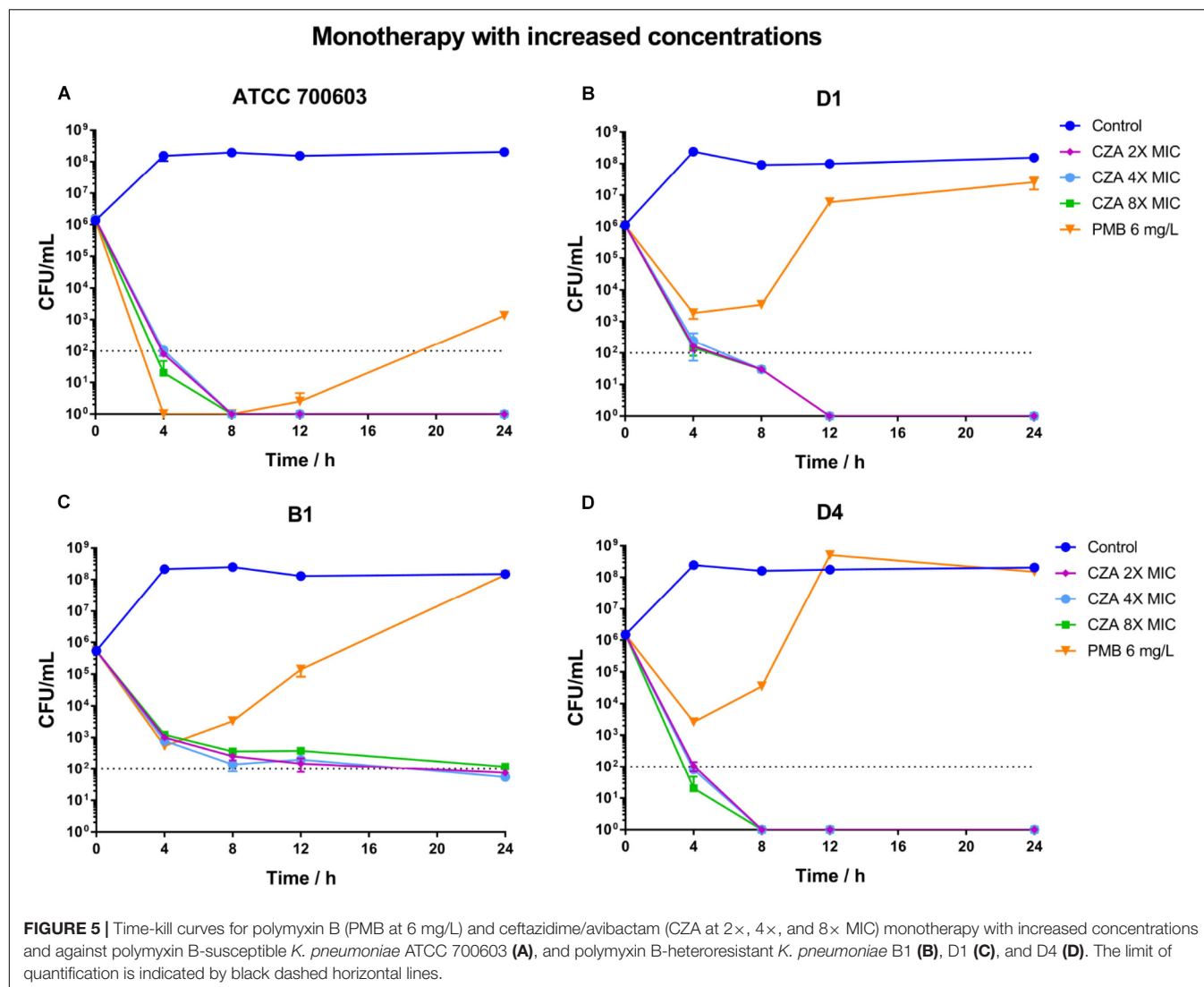
Recently, many researchers pointed out that the clinically undetected heteroresistance might have a profound impact on



treatment efficacy (Band et al., 2016, 2018; Band and Weiss, 2019). Polymyxin B has been widely used as a conventional lifesaver against superbugs for a long time, but the emergence of polymyxin heteroresistant will threaten the clinical use of polymyxins (Meletis et al., 2011; El-Halfawy and Valvano, 2015; Bardet et al., 2017; Wozniak et al., 2019). Therefore, it is critical to explore novel combination therapies which can delay or prevent the regrowth of polymyxin resistant subpopulations.

Here we evaluated the *in vitro* effect of the combination of polymyxin B with ceftazidime/avibactam against three polymyxin B-heteroresistant KPC-Kp (B1, D1, and D4). For polymyxin B-heteroresistant isolates, polymyxin B monotherapy resulted in a prompt killing effect, but followed by regrowth associated with the amplification of polymyxin

B resistant subpopulations. The same situation had been reported in polymyxin B or colistin monotherapy against other Gram-negative bacteria (Ly et al., 2015; Lenhard et al., 2017; Zhao et al., 2017; Nicoloff et al., 2019). Therefore, many researchers concerned that resistant subpopulations might affect treatment outcome (Band and Weiss, 2019). Ceftazidime/avibactam monotherapy at high concentrations (above $2 \times \text{MIC}$) prevented the regrowth of resistant subpopulations successfully. While ceftazidime/avibactam at low concentrations ($<2 \times \text{MIC}$) showed relatively poorer effect and only delayed the regrowth of resistant subpopulations. Previous studies have tested polymyxins (polymyxin B or colistin) in combination with ceftazidime/avibactam against KPC-Kp with positive results (Nath et al., 2018; Mikhail et al., 2019).



The similar killing effect against polymyxin B heteroresistant KPC-Kp was observed in our study. Nath et al. suggested that the addition of another antibiotic could be considered when ceftazidime/avibactam MIC values of isolates were close to the MIC breakpoint (Nath et al., 2018). Moreover, the addition of ceftazidime/avibactam improved the efficacy of polymyxin B and allowed for containment of all resistant subpopulations. Some animal models have demonstrated that heteroresistance might contribute to monotherapy treatment failure (Band et al., 2016, 2018). Our findings provided a potential polymyxin-based combination therapy, which held the promise to hamper the emergence of resistant subpopulations, and improved clinical outcomes in difficult to treat infections. The combination can also reduce the dose of both drugs. However, there might be some concerns about this combination therapy. Firstly, challenges in detection of polymyxin heteroresistance might set a barrier for clinicians to determine an appropriate time to start combination therapy. It is uncertain whether the combination therapy will still remain effective against heteroresistant strains after the

failure of polymyxin B monotherapy. Secondly, a suitable dose of polymyxin B in combination need to be reevaluated due to its unreliably plasma concentrations in monotherapy (Bergen et al., 2015). Lastly, it is important to keep a balance between the theoretical benefits of combination therapy and worries that antibiotic combination will increase the financial burden and potentially more toxic than monotherapy.

Our study also showed that the microdilution broth method might misclassify heteroresistant strains as susceptible, which was consistent with other reports (Band et al., 2018; Turlej-Rogacka et al., 2018; Ezadi et al., 2019). There is a possibility that the resistant subpopulation is at a low frequency so that the growth cannot be detected by conventional tests. Different from persistency, which confer antibiotic tolerance at the cost of growth (Brauner et al., 2016), the resistant subpopulation can rapidly replicate in the presence of antibiotic (El-Halfawy and Valvano, 2015; Band et al., 2016, 2018; Anderson et al., 2018). Therefore, expanding the time of incubation may be helpful to detect the heteroresistance. Interestingly, some reports

TABLE 1 | The change in log₁₀ CFU/ml at 4, 8, 12, and 48 h during time-kill experiments in combination with PMB and ceftazidime/avibactam^a.

Strains	MICs (mg/L)		FICI (n = 3)	Time (h)	Change in log ₁₀ CFU/ml									
	PMB	CZA			Control	Ceftazidime/avibactam at 0.25 × MIC plus PMB at (mg/L)			Ceftazidime/avibactam at 0.5 × MIC plus PMB at (mg/L)			Ceftazidime/avibactam at 1 × MIC plus PMB at (mg/L)		
						0.5	1	2	0.5	1	2	0.5	1	2
ATCC700603	0.5	0.5	0.46 ± 0.12	4	2.06	-6.13	-6.13	-6.13	-6.13	-6.13	-6.13	-6.13	-6.13	-6.13
				8	2.16	-4.16	-6.13	-6.13	-6.13	-6.13	-6.13	-6.13	-6.13	-6.13
				12	2.06	-3.03	-6.13	-6.13	-5.13	-6.13	-6.13	-6.13	-6.13	-6.13
				24	2.18	2.05	-6.13	-6.13	2.00	-6.13	-6.13	-6.13	-6.13	-6.13
B1	HR	8	0.38 ± 0.10	4	2.59	-3.03	-4.09	-3.93	-4.26	-5.74	-5.74	-3.34	-5.74	-5.74
				8	2.66	-1.88	-2.43	-2.69	-2.30	-5.74	-5.74	-5.74	-5.74	-5.74
				12	2.37	2.06	0.96	-2.23	-1.06	-5.74	-5.74	-5.74	-5.74	-5.74
				24	2.44	2.84	2.73	2.75	2.50	-5.74	-5.74	-5.74	-5.74	-5.74
D1	HR	4	0.79 ± 0.16	4	2.34	-2.55	-4.49	-6.04	-4.85	-6.04	-6.04	-6.04	-6.04	-6.04
				8	1.92	-1.84	-6.04	-4.56	-6.04	-6.04	-6.04	-6.04	-6.04	-6.04
				12	1.95	2.15	-3.39	-6.04	-3.20	-6.04	-6.04	-6.04	-6.04	-6.04
				24	2.15	2.07	2.18	-6.04	1.80	-6.04	-6.04	-6.04	-6.04	-6.04
D4	HR	2	0.35 ± 0.13	4	2.21	-2.71	-4.13	-4.44	-3.59	-6.18	-6.18	-6.18	-6.18	-4.88
				8	2.03	-1.93	-2.88	-4.18	-2.55	-6.18	-6.18	-6.18	-6.18	-6.18
				12	2.07	2.23	-2.27	-3.93	1.84	-6.18	-6.18	-6.18	-6.18	-6.18
				24	2.14	2.46	2.33	1.52	2.39	-6.18	-6.18	-6.18	-6.18	-6.18

^aPMB, polymyxin B; CZA, ceftazidime/avibactam; HR, heteroresistance. Bactericidal activity (≥ 3 log₁₀ CFU/ml reduction compared to the initial inoculum) is shown in bold. Additivity is defined as a reduction of between 1 and 2 log₁₀ CFU/ml and synergy is defined as a reduction of ≥ 2 log₁₀ CFU/ml caused by the combination compared to the results seen with the most active single agent in the combination. Additivity is highlighted with light gray shading and synergy with dark gray shading.

showed this increased resistance phenotype could revert from being entirely resistance to susceptible after removing antibiotic pressure (Band et al., 2016; Anderson et al., 2018). To our surprise, the resistant subpopulation in our study still dominated without selective pressure, even when the subculture time expanded. Later analysis demonstrated that several genetic changes might be responsible for it, involving the upregulation of PmrAB system and PhoPQ system and mutations. The changes in PmrAB system and PhoPQ system have been reported by other researches in different species (Jayol et al., 2015; Halaby et al., 2016; Charretier et al., 2018). Furthermore, *pmrA* G53R was detected in resistant cells of B1. The same mutation in *pmrA* have been described in colistin resistant *Enterobacter aerogenes*, *S. enterica* and *K. pneumoniae* (Sun et al., 2009; Diene et al., 2013; Olaitan et al., 2014). Charretier et al. (2018) revealed that the mutations in the PmrAB regulatory pathway in *Acinetobacter baumannii*, which resulted in the overexpression of PmrAB system, led to colistin heteroresistance. Alterations in the PhoPQ turned out to be related with colistin heteroresistance in *K. pneumoniae* (Jayol et al., 2015). Except for the PmrAB and PhoPQ systems, mutations in the *lpxM* and *yciM* genes also played roles in the emergence of colistin-resistant *K. pneumoniae* (Halaby et al., 2016). In our study, no mutations in above genes were detected, but there were several genetic variations in other genes were detected in the resistant subpopulations of both D1 and D4. The roles of these mutations remained unclear and also need further experiments to confirm.

Nevertheless, this study had limitations. The sample size is small and may not be suitable for other KPC-producing strains. Antibiotic concentrations are constant and may not accurately reflect the real pharmacokinetics of antibiotics in a clinical dose. Therefore, pharmacodynamic activity need to be evaluated. The investigation of molecular mechanisms related to heteroresistance in *K. pneumoniae* were preliminary and need further exploration.

CONCLUSION

In conclusion, our study provides evidence that the combination of ceftazidime/avibactam improved the antibacterial efficacy of polymyxin B against heteroresistant KPC-Kp and hindered the emergence of polymyxin resistant subpopulations. On top of that, an operational definition and uniform criteria for assessment of heteroresistant bacteria should be established to counteract heteroresistance.

ETHICS STATEMENT

This study was approved by the Institutional Review Board of Second Affiliated Hospital of Soochow University. This study was retrospective and patients were not identified during data collection. Informed consent was not needed for this study.

AUTHOR CONTRIBUTIONS

XM participated in the design of the study, performed the antibiotic susceptibility tests, PAP analyses, qRT-PCR and time-kill assays, interpreted the data, and drafted the manuscript. YH carried out the qRT-PCR assays and participated in data analysis. XY and YC participated in whole-genome sequencing. JZ and LC participated in *de novo* assembly and SNPs analysis. RC collected the clinical strains. YL, CC, and BH designed the study, participated in data analysis, and provided critical revisions of the manuscript for important intellectual content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.02029/full#supplementary-material>

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Hypoionic Shock Facilitates Aminoglycoside Killing of Both Nutrient Shift- and Starvation-Induced Bacterial Persister Cells by Rapidly Enhancing Aminoglycoside Uptake

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Bacterial persister cells are phenotypic variants that exhibit transient antibiotic tolerance and play a leading role in chronic infections and the development of antibiotic resistance. Determining the mechanism that underlies persister formation and developing anti-persister strategies, therefore, are clinically important goals. Here, we report that many gram-negative and gram-positive bacteria become highly tolerant to typical bactericidal antibiotics when the carbon source for their antibiotic-sensitive exponential growth phase is shifted to fumarate, suggesting a role for fumarate in persister induction. Nutrient shift-induced *Escherichia coli* but not *Staphylococcus aureus* persister cells can be killed by aminoglycosides upon hypoionic shock (i.e., the absence of ions), which is achieved by suspending the persisters in aminoglycoside-containing pure water for only 1 or 2 min. Such potentiation can be abolished by inhibitors of the electron transport chain (e.g., NaN₃) or proton motive force (e.g., CCCP). Additionally, we show that hypoionic shock facilitates the eradication of starvation-induced *E. coli* but not *S. aureus* persisters by aminoglycosides, and that such potentiation can be significantly suppressed by NaN₃ or CCCP. Mechanistically, hypoionic shock dramatically enhances aminoglycoside uptake by both nutrient shift- and starvation-induced *E. coli* persisters, whereas CCCP can diminish this uptake. Results of our study illustrate the general role of fumarate in bacterial persistence and may open new avenues for persister eradication and aminoglycoside use.

Keywords: persister, antibiotic tolerance, aminoglycoside, antibiotic uptake, fumarate, hypoionic shock

INTRODUCTION

Bacterial persistence is a state in which a sub-population of non-growing/slowly growing bacterial cells (i.e., persisters) resist killing by supra-lethal concentrations of bactericidal antibiotics (Balaban et al., 2004; Lewis, 2010). Persisters are distinct from antibiotic-resistant cells but genetically identical to their drug-susceptible kin, as their antibiotic tolerance is transient and non-inheritable (Keren et al., 2004; Lewis, 2007, 2010; Brauner et al., 2016). Because persisters have been implicated in chronic and recurrent infections (Lewis, 2010) and play a key role in the development of antibiotic resistance (Levin-Reisman et al., 2017), discovering the mechanism of persister formation and developing new strategies for persister eradication are important goals.

The formation of persisters has been attributed mainly to the entry of bacteria into a non-growing physiological state in which essential antibiotic targets are inactive and/or inaccessible to antibiotics. Genetic analyses reveal that many genes contribute to bacterial persistence (Hu and Coates, 2005; Spoering et al., 2006; Hansen et al., 2008; Lee et al., 2009; Lewis, 2010; Girgis et al., 2012; Ling et al., 2012; Shan et al., 2015; Kim et al., 2016). Well-studied components are toxin-antitoxin modules (Lewis, 2010; Germain et al., 2013; Maisonneuve et al., 2013), which produce toxins that halt cell growth and thus enable non-growing cells to tolerate antibiotics. Nevertheless, a recent study by Gerdes and colleagues raised the possibility that toxin-antitoxin modules are not involved in the formation of *Escherichia coli* persisters in unstressed conditions (Harms et al., 2017). Metabolic analyses indicate that some carbon sources are able to increase the tolerance of bacteria against one or multiple bactericidal antibiotics. For instance, Amato et al. (2013) found that diauxic shifts following exposure to fumarate or succinate can stimulate persister formation in exponential-phase *E. coli* cells (Amato and Brynildsen, 2014). Conversely, various metabolites such as glucose and mannitol may reverse the antibiotic tolerance of stationary-phase persister cells (Allison et al., 2011; Barraud et al., 2013; Meylan et al., 2017). The antibiotic tolerance of bacterial persisters appears to be tightly regulated by cellular respiration (Lobritz et al., 2015; Conlon et al., 2016; Meylan et al., 2017; Shan et al., 2017; Wang et al., 2018; Pu et al., 2019), which may affect both antibiotic uptake and downstream lethal actions of antibiotics (Lobritz et al., 2015; Meylan et al., 2017).

The use of existing antibiotics in a wiser manner, in addition to the discovery and/or development of new antibiotics, is a promising strategy for combating antibiotic-tolerant persisters (WHO, 2014; The Pew Charitable Trusts, 2016). Metabolite-stimulated aminoglycoside potentiation has been widely reported to eradicate different pathogenic persisters (Allison et al., 2011; Barraud et al., 2013; Peng et al., 2015; Meylan et al., 2017; Su et al., 2018). Iron chelators (Moreau-Marquis et al., 2009) and β -lactam aztreonam (Yu et al., 2012) were also found to potentiate the aminoglycoside tobramycin (Tom) to fight against *Pseudomonas aeruginosa* infections. Further, inhibitors of efflux pumps are potent drugs that suppress antibiotic efflux and thus increase the effective

intracellular concentrations of antibiotics (Mahamoud et al., 2007; Li and Nikaido, 2009). Other promising strategies for potentiating existing antibiotics have been reported, such as pH alternation (Lebeaux et al., 2014), the use of membrane-active macromolecules (Uppu et al., 2017), and osmotic perturbation (Falghouse et al., 2017).

To study the mechanisms underlying bacterial persistence and evaluate the efficacy of antibiotics in persister eradication, a few persister models have been established and exploited. One model involves type II persisters, also called spontaneous persisters (Balaban et al., 2019), which are formed stochastically in growing cultures (Balaban et al., 2004; Maisonneuve et al., 2013; Feng et al., 2014; Brauner et al., 2016). Another is based on starvation-induced persisters (Eng et al., 1991; Keren et al., 2004; Nguyen et al., 2005), as exemplified by those formed in stationary-phase cultures and requiring a long lag time to initiate regrowth after they are transferred to growth-favorable conditions (Balaban et al., 2004; Fridman et al., 2014; Brauner et al., 2016). A third model with nutrient shift-induced persisters, which are non-growing but metabolically active cells, was proposed recently (Amato et al., 2013; Amato and Brynildsen, 2014; Kim et al., 2016; Radzikowski et al., 2016). In addition, genetically modified and environmentally stressed bacteria with high antibiotic tolerance have been explored in mechanistic studies of bacterial persistence (Xiong et al., 1996; Christensen et al., 2001; Hong et al., 2012; Wu et al., 2012; Feng et al., 2014). These environmental factor-stimulated persisters can all be defined as triggered persisters (Balaban et al., 2019).

We recently reported that hypoionic shock (i.e., shock with an ion-free solution) can markedly potentiate aminoglycosides to kill stationary-phase *E. coli* persister cells (Jiafeng et al., 2015). We sought to expand upon our finding by examining the efficacy of this unique strategy in eradicating other persisters. Here, we report that hypoionic shock can dramatically enhance the bactericidal action of aminoglycoside antibiotics against both nutrient shift- and starvation-induced *E. coli* persisters by 2–6 orders of magnitude. This is achieved by enhancing antibiotic uptake and is apparently dependent on cellular respiration. Our work suggests potential strategies for persister eradication.

MATERIALS AND METHODS

Strains, Medium and Reagents

Various Gram-negative (*E. coli*, *P. aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Shigella flexneri*, *Salmonella typhimurium*, and *Aeromonas hydrophila*) and Gram-positive (*S. aureus*, *Bacillus subtilis*, *Bacillus thuringiensis*, and *Staphylococcus epidermidis*) bacterial strains were used in this study and their characteristics are described in **Supplementary Table S1**. For normal cell culturing, three mediums were used: M9 medium plus 5 g/L glucose, Luria-Bertani (LB) medium, or Mueller-Hinton broth. M9 medium with and without 2 g/L fumarate were used for nutrient shift- and starvation-induced *E. coli* persister formation, respectively. Yeast nitrogen broth medium was used for starvation-induced persister

formation in *S. aureus*. Antibiotics used in this study include tobramycin, streptomycin, gentamicin, kanamycin, ampicillin, ofloxacin, with their manufacturers and final concentrations for different treatments being described in **Supplementary Table S2**. Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) and its analog FCCP (carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone) were purchased from Sigma-Aldrich. All other chemical reagents are of analytical purity.

Antibiotic Tolerance Test for Nutrient Shift- or Starvation-Induced Persisters

Nutrient shift-induced persisters were prepared as previously reported (Radzikowski et al., 2016). In brief, over-night cultures of each bacterial strain were diluted at 1:100 into M9 medium plus 5 g/L glucose or LB medium (37 °C, 220 rpm) and cultured to mid-exponential phase at a cell density of $OD_{600} = 0.5$ – 0.6 . Cells were centrifuged and washed with M9 medium twice before transferred to M9 medium plus 2 g/L fumarate and agitated for 4 h before antibiotic tolerance test. Starvation-induced persisters were prepared as previously reported (Eng et al., 1991). Briefly, *E. coli* and *S. aureus* cells were diluted at 1:500 in Mueller-Hinton broth medium and cultured for 24 h (35 °C, 220 rpm) to a cell density of around 10^9 CFU/mL. Cells were centrifuged, re-suspended in M9 medium (for *E. coli*) and in yeast nitrogen broth medium without amino acids (for *S. aureus*) by dilution to a cell density of around 10^8 CFU/mL and agitated for 5 h. Antibiotic tolerance test was performed by adding each antibiotic at concentrations as described in **Supplementary Table S2** and further agitated the cells for 2 or 3 h. Antibiotic-treated cells were washed twice using phosphate-buffered saline (PBS; 0.27 g/L KH_2PO_4 , 1.42 g/L Na_2HPO_4 , 8 g/L NaCl, 0.2 g/L KCl, pH 7.4) by centrifugation (13000 g, 30 s), and then 5 μ L of tenfold serially diluted cell suspension were spot plated onto LB agar dishes for survival assay. The antibiotic sensitivity of each bacterium was evaluated by incubating the exponential-phase cell culture with ampicillin (100 μ g/mL), tobramycin (50 μ g/mL) or ofloxacin (5 μ g/mL) for 2 h before bacterial survival assay.

Aminoglycoside Potentiation by Hypoionic Shock Against Persisters

Nutrient shift- or starvation-induced persister cells were prepared as described above and hypoionic shock was performed as we previously reported (Jiafeng et al., 2015). Briefly, 100 μ L cell cultures were centrifuged (13000 rpm, 1 min) in Eppendorf tube, with the supernatant being fully removed. The cells were then subjected to hypoionic shock treatment by re-suspending the pellet with pure water (i.e., without the presence of ions; a negative control was set using 0.9% NaCl solution) containing aminoglycoside antibiotic at concentrations as described in **Supplementary Table S2**. Cell suspension was kept at 25°C for 3 min before washing twice with PBS before subsequent cell survival assay as described above. The effect of proton motive force (PMF) and electron transport was examined by agitating the cell culture in the presence of 20 μ M protonophore

CCCP or FCCP, 2,4-Dinitrophenol (DNP; 20 μ g/mL), rotenone (5 μ g/mL) or NaN_3 (200 μ g/mL) for 1 h before hypoionic shock treatment.

Aminoglycoside Uptake Assay

Tobramycin (gentamicin, kanamycin or streptomycin) extraction coupled with cell growth inhibition was explored for antibiotic uptake assay as follows. Briefly, 1 ml persister cells, after hypoionic shock treatment in the presence of each antibiotic at concentrations as described in **Supplementary Table S2**, were washed twice with PBS and re-suspended in 100 μ L cell wall-digestion buffer (30 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mg/mL lysozyme) for further incubation at room temperature for 2 h. Cells were subjected to three cycles of freezing treatment at -80°C , thermally denatured at 90°C for 10 min (Note: the bactericidal activity of each aminoglycoside after heating at 90°C for 15 min was verified to be almost fully retained; refer to **Supplementary Figures S5A, S6A**) and centrifuged for removing cell debris and denatured proteins. Afterward, 5 μ L supernatant was spotted on *E. coli*-seeded LB agar dish for further incubation at 37°C for 8–10 h and the diameter of cell growth inhibition zone was measured. In addition, tobramycin or gentamicin uptake by CCCP or FCCP pre-treated persister cells was measured similarly. A standard curve was prepared by directly adding each aminoglycoside at different concentrations (0, 15, 25, 50, 75, and 100 μ g/mL) into persister cell suspension with the cell-wall digestion buffer. The tobramycin uptake by *S. aureus* cells was measured using the same procedure except of applying a different cell wall-digestion buffer (30 mM Tris-HCl, pH 8.0) plus 20 μ g/mL lysostaphin [purchased from Sangon Biotech (Shanghai) Co., Ltd.; Cat no.: A609001].

Intracellular ATP Level Assay

A luciferase-based kit (Beyotime Biotechnology, Shanghai, China; S0026B) was used to measure ATP level according to the manufacturer's instruction. Briefly, *E. coli* persister cells, with or without pretreatment of 20 μ M CCCP for 1 h, was lysed using the lysis buffer and centrifuged (12000 g, 4°C , 5 min). The supernatant was quickly mixed with the working solution at equal volumes and then transferred into a 96-well plate before light recording on a FLUOstar Omega Microplate Reader using the Luminometer method.

Proton Motive Force Assay

A flow cytometry-based assay was applied to measure the PMF by using the fluorescence probe 3,3'-Diethyloxycarbocyanine Iodide [DiOC2(3); purchased from MaoKang Biotechnology, Inc., Shanghai, China] according to the manufacturer's instruction. Briefly, *E. coli* persisters, with or without CCCP pretreatment as described above, were diluted into PBS to a cell density of 10^6 cells/mL and incubated with DiOC2(3) (at a final concentration of 30 μ M) at room temperature for 30 min. Cells were subjected to flow cytometric analysis on FACSymphonyTMA5 (BD Biosciences) with an excitation at 488 nm and emission at both red and green channels.

RESULTS

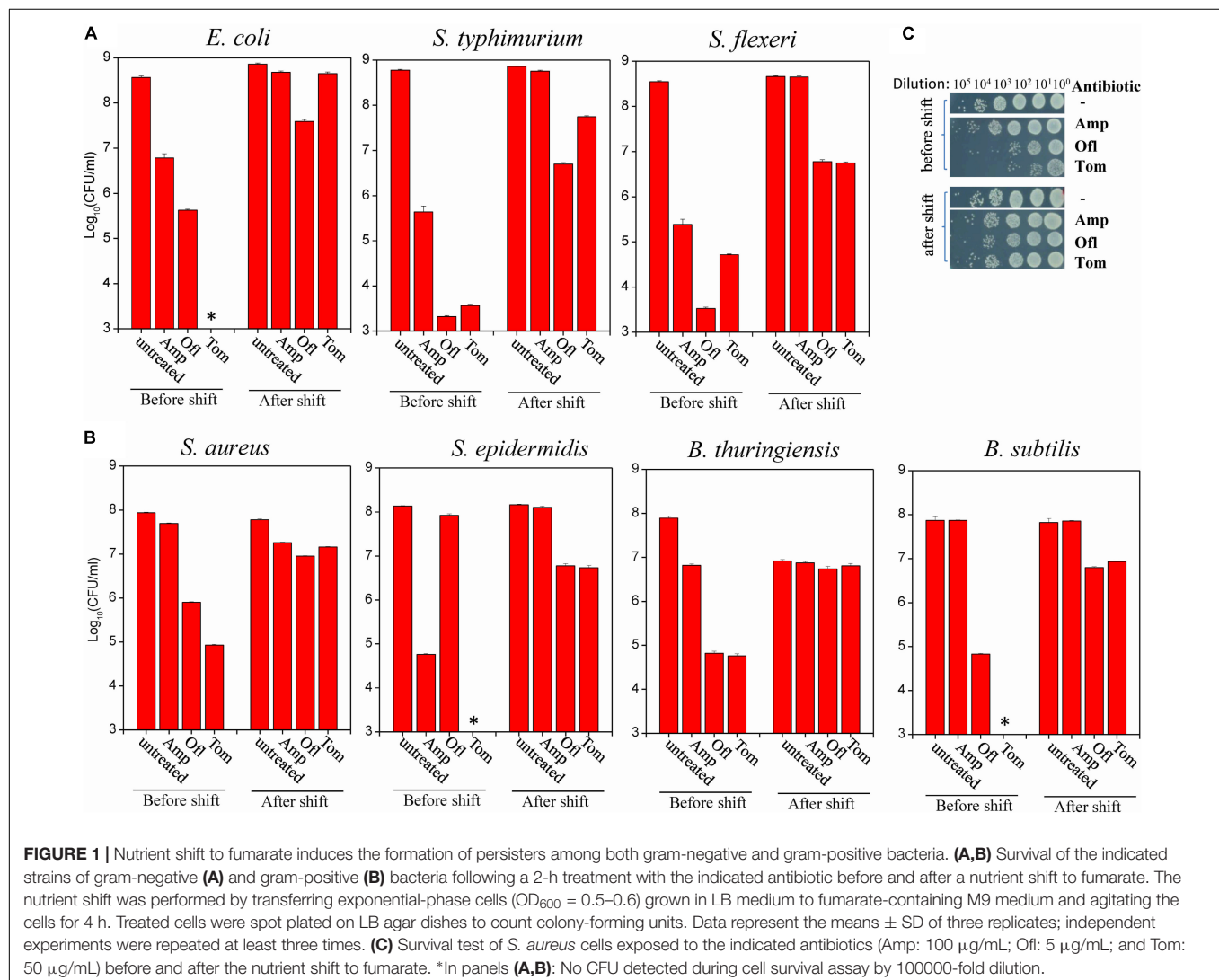
A Shift to Fumarate as a Carbon Source for Exponential-Phase Cells Induces Persister Formation in Many Strains of Gram-Negative and Gram-Positive Bacteria

A carbon source shift from glucose to fumarate was recently reported to induce the formation of *E. coli* persister cells (Kim et al., 2016; Radzikowski et al., 2016). Here, we examined whether such a nutrient shift from glucose to fumarate could induce persister formation in other bacterial strains, including both gram-negative and gram-positive pathogens, and, if so, whether an aminoglycoside coupled with hypoionic shock could kill those persisters.

Of the seven gram-negative bacterial strains (refer to **Supplementary Table S1**), we found that the tolerance of *S. typhimurium*, *S. flexneri*, and *E. coli* to typical bactericidal

antibiotics (ampicillin [Amp], ofloxacin [Of], and Tom) were significantly increased by different degrees after antibiotic-sensitive exponential-phase cells grown in LB medium were transferred to fumarate-containing M9 medium 4 h prior to antibiotic treatment (**Figure 1A**). Meanwhile, cell densities were largely held constant before and after the nutrient shift (as indicated in the “untreated” column in **Figure 1A**), i.e., exponential-phase cells in a growing state were switched to a non/slowly growing state, which is a prerequisite for persister formation. *A. baumannii* Ab6 and *K. pneumonia* KP-D367 were not tested because of their antibiotic resistance (**Supplementary Figures S1A,B**). Both *A. hydrophila* and *P. aeruginosa* PAO1, despite tolerance to antibiotics after culture in fumarate-containing M9 medium for 4 h (**Supplementary Figures S1C,D**), were able to grow substantially (refer to the colony density in the red frames of figures). These strains were not used in further studies because of this growth.

Of the four gram-positive bacterial strains (refer to **Supplementary Table S1**), we found that the antibiotic tolerance



of *S. aureus*, *S. epidermidis*, *B. thuringiensis*, and *B. Subtilis* to Amp, Ofi, and Tom were all increased after the nutrient shift to fumarate (Figure 1B; for *S. aureus*, also refer to Figure 1C). Meanwhile, their cell densities were largely held constant (as indicated in the “untreated” column in Figure 1B).

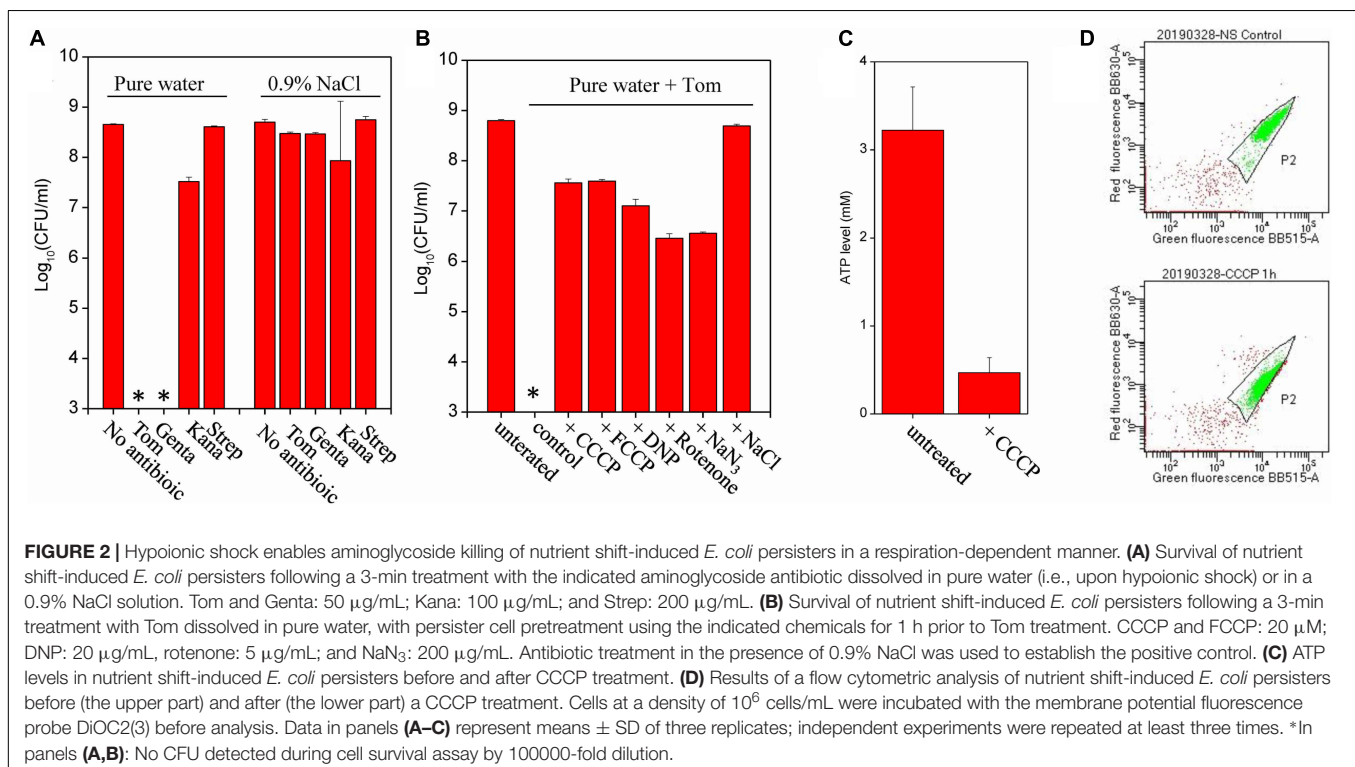
Hypoionic Shock Facilitates Aminoglycoside Killing of Nutrient Shift-Induced *E. coli* Persisters in a Respiration-Dependent Manner

Then, prompted by our earlier observations on stationary-phase *E. coli* persisters (Jiafeng et al., 2015), we examined whether hypoionic shock could facilitate eradication of nutrient shift-induced persisters by aminoglycoside antibiotics. For this purpose, fumarate-induced *E. coli* and *S. aureus* persisters (representing gram-negative and gram-positive bacteria, respectively) and four aminoglycoside antibiotics (Tom, gentamicin [Genta], kanamycin [Kana], and streptomycin [Strep]) were tested.

Cell survival assay revealed that *E. coli* persisters could be killed by Tom- or Genta-containing pure water after the cells were resuspended in the solution and incubated for only 3 min (Figure 2A). By contrast, Tom and Genta had little effect if they were dissolved in 0.9% NaCl solution (right panel, Figure 2A), which is consistent with our early observation that the presence of ions abolished aminoglycoside potentiation (Jiafeng et al., 2015). Notably, such nutrient shift-induced *E. coli* persisters were not killed by Kana and Strep upon hypoionic shock (Figure 2A), whereas cells

before the nutrient shift were killed (Supplementary Figure S2A). Time-dependent analysis revealed that the minimal time for hypoionic shock-enabled eradication of *E. coli* persisters was approximately 2 min (Supplementary Figure S2B). In contrast, fumarate-induced *S. aureus* persisters were not killed by combined treatment with aminoglycoside antibiotics and hypoionic shock (upper panel, Supplementary Figure S2C), although the same treatment enabled Tom to kill normally growing exponential-phase *S. aureus* cells [lower panel, Supplementary Figure S2C, and as reported earlier (Jiafeng et al., 2015)].

We sought to examine whether CCCP, an uncoupler of the proton gradient, could suppress hypoionic shock-potentiated aminoglycoside killing of persisters, given that the bacterial uptake of aminoglycosides requires a PMF across cytoplasmic membranes of bacteria [reviewed in Taber et al. (1987)]. For this purpose, we pretreated fumarate-induced *E. coli* persisters with CCCP for 1 h and then subjected the cells to combined treatment with Tom and hypoionic shock. Cell survival assay revealed that CCCP, as well as its functional analogs FCCP and DNP, efficiently suppressed hypoionic shock-induced Tom potentiation that could kill *E. coli* persisters (Figure 2B). We confirmed that such CCCP pretreatment decreased intracellular ATP levels (Figure 2C) and also the PMF (Figure 2D) in *E. coli* persisters, as monitored by luciferase assay and membrane potential probe-based flow cytometric analysis, respectively. In line with the results from CCCP pretreatment, rotenone and NaN_3 , two electron transport inhibitors that inhibit the transfer of electrons from iron-sulfur centers in complex I to ubiquinone and cytochrome c oxidase, respectively, were



found to significantly suppress hypoionic shock-induced Tom potentiation (**Figure 2B**). Similarly, we found that all of these uncouplers or inhibitors significantly suppressed hypoionic shock-induced Genta potentiation that could kill nutrient shift-induced *E. coli* persisters (**Supplementary Figure S2D**).

Hypoionic Shock Facilitates Aminoglycoside Killing of Starvation-Induced Persisters in a Respiration-Dependent Manner

We next examined whether hypoionic shock could facilitate aminoglycoside antibiotic killing of other persisters. Experimentally, we adopted the starvation-induced persister model described in an earlier report (Eng et al., 1991), in which stationary-phase *E. coli* and *S. aureus* cells were centrifuged and resuspended by 10-fold dilution in new medium without any nutrients, thus ruling out effects of the old medium and high cell density on antibiotic killing (Gutierrez et al., 2017).

First, we evaluated the antibiotic tolerance of starvation-adapted *E. coli* and *S. aureus* stationary-phase cells by agitating them in the presence of each aminoglycoside antibiotic for 3 h. Cell survival assay revealed that starvation adaptation caused *E. coli* to be highly tolerant to Kana and Strep and moderately tolerant to Tom and Genta (**Supplementary Figure S3A**) and *S. aureus* to be highly tolerant to Genta/Kana/Strep and moderately tolerant to Tom (**Supplementary Figure S3B**), results that are in line with those presented in an earlier report (Eng et al., 1991).

Next, we determined the efficacy of each aminoglycoside antibiotic coupled with hypoionic shock in killing

starvation-induced persister cells. Cell survival assay revealed that starvation-induced *E. coli* persisters were killed by each aminoglycoside antibiotic upon hypoionic shock (**Figure 3A**), with this efficacy lost in the presence of 0.9% NaCl. Time-dependent analysis revealed that the cell survival ratio was constant with combined treatment from 1 min to 5 min (**Supplementary Figure S3C**), indicating that the effect of hypoionic shock on aminoglycoside antibiotics occurs as early as 1 min. Again, starvation-induced *S. aureus* persisters showed little killing after the combined treatment (**Figure 3B** and upper panel, **Supplementary Figure S3D**). However, stationary-phase *S. aureus* cells before starvation adaptation were killed by each aminoglycoside antibiotic upon hypoionic shock (lower panel, **Supplementary Figure S3D**).

We further examined the effect of proton gradient uncouplers (CCCP, FCCP, and DNP) and electron transport inhibitors (rotenone and NaN₃) on hypoionic shock-induced aminoglycoside potentiation that could kill starvation-induced *E. coli* persisters. We found that both CCCP and FCCP abolished Tom potentiation by hypoionic shock and that rotenone and NaN₃ exhibited a smaller but still significant suppressive effect (**Figure 3C**). We confirmed that CCCP pretreatment reduced intracellular ATP levels in *E. coli* persisters (**Figure 3D**) but had no significant effect on the PMF (**Supplementary Figure S4**), presumably because the basal PMF in starvation-induced *E. coli* persisters is quite low, as reported previously (Allison et al., 2011). Intriguingly, DNP showed a weak suppressive effect in this assay. Similarly, we found that all of these uncouplers or inhibitors significantly suppressed hypoionic shock-induced Genta potentiation for starvation-induced *E. coli* persister cell killing (**Supplementary Figure S3E**).

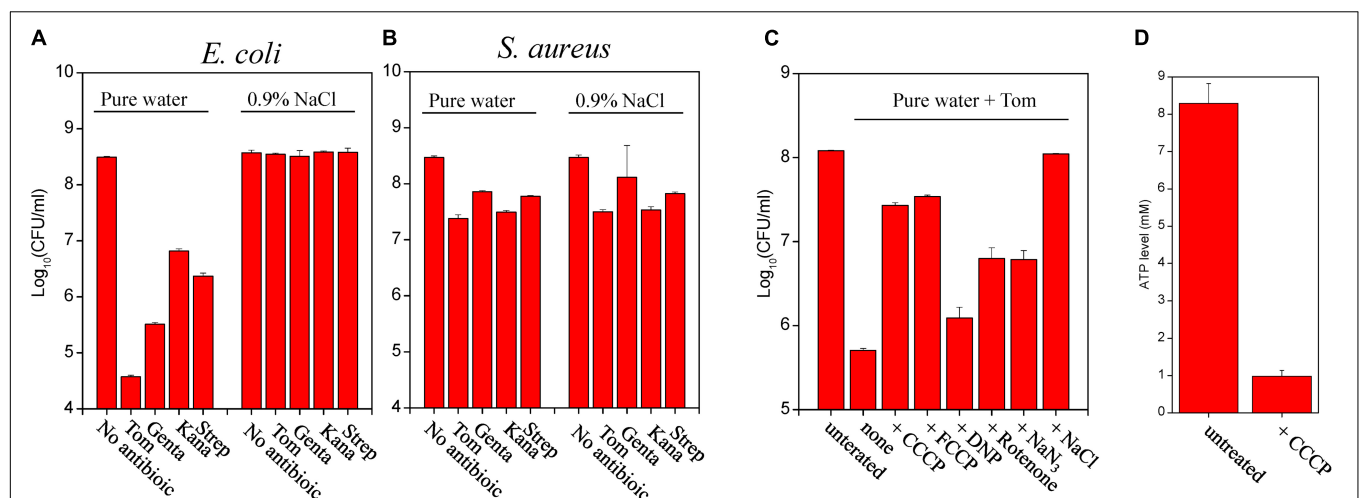


FIGURE 3 | Starvation induces formation of *E. coli* stationary-phase persister cells that can be eradicated by aminoglycosides upon hypoionic shock. **(A,B)** Survival of starvation-induced *E. coli* **(A)** and *S. aureus* **(B)** persisters following a 3-min treatment with the indicated aminoglycoside antibiotics dissolved in pure water (i.e., cells in hypoionic shock) or in a 0.9% NaCl solution. *E. coli* and *S. aureus* stationary-phase cells grown in MHB medium were resuspended in M9 medium and YNB medium (without amino acids), respectively, at a final cell density of 10⁵ CFU/mL and agitated for 5 h prior to antibiotic treatment. Tom and Genta: 50 μg/mL; Kana: 100 μg/mL; and Strep: 200 μg/mL. **(C)** Survival of starvation-induced *E. coli* persisters following a 3-min treatment with Tom dissolved in pure water. Cells were pretreated using the same experimental conditions described in **Figure 2B**. **(D)** ATP levels in nutrient shift-induced *E. coli* persisters before and after CCCP treatment. Data in Panels **(A–D)** represent means ± SD of three replicates; independent experiments were repeated at least three times.

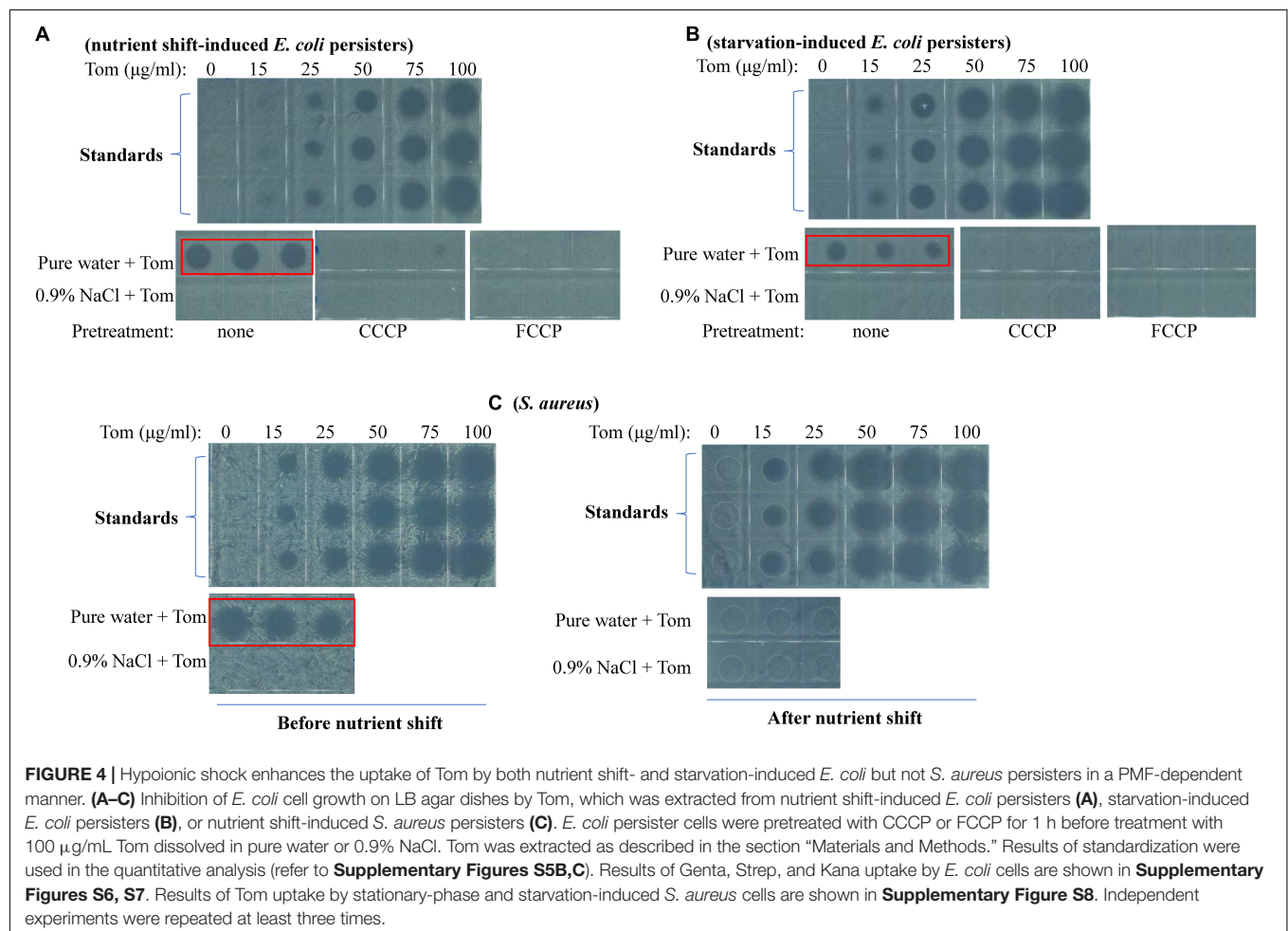
Aminoglycoside Potentiation Upon Hypoionic Shock Is Achieved via Enhancement of Aminoglycoside Uptake by Both Nutrient Shift- and Starvation-Induced *E. coli* Persisters

In view of the fact that pretreatment with CCCP or FCCP can abolish potentiation (Figures 2B, 3C) and that aminoglycoside uptake is dependent on a PMF [reviewed in Taber et al. (1987)], we hypothesized that hypoionic shock-induced aminoglycoside potentiation is accomplished by enhancing bacterial uptake of antibiotics. Taking advantage of the high thermal stability of Tom (Supplementary Figure S5A) and the irreversible nature of aminoglycoside uptake by *E. coli* cells (Nichols and Young, 1985), we explored a protocol to measure the bacterial uptake of Tom. To this end, Tom taken up by *E. coli* cells was extracted by cell wall digestion coupled with cycled freezing/thawing and thermal denaturation, and then the relative level of Tom in the lysate was measured by bacterial cell growth inhibition assay (for details, refer to the section “Materials and Methods”).

Cell growth inhibition assay revealed that Tom extracted from nutrient shift-induced *E. coli* persisters upon hypoionic shock significantly suppressed bacterial cell growth on LB agar

plates (red frame, Figure 4A). In contrast, no significant cell growth inhibition was observed if Tom was extracted from the persister cells not in hypoionic shock or pretreated with CCCP or FCCP (Figure 4A). A regression analysis (Supplementary Figure S5B) based on standards (upper panel, Figure 4A) showed that the concentration of Tom extracted from nutrient shift-induced *E. coli* persister cells in hypoionic shock was up to 57 ± 4 $\mu\text{g/mL}$, whereas that extracted from the cells treated in NaCl-containing solution or pre-treated with CCCP was less than 15 $\mu\text{g/mL}$. Similarly, whereas hypoionic shock dramatically enhanced the uptake of Tom by starvation-induced *E. coli* persister cells (red frame, Figure 4B), this enhancement was diminished by the presence of 0.9% NaCl, CCCP, or FCCP pretreatment.

We also examined the bacterial uptake of Genta, Strep, and Kana during hypoionic shock. First, we verified the high thermal stability of these aminoglycosides (Supplementary Figure S6A). Then, we extracted each and measured their inhibitory effects on cell growth. Data presented in Supplementary Figure S6B revealed that Genta extracted from nutrient shift-induced *E. coli* persisters in hypoionic shock dramatically suppressed bacterial cell growth on LB agar plates (as indicated by the red frame). In contrast, the extracted Kana only slightly inhibited cell growth (Supplementary Figure S6C), and the extracted Strep hardly



showed any inhibitory effect (**Supplementary Figure S6D**). These results agreed with the weak bactericidal actions of these antibiotics in persister cells (**Figure 2A** and **Supplementary Figure S2A**, respectively). Similarly, Genta extracted from starvation-induced *E. coli* persisters significantly suppressed cell growth (**Supplementary Figure S7A**), whereas extracted Kana and Strep exhibited hardly any inhibitory effects (**Supplementary Figures S7B,C**). These results were in accordance with the strong bactericidal action of Genta and relatively weak killing action of Kana and Strep (**Figure 3A**).

Hypoionic Shock Enhances Tom Uptake by Both Exponential- and Stationary-Phase *S. aureus* Cells but Not by Nutrient Shift- and Starvation-Induced *S. aureus* Persisters

An intriguing observation in our study is that neither nutrient shift- nor starvation-induced *S. aureus* persisters were sensitive to hypoionic shock-induced Tom potentiation, whereas exponential- and stationary-phase *S. aureus* cells were sensitive (**Supplementary Figures S2C, S3D**). To clarify the reason for this, we measured Tom uptake by these different *S. aureus* cells. We observed that Tom extracted from exponential-phase *S. aureus* cells (i.e., cells obtained before the nutrient shift) dramatically inhibited cell growth on LB agar plates (red frame, left panel, **Figure 4C**), whereas Tom extracted from nutrient shift-induced *S. aureus* persister cells showed little inhibitory effect (right panel, **Figure 4C**). Similarly, we observed significant cell growth inhibition by Tom extracted from stationary-phase *S. aureus* cells (i.e., cells before starvation induction) (red frame, **Supplementary Figure S8A**), but not by Tom from starvation-induced *S. aureus* persister cells (**Supplementary Figure S8B**). These results suggest that the insensitivity of both types of *S. aureus* persisters to hypoionic shock-induced Tom potentiation is most likely due to the failure of hypoionic shock to enhance bacterial uptake of Tom.

DISCUSSION

This work resulted in several notable findings. First, we showed the general role of fumarate in inducing persisters among gram-negative and gram-positive bacteria, including those of many pathogens (**Figure 1**). Second, we found that hypoionic shock facilitated aminoglycoside antibiotic eradication of not only fumarate-induced (i.e., nutrient shift-induced) *E. coli* persisters but also starvation-induced *E. coli* persisters (**Figures 2, 3**). Importantly, we showed that hypoionic shock-induced aminoglycoside potentiation was achieved by enhancing aminoglycoside uptake and that this potentiation could be abolished by proton gradient uncouplers (**Figure 4**). In addition, we observed distinct activities of aminoglycoside antibiotics against cells with different growth statuses in hypoionic shock (**Figures 2A, 3A** and **Supplementary Figures S2A,C, S3D**). These findings advance our understanding of persister formation and may

open avenues to the development of new anti-persister antibiotic strategies.

Hypoionic Shock Potentiates Aminoglycosides to Kill Bacterial Persisters by Enhancing Aminoglycoside Uptake in a Respiration-Dependent Manner

We recently reported that hypoionic shock enabled aminoglycosides to kill stationary-phase *E. coli* persisters (Jiafeng et al., 2015). Here, we found that aminoglycoside antibiotics exhibited different actions against *E. coli* and *S. aureus* cells with different growth statuses upon hypoionic shock. These actions can be summarized as follows. (1) Kana and Strep eradicated exponential-phase *E. coli* cells but not nutrient-shifted *E. coli* cells (**Supplementary Figure S2A**), whereas Tom and Genta killed both (**Figure 2A**); (2) Tom killed exponential-phase *S. aureus* cells but not nutrient-shifted *S. aureus* cells, whereas the other three aminoglycoside antibiotics had little effect on either of these cell types (**Supplementary Figure S2C**); and (3) each aminoglycoside antibiotic eradicated stationary-phase *S. aureus* cells but not starvation-induced *S. aureus* cells (**Supplementary Figure S3D**). Notably, these distinct bactericidal actions of aminoglycoside antibiotics induced by hypoionic shock (**Figures 2A, 3A,B** and **Supplementary Figures S2A,C, S3D**) agreed well with the amount of aminoglycoside taken up by persister cells (**Figure 4** and **Supplementary Figures S6–S8**), which strongly suggests that hypoionic shock-induced aminoglycoside potentiation is achieved by enhancing aminoglycoside uptake.

Hypoionic shock-induced aminoglycoside potentiation that can kill *E. coli* persisters appears to depend on the cellular respiration of bacteria based on the following evidence. First, it is well-known that aminoglycoside uptake depends on a PMF, which is generated through respiration (Taber et al., 1987). Second, recent studies have suggested that the downstream lethal action of aminoglycosides depends on the respiration of bacterial cells (Lobritz et al., 2015; Meylan et al., 2017). In our study, CCCP or FCCP alone was able to abolish hypoionic shock-induced aminoglycoside potentiation (**Figures 2B, 3C** and **Supplementary Figures S2D, S3E**) and uptake (**Figure 4** and **Supplementary Figures S6B, S7A**). In addition, sodium azide and rotenone (inhibitors of the electron transport chain) significantly suppressed such potentiation (**Figures 2B, 3C** and **Supplementary Figures S2D, S3E**). These observations indicate that hypoionic shock, although lasting for only a couple of minutes, dramatically enhances bacterial uptake of aminoglycosides in a respiration-dependent manner.

Based on these observations, we hypothesize that certain channels on the cytoplasmic membrane of bacterial cells may be responsible for hypoionic shock-induced aminoglycoside potentiation. These channels can be activated for aminoglycoside uptake in response to hypoionic shock and may exhibit selectivity in transporting structurally different aminoglycosides, as demonstrated by the potentiation of some aminoglycosides and not others in this study. In addition, the protein level

and/or transportation activity of these channels could be tightly regulated by growth conditions; therefore, they are functionally dependent on cellular respiration and physiological status. As such, an aminoglycoside (e.g., Tom) coupled with hypoionic shock can kill exponential-phase *S. aureus* cells but not these same cells after a nutrient shift to fumarate (**Supplementary Figure S2C**). In addition, it should be pointed out that ribosome is still the acting target of aminoglycoside during such hypoionic shock as revealed in our earlier study using the streptomycin-resistant *E. coli* strain MC4100 (Jiafeng et al., 2015).

Clinical Potential of Hypoionic Shock-Induced Aminoglycoside Potentiation to Eradicate Persisters

Considering that the clinical application of aminoglycosides has dropped substantially in recent decades due to their toxicity and the rise of antibiotic resistance (Mingeot-Leclercq and Tulkens, 1999; Mingeot-Leclercq et al., 1999), improving the efficacy of aminoglycosides by hypoionic shock while limiting their side effects would be a clinically valuable approach. An antibiotic potentiation strategy would entail exposing subjects to aminoglycosides for only 1 or 2 min, therefore reducing the toxicity associated with aminoglycosides use. Nevertheless, this approach cannot be directly applied to curing infections in animals and humans, largely because of the ubiquity of ions throughout the animal body (e.g., Na^+ , K^+ , Cl^- , and charged amino acids) that could abolish the potentiation effect (Jiafeng et al., 2015). If the biochemical mechanism underlying hypoionic shock-induced aminoglycoside potentiation can be discovered (e.g., if the membrane channels for aminoglycoside uptake during hypoionic shock can be identified and fully characterized), however, this would be helpful for developing new anti-persister strategies that are based on the mechanism rather than on hypoionic shock. Studies to identify this mechanism are currently underway in our laboratory.

Metabolite-stimulated aminoglycoside potentiation has recently been shown to kill stationary-phase *E. coli*, *P. aeruginosa*, and *Edwardsiella tarda* persister cells (Allison et al., 2011; Barraud et al., 2013; Peng et al., 2015; Meylan et al., 2017; Su et al., 2018). This approach has even been validated for eradication of persisters in animal models (Allison et al., 2011; Peng et al., 2015). Apparently, metabolic stimulation dramatically changes the physiological states of persister cells, boosting their respiration and reprogramming their metabolic network. It follows that persister cells might regrow during a lengthy period of metabolic stimulation (usually a couple of hours), and, if this occurs, cell regrowth would reduce the benefits of aminoglycoside potentiation to kill persisters. In comparison, our method of hypoionic shock-induced aminoglycoside potentiation requires only 1 or 2 min of stimulation [**Supplementary Figures S2B, S3C**; or refer to our earlier report (Jiafeng et al., 2015)]. Another advantage is that hypoionic shock enables aminoglycosides to eradicate normally growing bacterial cells [**Supplementary Figures S2A,C**; or refer to our earlier report (Jiafeng et al., 2015)].

A General Role for Fumarate in Bacterial Persistence

Nutrient shift-stimulated bacterial persistence has been widely reported for *E. coli* cells that are grown in a batch culture containing two carbon sources and exhibiting diauxic growth phases (Amato et al., 2013; Amato and Brynildsen, 2014, 2015; Kotte et al., 2014). Among the carbon sources, fumarate is highly potent in increasing the formation of persisters in exponential-phase *E. coli* cells (Amato et al., 2013; Amato and Brynildsen, 2014, 2015; Kim et al., 2016; Radzikowski et al., 2016). Nevertheless, fumarate was found to impair persister formation in *P. aeruginosa* stationary-phase cells exposed to Tom by activating cellular respiration and generating a PMF through stimulation of the tricarboxylic acid (TCA) cycle (Meylan et al., 2017). These actions might be linked to the role of fumarate as a metabolite of the TCA cycle and/or as an electron acceptor (Unden et al., 2014, 2016).

Here, we have shown that a nutrient shift to fumarate is able to increase persister formation in exponential-phase gram-negative (as represented by *E. coli*, *S. typhimurium*, and *S. flexneri*) and gram-positive (as represented by *S. aureus*, *S. epidermidis*, *B. thuringiensis*, and *B. subtilis*) bacterial cells. According to the recent definition by Balaban et al. (2019), these fumarate-induced persisters should be considered type I/triggered persisters. On the other hand, the concentration of intracellular fumarate was shown to be proportional to the frequency of persisters among exponential-phase *E. coli* cells (Kim et al., 2016), illustrating its critical role in the formation of type II/spontaneous persisters [according to the definition in Balaban et al. (2019)]. Based on our findings, we propose that intracellular fumarate may converge on both external and intrinsic signals in bacterial cells and together, these signals determine persister formation, conceivably by finely tuning the electron transport chain and/or TCA cycle (Unden et al., 2014, 2016).

CONCLUSION

In summary, hypoionic shock appears to facilitate aminoglycoside antibiotic killing of various types of *E. coli* persister cells, including those that are induced by nutrient shifts and starvation, as observed here, and those in the stationary phase as we previously reported (Jiafeng et al., 2015). Such aminoglycoside potentiation by hypoionic shock is most likely achieved by rapid enhancement of aminoglycoside uptake, but the precise mechanism is unknown and merits further exploration. In addition, we found that fumarate induces persisters among both gram-negative and gram-positive bacteria. Outstanding questions to be investigated include why only certain types of aminoglycoside antibiotics can be potentiated to kill *E. coli* persisters with hypoionic shock, why the sensitivity of *S. aureus* cells before and after a nutrient shift (or starvation adaption) is different in response to hypoionic shock-induced aminoglycoside potentiation, and why fumarate is able to decrease the tobramycin sensitivity of *P. aeruginosa* cells in exponential-phase growth (**Supplementary Figure S1C**).

but increases their sensitivity in the stationary phase (Meylan et al., 2017).

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

XF and YG designed the research. ZC, BL, FS, and WY performed the research. YW managed the project. XF, ZC, and YG analyzed the data. XF wrote the manuscript. BL, FS, and WY helped ZC to perform the research in the **Figure 4C** and **Supplementary Figures S2D, S3E, S8A,B**.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.02028/full#supplementary-material>

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Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* From Animals and Animal Products in the UK

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Livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) is an emerging problem in many parts of the world. Although animal-adapted LA-MRSA has been known for many years, recent reports suggest a possible increasing trend in the zoonotic transmission of LA-MRSA in Europe. Since its emergence in the early 2000's, several investigations have indicated that persons in prolonged, repeated contact with affected livestock are at a higher risk of becoming colonized with LA-MRSA. LA-MRSA monitoring in livestock is voluntary under current EU legislation, and not all member states, including the UK, participate. UK LA-MRSA isolates have been detected through scanning surveillance, where samples are submitted from clinically diseased livestock for diagnostic investigation, and research studies. Surveys conducted on retail beef, pig and poultry meat on sale in the UK have also detected LA-MRSA. Taken together these results suggest that LA-MRSA is present in the UK, possibly at low prevalence level, as suggested by available evidence. In this review, we examine the data available from UK livestock and animal products, and make recommendations for future. We also review the findings from whole genome sequencing (WGS) of the possible lineage of some UK livestock isolates.

Keywords: MRSA, United Kingdom, livestock, animal products, animal

INTRODUCTION

Livestock associated MRSA (LA-MRSA) was first described in 2005 (Voss et al., 2005) where a new clone of MRSA of sequence type (ST) 398 was identified and grouped within clonal complex (CC) 398¹. The LA-MRSA CC398 lineage, which apparently emerged in European pigs between 2003 and 2005, has since detected in other animal species in many European countries and also in

¹ <http://saureus.mlst.net>

North America, where it can colonize the animal but only rarely cause infections (Voss et al., 2005; Khanna et al., 2008; Smith et al., 2009, 2013; Goerge et al., 2017). Asymptomatic colonization is common although it can cause a variety of human and animal infections including fatal courses, as described for *S. aureus* and MRSA where heavily colonized carriers are more likely to be infected than transient or intermittent carriers (Bradley, 2007; Cuny et al., 2015). However, the human disease burden of LA-MRSA is lower compared to other MRSA lineages possibly because patients affected by MRSA CC398 generally show different demographics in that they are younger, or stay for a shorter time in hospital, and the clinical characteristics are usually less severe or complicated (Becker et al., 2017); but LA-MRSA CC398 is thought not to be inherently less pathogenic for humans than *S. aureus* (Cuny et al., 2013). In fact, LA-MRSA is an emerging category of *S. aureus* throughout the world (Smith, 2015).

Several studies have speculated that CC398 MSSA originated in humans but lost human associated factors such as Panton-Valentine Leukocidin (PVL)-associated phages, toxic shock syndrome toxin I and exfoliative toxins, which are markers of community associated (CA)-MRSA and hospital associated (HA)-MRSA strains (Kadlec et al., 2012; Mohamed et al., 2012; Ballhausen et al., 2017), and acquired antibiotic resistance genes such as *mecA* and *tetM* as they adapted to livestock (Fitzgerald, 2012a,b; Price et al., 2012). Furthermore, several investigations have shown that persons in contact with livestock may be at increased risk of becoming colonized with LA-MRSA. LA-MRSA CC398 colonization has been detected in 24–86% of pig-, 31–37% of cattle-, and 9–37% of poultry-farmers, as well as 44–45% of pig-care veterinarians in European countries (Goerge et al., 2017). Nevertheless, colonization is thought to be dependent on frequency and intensity of animal contact and the duration of exposure, as livestock are thought to be transiently rather than permanently colonized (Bangerter et al., 2016). Also, human contamination of carcasses or meat product at abattoir or meat processing plants may occur and be a source of MRSA, which are not livestock associated (Hadjirin et al., 2015).

Although CC398 is the main lineage associated with MRSA isolated from livestock other clonal complexes, and sequence types (STs) which are not within CC398, have also been associated with livestock and animal products, both in the UK and elsewhere. In this review we focus on the findings from UK livestock and animal products.

LA-MRSA IN THE UNITED KINGDOM

During the last decade, LA-MRSA (mainly MRSA CC398), has become increasingly common among pigs in several European countries (Verkade and Kluytmans, 2014); it has also been reported from humans and animal products (European Food Safety Authority, and European Centre for Disease Prevention, and Control, 2015). Surveillance for LA-MRSA in animals and food is voluntary in the European Union, although the European Food Safety Authority (EFSA) recommends routine surveillance for LA-MRSA in broiler flocks, fattening pigs and dairy cattle.

The recommendation also includes veal calves under 1 year of age and fattening turkey flocks in countries where production exceeds 10 million tonnes slaughtered/year. Prevalence data for LA-MRSA is available from all member states that participate in the routine systematic surveillance in annual reports compiled by EFSA (European Food Safety Authority and European Centre for Disease Prevention, and Control, 2017). However, the production levels in the UK for veal calves are below these thresholds and there is currently no structured surveillance of LA MRSA in UK livestock. Despite increasing reports of LA-MRSA from animals and food sampled in continental Europe, there have been limited reports of its recovery in the UK (Table 1).

A single structured prevalence study for LA-MRSA in pig herds was performed in the UK in 2008 as part of a wider study to determine the prevalence of positive pig herds in EU Member States (MS). The prevalence in MS ranged from 0 to 46% in breeding herds, and 0 to 51% in production/fattening herds (European Food Safety Authority, 2009). In the UK, a total of 258 pig holdings were tested and none was found positive for LA-MRSA CC398. This yielded 95% confidence intervals of 0.0–3.8% estimated prevalence in UK pig breeding herds and 0.0–1.8% in UK pig production herds (European Food Safety Authority, 2009). Following the 2008 study there have been sporadic reports of LA-MRSA in UK livestock although no further prevalence studies have been carried out. The majority of LA-MRSA detections have been made through scanning

TABLE 1 | Published reports of LA-MRSA from livestock and animal-derived food products in the United Kingdom.

Type	Clonal information	Species	Studies
Farm	ST398	Pig	Hartley et al., 2014
Farm	ST398	Pig	Hall et al., 2015
Farm	ST398	Pig	Sharma et al., 2016
Farm	CC30	Pig	Lahuerta-Marin et al., 2016
Farm	CC398	Various	Anonymous, 2017*
Farm	ST398	Turkey	GOV. UK, 2013
Farm	CC9/CC398	Turkey	Sharma et al., 2018
Game Farm	CC9/CC398	Pheasant	Sharma et al., 2018
Farm	CC398	Beef cattle calf	Stone, 2017
Meat	CC9, CC22	Chicken, pork and beef	Dhup et al., 2015
Meat	ST398	Pork and chicken	Hadjirin et al., 2015
Meat	ST398	Chicken, turkey and pork	Fox et al., 2017
Milk bulk tank	CC130, CC705, ST425	Dairy cattle	Garcia-Alvarez et al., 2011
Milk bulk tank	CC398	Dairy cattle	Paterson et al., 2012
Milk bulk tank	CC130, ST398, ST425	Dairy cattle	Paterson et al., 2014
Equine hospital	ST398	Horse	Loeffler et al., 2009
Equine hospital	CC398	Horse	Bortolami et al., 2017

*Provides summary of all LA-MRSA isolations from UK government laboratories since February 2013, including some isolates already listed in this table, which have been referenced separately e.g., CC30.

surveillance activities. Scanning surveillance comprises the submission of clinical samples from livestock, by veterinarians, to government laboratories, where microbiological and other investigations are performed to identify the possible causative agent. Scanning surveillance and other studies have reported the presence in the UK of LA-MRSA CC398, CC9, CC9/CC398 hybrid, CC22, CC30, CC130, CC705, and ST425 (Table 1). These isolates have been reported from horses (Loeffler et al., 2009; Bortolami et al., 2017), dairy cattle (Garcia-Alvarez et al., 2011; Paterson et al., 2012, 2014) beef cattle (Stone, 2017), poultry and pheasant (GOV. UK, 2013; Stone, 2017; Sharma et al., 2018), pigs (Hartley et al., 2014; Hall et al., 2015; Lahuerta-Marin et al., 2016; Sharma et al., 2016), pork meat (Dhup et al., 2015; Hadjirin et al., 2015; Fox et al., 2017), beef meat (Dhup et al., 2015), chicken and turkey meat (Dhup et al., 2015; Fox et al., 2017).

Government institutes involved in scanning surveillance includes the Animal and Plant Health Agency (APHA) in England and Wales, Agri-Food and Biosciences Institute (AFBI; Northern Ireland) and Scotland's Rural College (SRUC). Generally *S. aureus* isolated by routine microbiology from diagnostic samples such as mastitis in cattle are tested for penicillin or ampicillin sensitivity by disc diffusion; only some pig and poultry samples are tested. Any penicillin/ampicillin resistant isolate is tested for cefoxitin and oxacillin antibiotic sensitivity for identification of presumptive *mecA* or *mecC* harboring MRSA. For any phenotypically positive isolates, a multiplex PCR that can detect *mecA*, as well as *S. aureus* species specific genes *nuc* and 16S rRNA (European Union Reference Laboratory for Antimicrobial Resistance, 2009) are used. For *mecA* negative samples a PCR is used for amplification of *mecA* and *mecC*, identification of *S. aureus* by amplification of the *spa* gene, and detection of the Panton-Valentine Leukocidin (PVL or LukF PV) encoding gene (Stegger et al., 2012). In any *mecA* or *mecC* positive sample, *spa*-typing and WGS is performed (Sharma et al., 2016, 2018). Between 2013 and 2015, nine LA-MRSA were identified after screening more than 1000 *S. aureus* isolated from diagnostic submissions submitted through scanning surveillance to APHA and AFBI (160); none had been reported from Scotland during this period. A low frequency of detection has continued since 2015. Details of the UK isolates gathered from scanning surveillance and other studies are provided in Table 1. The disease surveillance and microbiological laboratories based in Northern Ireland and Scotland are involved in isolation of LA-MRSA from these countries.

BACKGROUND OF UK LA-MRSA ISOLATES

Isolation From Scanning Surveillance of Livestock

The first confirmation of CC398 LA-MRSA on a pig farm in the UK was reported in 2014, and the piglet harboring CC398 LA-MRSA was one of a group of five piglets submitted to the Omagh disease surveillance laboratory, AFBI, with a history of pneumonia and wasting (Hartley et al., 2014). Also in 2014,

CC398 LA-MRSA was isolated from one 10-day-old piglet with skin lesions submitted to an APHA veterinary investigation center (Hall et al., 2015). CC398 LA-MRSA has been isolated from the caecal content of healthy pigs at abattoir from 2 of 56 pig farms in England that were sampled during 2014–2015, as part of a research project based at APHA (AbuOun et al., 2017). All three APHA isolates belonged to *spa*-type t011 and showed similar characteristics to other UK and European CC398 LA-MRSA strains by WGS (Sharma et al., 2016). In 2015, three pigs with signs of ill-thrift (low rate of growth) from a farm in Northern Ireland were submitted to the AFBI Veterinary Sciences Division for post-mortem investigation. *S. aureus* was obtained from different tissues in the three animals and all nine detected *S. aureus* isolates were identified as CC30 MRSA (Lahuerta-Marin et al., 2016). The APHA also reported detection of LA-MRSA CC398 in 2016 through scanning surveillance from pigs during a non-clinical investigation, and in 2017 from pigs during a clinical investigation (Anonymous, 2017). In addition, CC398 LA-MRSA, isolated from seven clinical investigations in pigs from Northern Ireland, have been reported between 2014 and 2017 (Anonymous, 2017).

The first isolation of CC398 LA-MRSA at farm level was reported in November 2013 in turkeys on a poultry farm (GOV. UK, 2013), where all isolates were shown to belong to *spa*-type t011 with WGS indicating the same clone to be disseminated across the farm (Sharma et al., 2016). LA-MRSA CC398 isolates were reported by APHA from UK fattening turkeys in 2016 from England. The turkey was incidentally diagnosed with LA-MRSA CC398 *spa*-type t899 while under investigation for an unrelated upper respiratory tract infection (Stone, 2017). A CC398 LA-MRSA, also of *spa*-type t899, isolated from a pheasant during a clinical investigation in Scotland was reported in 2017 (Anonymous, 2017). Both avian isolates were shown by WGS to belong to the CC9/CC398 hybrid genotype (Sharma et al., 2018).

Government surveillance reported LA-MRSA CC398 isolates detected by APHA from a spontaneously aborted calf from England/Wales in 2016, and from a single clinical investigation of cattle from Northern Ireland between 2014 and 2017 (Anonymous, 2017; Stone, 2017).

Isolation From Retail Meat

Three point-prevalence surveys were conducted on meat products by Dhup in 2011, and Hadjin and Fox in 2015, at UK retail outlets; all three studies examined pork meat. CC398 LA-MRSA was identified in 3 of 52 (5.8%) (Hadjirin et al., 2015) and 3 of 63 (4.7%) pork products (Fox et al., 2017) on sale in 2015, while CC9 LA-MRSA was identified in 1 of 30 (3.3%) pork products on sale in 2011 (Dhup et al., 2015). It is noteworthy that only one of the three products from the Fox et al. study (Fox et al., 2017) was specified as UK origin.

Although LA-MRSA has not been detected directly on UK broiler farms, two out of three studies that examined chicken meat at retail isolated LA-MRSA. In 2011, CC22 a common HA-MRSA, was reported from 2 of 30 samples (6.7%) suggesting contamination from human sources; while one CC9 isolate (3.3%) that lacked the immune evasion cluster characteristic of

LA-MRSA, was detected (Dhup et al., 2015). CC398 LA-MRSA was also reported in four of 50 (8.0%) chicken meat samples and two of 11 (18.2%) turkey meat samples in 2015; although these samples were bought from retail meat outlets in North West England only three were from UK, two were from continental Europe, the origin of one was not specified (Fox et al., 2017).

In beef, CC22 MRSA was identified in 2011 from 1 of 30 meat samples (3.3%), suggesting contamination from human source during processing (Dhup et al., 2015).

When results from the 2011 retail study by Dhup et al. (2015) was compared with that from 2015 by Fox et al. (2017), which was performed in the same geographic region, it indicated a possible increase in prevalence of LA-MRSA in the UK. The authors suggested this could be due to the fact that 60% of meat consumed in the UK is imported from European countries where CC398 contamination has been reported in up to 60% of samples (Fessler et al., 2011; European Food Safety Authority, and European Centre for Disease Prevention, and Control, 2015; Dhup et al., 2015).

Isolation From Milk

In bulk milk from dairy cattle, the first isolation of CC398 LA-MRSA was reported in 2012 (Paterson et al., 2012). A survey of 1500 bulk tank milk (BTM) samples from about 1500 farms was undertaken to determine the prevalence of both *mecA* and *mecC* MRSA. Seven *mecA* CC398 isolates were identified, including three from the same farm. A total of five geographically dispersed farms in the UK were positive for LA-MRSA CC398 (Shore et al., 2012). Following the detection and reporting of *mecC* MRSA from dairy cattle in England (Garcia-Alvarez et al., 2011), the same authors conducted a study to determine the occurrence of *mecC* MRSA in bovine bulk milk in Great Britain. A total of 1090 dairy farms were evaluated for the presence of *mecC* MRSA in BTM samples (Paterson et al., 2014). *mecC* MRSA was identified in 10 of 465 dairy farms (2.15%, [95% CI 1.17–3.91]) from England and Wales but not from 625 farms sampled from Scotland. However, only one CC398 LA-MRSA was identified (0.06%) from a farm in England. Three of the ten *mecC* MRSA isolates were CC130 and seven were ST425 (Paterson et al., 2014).

Isolation From Horses

In 2009, CC398 LA-MRSA was reported from two horses (one with a history of travel outside UK) detected in a screening study at an equine hospital performed by the Royal Veterinary College (Loeffler et al., 2009). This was the first isolation of CC398 LA-MRSA reported from UK animals. Further analysis of these two isolates by WGS indicated that they resembled other *spa* type 11 strains, clustering closely to another horse isolate from Belgium (Sharma et al., 2016). In a study from University of Liverpool, presence of LA-MRSA CC398 isolates, predominantly of *spa*-type 11 was reported from surveillance at an UK Equine Veterinary Hospital from 2011 to 2016; 65 of the 829 samples collected from environmental sites, surgical site implants and hand-plates were CC398 (Bortolami et al., 2017).

WHOLE GENOME SEQUENCING OF UK LIVESTOCK ISOLATES

MRSA lineages can be identified with molecular tests such as pulsed-field gel electrophoresis (PFGE), *Staphylococcus* protein A or *spa* typing or multiple locus variable number tandem repeat analysis (MLVA), multilocus sequence typing (MLST), and *Staphylococcal* cassette chromosome (SCCmec) typing. DNA microarrays, which have been used for determining virulence or antimicrobial gene presence in diverse bacteria (Hopkins et al., 2007; Carter et al., 2008; Wragg et al., 2009), have also been applied to MRSA, as have WGS (Schouls et al., 2009; Monecke et al., 2011; Piccinini et al., 2012; Shore et al., 2012; Sharma et al., 2016; Sabat et al., 2017). The latter has been particularly useful and is increasingly being used instead of PFGE for purposes such as tracing outbreaks, as well as identifying the most likely source of acquisition, which is an essential component of an effective surveillance system to describe epidemiological trends and infection control strategies (Rebic et al., 2016; Sharma et al., 2016). In addition, although phenotypic methods are easier to perform and interpret, and they are cost effective and widely available, they may be less discriminatory. Genotypic methods, although more expensive and technically demanding, can provide more detailed characterisation of the isolate (Anjum, 2015; Rebic et al., 2016). Specifically, WGS is a more rapid method for identifying genetic determinants such as virulence and AMR genes, as well as studying phylogenetic relationships between groups of isolates based on the core genome of hundreds of genes, rather than DNA microarray. The latter is based on a finite panel of selected genes and it can be time consuming to update with new genes; also it is becoming easier to apply WGS routinely as the associated costs are being reduced and bioinformatic tools are becoming more readily available (Anjum, 2015; Anjum et al., 2017).

Phylogenetic comparison of WGS data derived from a subset of the LA-MRSA CC398 isolates from livestock detected in United Kingdom, with isolates across Europe and North America, has indicated a possible European origin of UK LA-MRSA isolates. All UK isolates included in the phylogenetic comparison belonged to *spa*-types t011 and t034 (Sharma et al., 2016). Also, these isolates showed a multi-drug resistance genotype i.e., presence of three or more antimicrobial resistance genes including the tetracycline resistance gene, which is often present in LA-MRSA isolated from animals (Sharma et al., 2016). They did not harbor any human associated virulence factors such as the human ϕ Sa3 Immune Evasion Cluster (IEC) which contains a number of genes including staphylococcal complement inhibitor, staphylokinase and toxins that promotes survival within the human host (Xu et al., 2014); nor did they harbor the avian prophage carrying the SAAV_2008 and SAAV_2009 genes which have been detected in CC398 isolates from poultry, and produces resistance to killing by avian phagocytes (Argudin et al., 2013). However, as many of the isolates were from diseased livestock, further exploration of the WGS data is warranted to help identify possible animal-associated virulence factors, which may be another marker of adaptation in animals. A study which used genome-wide

high-throughput screening to identify essential genes for CC398 LA-MRSA survival identified 24 genes important for survival in porcine blood; none of the genes were directly related to virulence factors (Christiansen et al., 2014).

More recently WGS of two *spa*-type t899 isolates, reported as incidental findings from a turkey and a pheasant in the UK, was performed. Both avian isolates harbored the *sac* *scn* *chp* genes associated with the ϕ Sa3 Immune Evasion Cluster. Also, both isolates were multi-drug resistant, although the heavy metal resistance gene *czrC*, encoding zinc resistance, was not detected in either isolate (Sharma et al., 2018). This is in contrast to previous findings where all nine UK isolates from pigs, cattle and turkey examined by WGS harbored the *czrC* gene (Sharma et al., 2016). Also, neither isolate harbored the avian prophage genes. Phylogenetic reconstruction indicated both isolates clustered with other *spa*-type t899 isolates, which display a unique genotype of being a CC9/CC398 hybrid. The English t899 turkey strain was closely related to t899 isolated from turkey in Germany and France, and from UK retail chicken meat, which had previously been classed as CC398 (Fox et al., 2017). The Scottish pheasant isolate, although still within the same phylogenetic cluster, was more distantly related (Sharma et al., 2018).

WGS has also been applied to isolates recovered from three pigs with signs of ill-thrift from a farm in Northern Ireland and shown to be phenotypically penicillin, cefoxitin and tetracycline resistant. The eight isolates identified as MRSA belonged to a novel CC30 clone, being positive for *lukM* and *lukF-P83* genes, a marker for virulence restricted to animal lineages which has been implicated in the pathogenesis of mastitis cattle and exudative dermatitis in squirrels (Lahuerta-Marin et al., 2016).

DISCUSSION

The fact that in the UK only a handful of LA-MRSA were identified after screening more than 1000 *S. aureus* isolated from diagnostic submissions from livestock submitted through scanning surveillance between 2013 and 2015 (Sharma et al., 2016), indicates it may be an emerging problem with currently a possible low prevalence. In some cases, LA MRSA was reported as an incidental finding and there was little evidence of it causing significant disease in animals. However, scanning surveillance is biased to investigations of clinically significant *S. aureus* isolates, which have originated primarily from cattle, with few isolates from pigs or poultry, where it may be present asymptotically in the healthy population. In specific screening programs of pigs, MRSA is most commonly sampled through collection of nasal swabs; skin swabs can also be taken and sampling both skin behind the ear in conjunction with nasal swabs has been shown to be the most sensitive method for detection of MRSA in live pigs, suggesting a targeted approach to sampling is required (Pletinckx et al., 2012; Agerso et al., 2014). Furthermore, pooling of swabs can increase the sensitivity of detection of MRSA in herds compared to single swabs (Friese et al., 2012). Also, the MRSA status in individual pigs from a MRSA-positive herd can change, as pigs may be transiently rather than permanently colonized (Bangertner et al., 2016).

Although the frequency of reports in livestock in the UK remains low, the geographic and species dispersal suggests a widening distribution. Given the lack of available data, there is a need for surveillance of healthy livestock to establish how prevalent LA-MRSA is in UK livestock, and what the risks are for humans. The guidelines for harmonization of sampling strategies, outlined by EFSA, provide valuable guidance for the effective detection of LA-MRSA within the livestock industry (European Food Safety Authority, 2012).

Livestock-associated methicillin-resistant *Staphylococcus aureus* was also reported at a low-to-moderate prevalence in UK animal products such as bulk tank milk and retail meat, although the latter may partly reflect EU meat. It is also noteworthy that phylogenetic comparisons of UK livestock isolates through WGS, suggests a possible European origin with multiple incursions (Sharma et al., 2016, 2018).

Nevertheless, although frequency has increased since 2007, and CC398 is the dominant type, LA MRSA is rarely identified from human samples in the EU (Kinross et al., 2017). A recent assessment by the Food Standards Agency also reported a very low risk in the UK food chain and noted that there have been no reported foodborne outbreaks worldwide². The need to revisit the assessment in the light of new data was recognized due to uncertainties on the prevalence of LA-MRSA in food and livestock. Furthermore, evidence from EU countries suggests a risk of transmission to people in contact with livestock but the zoonotic risk remains to be fully explored. The current lack of information suggests the need for systematic surveillance to understand reservoirs and transmission routes, applying a One Health approach and MRSA typing across sectors.

Harmonized structured surveillance would reduce uncertainties in risk assessment and linked epidemiological studies could be used to inform options for control and cost effectiveness. Furthermore, surveillance can be used to monitor the effectiveness of risk management at national and international levels as has been performed for zoonoses such as *Salmonella*.

AUTHOR CONTRIBUTIONS

MA and SE conceived the study. All authors wrote the manuscript.

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²<https://pdfs.semanticscholar.org/4fca/b9277652e3d8cc34a8150b31de865a838e67.pdf>

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Crisis of Antimicrobial Resistance in China: Now and the Future

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The crisis of antimicrobial resistance is worsening and has become a major public safety problem in China, seriously endangering human and animal health and ecological environment. Gram-negative bacterial resistance in China is severe: the related pathogens mainly include carbapenem-resistant *Acinetobacter*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. Surging antimicrobial consumption and irrational use of antimicrobials are the main causes of resistance. In China, a variety of strategies are implemented to control the antimicrobial resistance in hospitals, agriculture and environment. However, there is still a long way to go to strengthen the drug resistance surveillance, to reduce the emergence of drug-resistant bacteria, and to find new antimicrobials and therapies for drug-resistant bacteria. Controlling the antimicrobial resistance crisis takes great efforts from the whole society.

Keywords: crisis, antimicrobial resistance, China, resistance pattern, combating drug resistance

Antimicrobials have saved tens of millions of lives since penicillin was used clinically in 1940s. They have made an outstanding contribution to prolong the average life span. However, many bacteria have developed severe resistance to antimicrobials with the increased antimicrobial consumption worldwide. The development rate of bacterial resistance is much faster than that of new antimicrobials. If uncontrolled, humans will enter the “post-antibiotic era.” China is one of the top consumers of antimicrobials in the world with 1.3 billion population. Therefore, it is more challenging for China to face the antimicrobial resistance.

THE RESISTANCE PATTERN OF ANTIMICROBIALS IN CHINA

Infections caused by multidrug-resistant organisms (MDROs), especially carbapenem-resistant Gram-negative bacteria often cause high mortality due to limited treatment options. Bacterial resistance data from multiple hospitals in China have been collected by China Antimicrobial Resistance Surveillance System (CARSS) (National Health and Family Planning Commission of the People's Republic of China, 2017). In 2016, a total of 2727605 strains from 1273 hospitals were collected. Imipenem-resistant *Acinetobacter baumannii* increased from 45.8% in 2012 to 59.2% in 2016. The resistance rate of *Escherichia coli* to imipenem and third-generation cephalosporins (3GC) decreased slightly from 2.2 and 69.7% in 2012 to 1.2 and 56.3% in 2016, respectively. From 2012 to 2016, the resistance rate of *Klebsiella pneumoniae* fluctuated with the rising trend, reaching 34.5 and 8.7% in 2016 to 3GC and carbapenems, respectively. In 2016, the prevalence of carbapenem-resistant *Pseudomonas aeruginosa* (CRPA), methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* was 22.3, 34.4, and 2.0% respectively. There was no *S. aureus* with vancomycin resistance. The China Antimicrobial Surveillance Network (CHINET, 2018) began monitoring bacterial resistance nationwide since 2005.

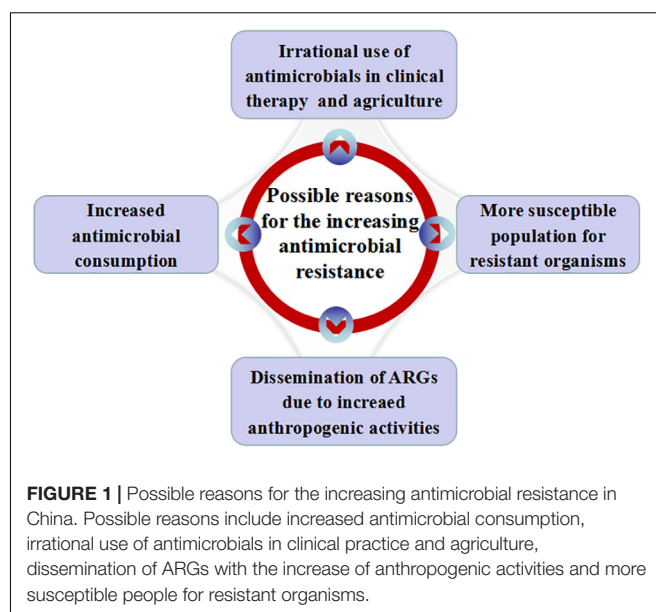
Their data helped us to understand the status and changes of bacterial resistance in China. Between 2005 and 2017, the number of bacterial strains isolated annually ranged between 22774 and 190610. Carbapenem-resistant *Acinetobacter*, of which over 90% were *A. baumannii* (CRAB), increased from 31 to 71.4%, with 60% being multidrug-resistant. *Enterobacteriaceae* were still highly sensitive to carbapenems, as the carbapenem resistance rate of most bacteria in this family was less than 10%. There was a slightly downward trend in the prevalence of *P. aeruginosa* and in its resistance rate to carbapenems (ranging from 20 to 30%, CRPA). Remarkably, there was a tenfold increase of the carbapenem-resistant *K. pneumoniae* (CRKP), from 2.4 to 24% in the past 13 years, most of which were isolated from sputum specimen. The geographical distribution of CRAB, CRKP and CRPA was mainly concentrated in central and eastern China and Yunnan Province (CHINET, 2018). On the contrary, MRSA isolates decreased from 69% in 2005 to 35.3% in 2017. The prevalence of vancomycin-resistant *Enterococci* (VRE) in China is still low (Hu et al., 2016; CHINET, 2018). In 2014, the first World Health Organization (WHO) global report on antimicrobial resistance surveillance showed that CRKP has appeared in almost all parts of the world (World Health Organization [WHO], 2014). A report on antimicrobial resistance surveillance in Europe showed that the resistance rate of *K. pneumoniae* against carbapenems was 6.1% in 2016, without a significant change from 2013 to 2016. The prevalence of CRKP in Greece was the highest, up to 61.9% (ECDC, 2016). In summary, just like the global trend, Gram-negative bacterial resistance in China is severe. In particular, the rapid growth of CRKP should draw widespread attention.

Drug resistance surveillance network for zoonotic bacteria in China was established in 2008 (Zhang et al., 2017). The key monitored strains are *E. coli*, *Salmonella*, *S. aureus*, *Enterococcus*, *Campylobacter*, *Streptococcus*, *Haemophilus parasuis*, and *Pasteurella*, etc. The serotype identification and drug resistance testing have been completed in more than 30,000 strains of bacteria. In recent years, bacteria such as *E. coli* and *Salmonella* carried by animals have been found to be resistant to colistin. The situation is worse in poultry than in swine. *E. coli* and *Salmonella* are also highly resistant to tetracycline. The resistance rate of *E. coli* to florfenicol has been as high as 100%, and to enrofloxacin about 50–70% (Cai, 2017).

WHAT DRIVES THE ANTIMICROBIAL RESISTANCE IN CHINA?

Some possible reasons for the increasing antimicrobial resistance in China are illustrated in **Figure 1**.

First, antimicrobial consumption promotes antimicrobial resistance. A study by Klein et al. (2018) showed that antimicrobial consumption increased by 79% [2.3–4.2 billion DDDs (defined daily doses)] in China between 2000 and 2015, higher than the increase of global antimicrobial consumption, which was 65% over the 15 years. The antimicrobial consumption rate in DDDs per 1,000 inhabitants per day in China (65%) also grew faster than that of the globe (39%). In China, the higher



population density, the decreased air quality due to the emission of gasoline and other fuels in the urbanization, and the high prevalence of chronic obstructive pulmonary disease (COPD) (8.6%, 99.9 million people) (Wang et al., 2018) make people become more susceptible to bacterial infections. In addition, more and more patients seek medical advice and have higher healthcare expectation with the continuous improvement of the social security system, living standards and health literacy. Therefore, the demand for antimicrobials has increased.

Secondly, irrational use of antimicrobial agents in clinical practice (especially among children) and agriculture (including livestock, aquatic products, and crops, etc.) (Zeng et al., 2017). Some general practitioners or rural doctors are unfamiliar with the principles and methods for the rational application of antimicrobials, they may wrongly prescribe antimicrobials in the ways such as incorrect dosing, topical application of systemic antimicrobials, and improper antimicrobial prophylaxis, etc. Most often, patients with viral infections (flu or common cold) may be prescribed antimicrobials. A study from Poland also showed increased antimicrobial consumption in viral infection season (Ciszewski et al., 2017). Financial incentives, such as mark-ups on drug price, is considered to be the main driver of over-prescribing in China (Qiao et al., 2018). Many people have low literacy about antimicrobials, and they pursue antimicrobials through a pharmacy without prescription (online or on site) (Wang et al., 2016). Antimicrobials are widely used in livestock as prophylactic and therapeutic agent for infections and as growth promoters. Antimicrobial use in livestock is even slightly higher than in humans (Zhang et al., 2015). Antimicrobials have contaminated the food and drinking water supply in China because a large number of antimicrobials are used improperly in livestock in rural China (Hao et al., 2015). In 2015, a survey on the antimicrobial body burden of Chinese schoolchildren found that 58.3% of 1064 urine samples were tested positive for antimicrobials, and that the contaminated environment and

food may be the main sources of exposure (Wang et al., 2015). This may have induced bacterial resistance and unbalanced flora distribution, damaged the immune function and nervous system, and produced other adverse drug reactions.

Thirdly, antibiotic resistance genes (ARGs) are a natural component of all environments. However, anthropogenic activities have led to the dissemination of ARGs as an emerging environmental contaminant (Zhu et al., 2017; Qiao et al., 2018). Now, ARGs are widely distributed in China in environments including clinical areas, surface water, animal wastes, sewage treatment plant effluents and soils (Qiao et al., 2018). Antimicrobials and ARGs can spread among the environment, humans and animals, which is closely associated with the increasing prevalence of antimicrobial resistance and is threatening human health. A recent study even found that smog metagenomes in Beijing contained multiple carbapenem-resistant genes. The relative abundance was similar to that in the human gut and sewage (Pal et al., 2016).

Fourthly, bacterial defense dysfunction occurs in some populations as a result of the aging Chinese society, the long-term use of steroids and immunosuppressants, the extensive development of bone marrow and organ transplantation, the increased number of invasive treatments, and the prevalence of acquired immunodeficiency syndrome (AIDS). In these populations, their demand for antimicrobials is greater and they are more likely to become critically ill. They are more susceptible to resistant organisms because of risk factors including ICU admission, being elderly, indwelling devices (such as central venous catheters, catheters, endotracheal tubes) and invasive procedures (Kaye and Pogue, 2015). It is very difficult to treat infections caused by multi-drug resistant strains in these patients. Maybe it is also one of the reasons for the severe bacterial resistance in China.

HOW TO DEAL WITH THE ANTIMICROBIAL RESISTANCE IN CHINA?

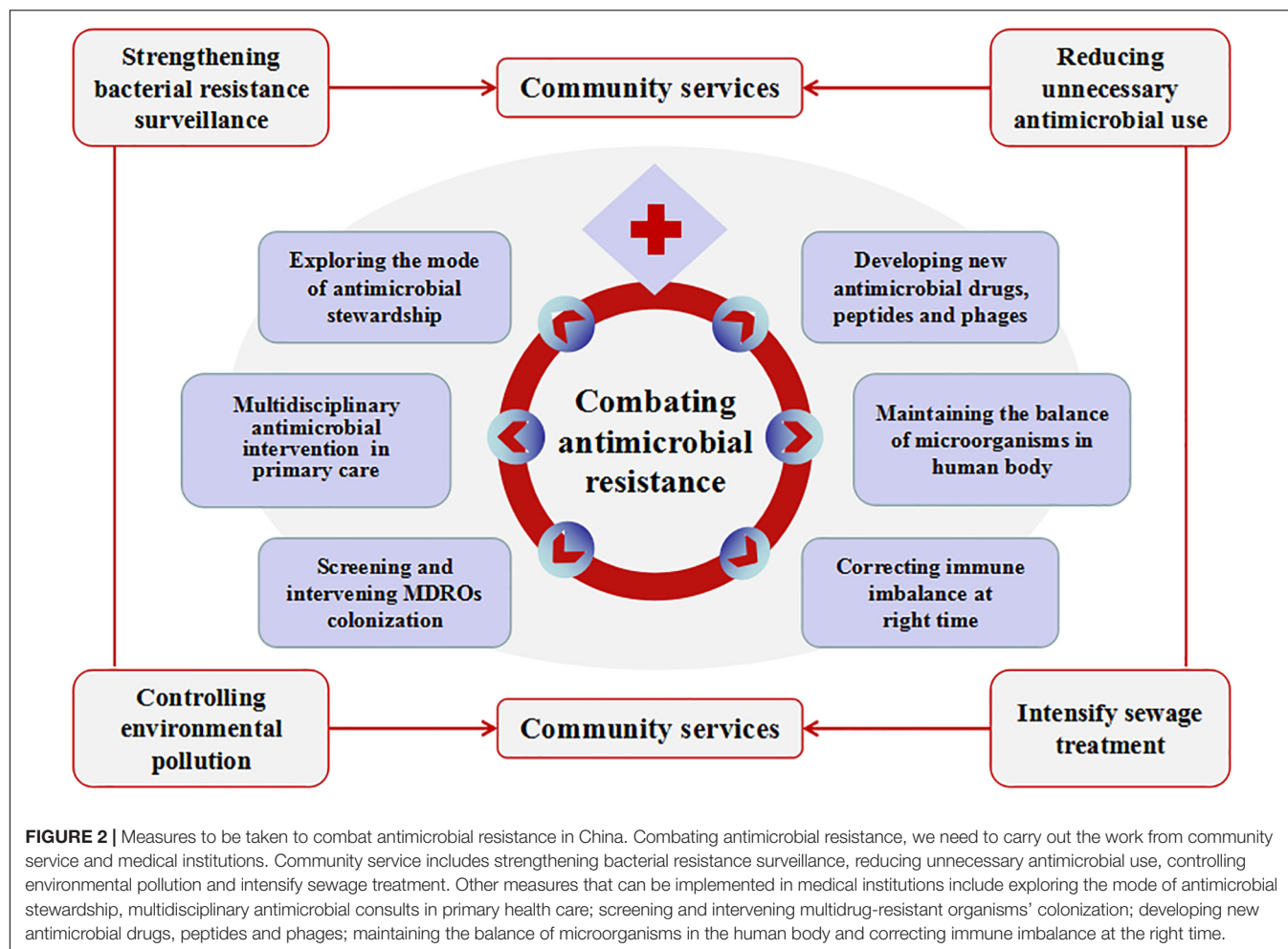
The theme for World Health Day 2011 was “combating drug resistance: no action today, no cure tomorrow,” which reinforced all countries around the world to take more active actions against bacterial resistance. The 2015 World Health Assembly adopted the global action on antimicrobial resistance. The 2016 UN High Level Meeting on antimicrobial resistance and the G20 Summit have made strong commitments to control antimicrobial resistance. What have we done? What else do we need to do?

The Chinese government attaches great importance to the issue of antimicrobial resistance and takes multiple measures to strengthen the antimicrobial stewardship. A series of documents were promulgated such as Administrative Measures for the Clinical Use of Antibacterial Drugs (2012) (Ministry of Health of the PRC, 2012), Antimicrobial Management will be Enhanced in Multiarears (2015) (National Health and Family Planning Commission of the PRC, 2015a), Five Year Action Plan for the Comprehensive Management of Veterinary Drugs in China (2015–2019) (Ministry of Agriculture

and Rural Affairs of the PRC (2015–2019), 2015), National Action Plan to Contain Antimicrobial Resistance (2016–2020) (National Health and Family Planning Commission of the PRC (2016–2020), 2016), and Work Program for the Reduction of the Use of Antimicrobials in Animals (2018–2021) (Ministry of Agriculture and Rural Affairs of the People's Republic of China (2018–2021), 2018), etc. These documents fully demonstrate that the state will strengthen supervision over the manufacture, circulation and use of antimicrobials and support the development of new antimicrobials. Therefore, regular training on rational use of antimicrobials, enhancement of antimicrobial stewardship, and strict implementation of infection control measures, especially hand hygiene have been carried out in the medical institutions at all levels to reduce the unnecessary consumption of antimicrobials and to delay the emergence and spread of resistant bacteria.

The management of clinical use of antimicrobials in China has gone through several stages: promulgation and implementation of guidelines for clinical use of antimicrobials (2004), specialized rectification (2011–2013), continuous improvement (2014–2017) with updated guidelines for clinical use of antimicrobials (2015), and refined and standardized management training since 2018 (Ministry of Health of the PRC, 2004; National Health and Family Planning Commission of the PRC, 2011, 2015b; National Health Commission of the People's Republic of China, 2018). These initiatives have achieved remarkable results through continuous efforts in recent years. According to the data from the Center for Antibacterial Surveillance (National Health and Family Planning Commission of the People's Republic of China, 2017), the use of antimicrobials in outpatient settings decreased from 17.2% in 2011 to 10.3% in 2016, in inpatient settings from 59.4% in 2011 to 37.5% in 2016. Antimicrobial drug use intensity decreased from 85.10 DDD in 2005 to 50.03 DDD in 2016. However, the sales of antimicrobials to children increased from ¥5 billion (US\$ 781 million) in 2005 to ¥12 billion (\$1.87 billion) in 2015 according to an analysis of Chinese children's drug market in 2017. The National Health Commission issued the document on May 10, 2018 to strengthen the clinical application and management of antimicrobials for key populations such as children (National Health Commission of the People's Republic of China, 2018).

All the above shows that China's firm determination to fight against bacterial resistance, but there are still many areas to be improved. **Figure 2** is a schematic diagram. First, bacterial resistance surveillance in China is limited by its scope and uneven levels, especially antimicrobial resistance surveillance of animal-derived bacteria. Surveillance network of antimicrobial utilization and resistance patterns from pharmacies, clinics, hospitals, environment, agriculture and animal husbandry should be established at both local and national levels. Efforts should be made to strengthen the “big data” of bacterial resistance in all these sources, and to further address the intrinsic connections hidden behind the data. Nowadays, the development of whole-gene sequencing (WGS) technology can help researchers predict antimicrobial resistance more efficiently, thus assisting in clinical diagnosis



and treatment decisions. WGS-powered online database, which developed by the Chinese scientists called BacWGSTdb, aims to pioneer the movement of WGS from proof-of-concept studies to routine use in clinical microbiology laboratory, offers a rapid and convenient platform to analyze epidemiological outbreak and the phylogeny of the bacterial genome, so as to provide information guarantee and decision-making support for prevention and control of infectious disease outbreaks and major bio-safety accidents (Ruan and Feng, 2016; Quainoo et al., 2017; Rossen et al., 2018). Second, there is still irrational use of antimicrobials in healthcare (especially among children) and agriculture (especially in animal husbandry). The standards and regulations for environmental discharge of antimicrobials still need to be improved. Chinese government has launched some measures to halt financial incentives such as the separation of prescription sales from physicians' income and the "zero mark-up" policy on drug sale. There is a huge amount of antimicrobial consumption in animal husbandry. Animal breeders, especially farmers, lack understanding of antimicrobial resistance and its hazards. Animal-use antimicrobials should be purchased for treatment with prescriptions from a veterinarian. Reducing the use of unnecessary antimicrobials in livestock

farms has not significantly harmed animal health or farmers' incomes (Dierikx et al., 2016). Strengthen scientific breeding and management of livestock and poultry should help to reduce antimicrobial consumption in animals. Public awareness of the prevention and control of the bacterial resistance needs to be gradually raised. Third, it is necessary to continue to strictly control the sources of antimicrobial pollution from various aspects such as non-therapeutic use of antimicrobials and discharge of antimicrobial-containing sewage. The control of waste residue in antimicrobial industry should be taken into concern. Advanced oxidation processes (AOPs) might be used to improve the removal of ARGs in municipal sewage effluent (Zhang et al., 2016). ARG metagenomic data make it possible to track ARG contamination sources (Li et al., 2018), which is important for the control of ARG contamination. Fourth, we should actively explore the mode of antimicrobial stewardship suitable for the institutional development. Improve the organizational structure of antimicrobial clinical application, clarify the responsibilities and strengthen the fine management. Provide regular training and education for the rational application of antimicrobials. Establish a long-term mechanism of multidisciplinary case discussion and a

multidisciplinary consulting team that consists of department of infectious diseases, clinical microbiological laboratory, department of pharmacy and department of nosocomial infection control. Under the support of information technology, strengthen the stratified management of antimicrobials, dynamically monitor the use of antimicrobial agents, evaluate the suitability and rectification of antimicrobial use. Transfer the management of antimicrobials from administrative intervention to multidisciplinary collaboration focusing on competency and patient-centered care. In addition, strengthen international cooperation is another important way to better implement antimicrobial stewardship and to stem the tide of antimicrobial resistance (Hoffman et al., 2015). Fifth, at present, many policies and training of antimicrobial agents in China are concentrated in hospitals above the level-II, but about 60% of patients in the country are treated in community hospitals/rural hospitals (level-I). To tackle this mismatch, multi-disciplinary intervention methods such as professional training in the diagnosis and treatment of infectious diseases, use of social media tool such as WeChat and on-site training platforms organized by tertiary hospitals are offered to healthcare professionals and patients in community hospitals/rural hospitals to create a model for antimicrobial intervention in primary health care institutions in China. Sixth, active screening and enhanced interventions for MDRO's colonization in high-risk patients should be an important way to reduce drug-resistant bacterial infections. Strict isolation enhanced environmental disinfection and hand hygiene should be implemented in MDRO-colonized patients. Currently, different de-planting measures have been adopted according to different colonizing bacteria and different planting sites. Oral cleaning is performed with chlorhexidine, nasal cleaning is performed with iodide or mupirocin: feces with intestinal drug-resistant bacteria are processed separately. Minimizing hospital length of stay and reducing the conversion rate from colonization to infections may be one of the most important infection control measures. Seventh, the scientific challenges are the main problems in the development of new antimicrobial agents. Low profit for pharmaceutical companies on research and development of new drugs is another obstacle. The use of antimicrobial peptides as adjuvants to antimicrobials could probably slow down the development of drug resistance (Lázár et al., 2018). New research suggested that immunomimetic designer cells might be used to cure resistant bacterial infections in the future (Liu et al., 2018). Promoting the development and use of vaccines may reduce the demand for antimicrobials, especially in animal feed. The rapid development of DNA sequencing and artificial intelligence (AI) makes it possible to

screen phages rapidly and efficiently. Phage therapy is promising to be a powerful weapon against super drug-resistant bacteria. Eighth, there are trillions of bacteria and other microorganisms in the human body. They coexist in symbiosis with human beings. Many factors, such as irrational use of antimicrobials, unhealthy diet or long-term stress and anxiety could destroy the balance of microorganisms and lead to various diseases. This is consistent with the Traditional Chinese Medicine rationale "When there is sufficient healthy qi inside, pathogenic factors have no way to invade". If people live in harmony with bacteria, fungi or viruses, people are less likely to get sick. In recent years, fecal microbiota transplantation (FMT) and probiotics are increasingly used in the treatment of various diseases such as *Clostridium difficile* infection, inflammatory bowel disease, constipation, diabetes and obesity, etc. (Rossen et al., 2015). These methods are all based on the restoration of dysregulated intestinal flora to cure the diseases. Maybe it will provide new insights for reducing the emergence of resistant bacteria and finding new treatments in the future. Ninth, immunity dysfunction, especially immune suppression during late stage of infection plays an important role in the development and prognosis of severe infections (Delano and Ward, 2016). If the immune status of patients with severe infections can be accurately monitored and effectively intervened, the prognosis of these patients could be significantly improved, then the dilemma of "no drug available" could be changed. There will be new discoveries and breakthroughs in the study of infection and immune balance.

In short, antimicrobial resistance in China must be curbed. It calls for the power of the whole society to control the antimicrobial resistance now and in the future. Antimicrobials are precious resources for us humans, and it is everyone's responsibility to protect them.

AUTHOR CONTRIBUTIONS

XL and JQ conceived and designed the study. JQ and YH wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

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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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Bactericidal and Anti-biofilm Activity of the Retinoid Compound CD437 Against *Enterococcus faecalis*

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Enterococcus faecalis (*E. faecalis*), a biofilm-forming pathogen, causes nosocomial infections. In recent years, drug resistance by enterococci has become increasingly severe due to widespread antibiotic abuse. Therefore, novel antibacterial agents are urgently needed. In this study, the synthetic retinoid compound CD437 was found to have potent bactericidal effect on *E. faecalis*. In addition, CD437 exhibited synergistic effects when administered in combination with gentamicin and additive effects when combined with ceftriaxone sodium. CD437 also inhibited biofilm formation by *E. faecalis* and exerted bactericidal effect on mature biofilm. Moreover, CD437 exhibited antibacterial and anti-biofilm effects against *Staphylococcus*. No bactericidal action of CD437 was observed against the gram-negative bacillus, but *Pseudomonas aeruginosa* biofilm extracellular polymeric substances (EPS) matrix formation was reduced. Overall, these findings indicate that CD437 has the potential to be developed as a novel antibacterial drug.

Keywords: retinoid, *Enterococcus faecalis*, antibacterial, resistance, biofilm, synergism

INTRODUCTION

Enterococcus faecalis (*E. faecalis*) is a gram-positive, facultative anaerobic oval coccus that can form chains of varying length. It can survive under harsh conditions, including high salt concentrations and a range of temperatures (from 10°C to >45°C) (Arias and Murray, 2012). It is widely distributed in the nature and the gastrointestinal tract of humans, animals, and insects. *E. faecalis* is an important pathogen of nosocomial infections, mainly causing urinary tract infections, bacteremia, artificial joint infections, abdominal–pelvic infections, and endocarditis (Arias et al., 2010; Tornero et al., 2014).

Because antibacterial-drug use has increased rapidly in recent years, enterococci resistance has become increasingly serious and prevalent. Therefore, several drugs that target gram-positive bacteria are not effective against *E. faecalis*. Intrinsic properties of *Enterococcus* cause resistance against common antibiotics, such as the production of the low-affinity penicillin-binding protein Pbp5 that results in reduced sensitivity to penicillin and ampicillin (Kristich et al., 2014). Poor cell wall permeability makes *Enterococcus* highly resistant to clinically achievable aminoglycoside concentrations, rendering this antibiotic unusable as a single agent (Garcia-Solache and Rice, 2019). Enterococcal resistance to linezolid is associated with mutations in the central loop of domain V of the 23S rRNA (Mendes et al., 2014). In addition, the transferable oxazolidinone resistance gene *cfr* mediates acquired drug resistance (Diaz et al., 2012). Glycopeptide-resistant enterococci are known to synthesize new peptidoglycan precursors, including D-Ala-D-lactate or D-Ala-D-Ser,

which replace the normal D-Ala-D-Ala termini (Depardieu et al., 2007). Nine gene clusters that cause glycopeptide resistance have been found in *Enterococcus*, including VanA- and VanB-type resistance clusters (Garcia-Solache and Rice, 2019).

Biofilms are communities of microorganisms encased in extracellular polymeric substances (EPS) (Karatan and Watnick, 2009). The formation of biofilms makes bacteria more capable of adapting to the external environment, which can increase resistance to antibiotics by 1000-fold (Coenye and Nelis, 2010; Pereira et al., 2014), and it is estimated that approximately 65–80% of infections in humans are biofilm mediated. Bacteria can adhere to the heart valves, wounds, and various catheters (Pereira et al., 2014). In *E. faecalis*, biofilm formation commonly occurs on urinary catheters, which can result in a strong immune response (Stickler, 2008; Guiton et al., 2010).

In addition to drug resistance and biofilm formation, the production of bacterial toxins and enzymes increases treatment difficulty for enterococci infections. *E. faecalis* expresses toxins such as lysin and hemolysin, which contribute to cell lysis and virulence, and produces gelatinase and serine protease that can degrade host tissues, regulate biofilm development, and promote microbial invasion. Additionally, adhesin and collagen-binding proteins of *E. faecalis* support biofilm formation in intravascular infections (Kristich et al., 2014). These factors complicate the treatment of *E. faecalis* infections and increase the urgency for new antibiotics.

Recently, it has been reported that the synthetic retinoid compound CD437 can effectively kill growing and persistent methicillin-resistant *Staphylococcus aureus* (MRSA) cells by disrupting the lipid bilayer (Kim et al., 2018). We also speculated that CD437 has bactericidal effect on *E. faecalis*, another gram-positive coccus. In this study, we investigated whether CD437 can inhibit the formation of bacterial biofilms and whether it can eradicate highly resistant biofilms. In addition, we examined the bactericidal action of CD437 and determined its effect in combination with antibiotics.

MATERIALS AND METHODS

Bacterial Isolates, Cultural Conditions, and Reagents

E. faecalis ATCC 29212, *A. baumannii* ATCC 1195, *K. pneumoniae* ATCC 700603, *E. coli* ATCC 25922, *P. aeruginosa* PAO1 (ATCC 15692) were obtained from the American Type Culture Collection, *S. epidermidis* RP62A (ATCC 35984) and ATCC 12228 were given by Di Qu (Shanghai Medical College of Fudan University), and *S. aureus* RJ-2 (ST59, clinical isolate of MRSA) were provided by Li Min (Shanghai Jiao Tong University). The clinical isolates of *E. faecium*, *S. epidermidis*, and *P. aeruginosa* were collected from patients in the Third Xiangya Hospital of Central South University, China. *S. aureus*, *S. epidermidis*, and *E. faecium* strains were grown in tryptic soy broth (TSB) (Solarbio, Beijing, China) or brain-heart infusion (BHI) broth (Solarbio), respectively, at 37°C. Other strains were grown in Luria Bertani (LB) broth (Solarbio). Vancomycin, gentamicin, levofloxacin, and ceftriaxone sodium were purchased

from Aladdin (Shanghai, China). CD437 was purchased from MedChem Express (Monmouth Junction, NJ, United States). All compounds were prepared as 10 mg/mL stock solution in DMSO or double-distilled H₂O (DDH₂O).

Minimal Inhibitory Concentration and Minimal Bactericidal Concentration Determination Assay

The minimal inhibitory concentration (MIC) was determined using the standard micro-dilution method recommended by the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute [CLSI], 2012). To determine the minimal bactericidal concentration (MBC), bacterial cultures from wells with antibiotic concentrations equal to or higher than the MIC were streaked on blood agar plates and incubated at 37°C for 48 h. The MBC was defined as the lowest concentration for which no visible bacterial colonies were observed on the plates after 24 h (Huang et al., 2012). The assay was conducted in triplicate.

Screening of Drug Resistance in Serial Passage

Serial-passage experiments were performed in 96-well cell culture microtiter plates (Corning costar, Cambridge, MA, United States) as a series of MIC experiments using a wide range of CD437 concentrations. CD437 was serially diluted in Mueller Hinton (CaMH) broth, and 50 µL of overnight *E. faecalis* suspension (approximately 10⁶ CFU/mL) was added to wells containing 50 µL of the serially diluted compound. The initial concentration of CD437 was twice that of the MIC. After incubation at 37°C for 24 h, optical density (OD) was measured at 630 nm using a spectrophotometer (iMark™ Microplate Absorbance Reader, BIO-RAD, Hercules, CA, United States). Bacterial growth was defined as OD_{630nm} > 0.1, and 2 µL of culture with the highest drug concentration that allowed bacterial growth was diluted 1000-fold in CaMH and used as an inoculum for the next passage. The remaining culture was stored in 16% glycerol at –80°C (Friedman et al., 2006). Reduced sensitivity to CD437 was determined by re-measuring the MIC of CD437 against resistant mutants from each glycerol-frozen parent. The same protocol was used for ceftriaxone sodium and vancomycin as the controls.

Time-Kill Assay

A single *E. faecalis* colony was inoculated into 25 mL of BHI medium and grown overnight at 37°C, with shaking at 180 rpm. Bacterial suspension (5 mL) was collected in centrifuge tubes and centrifuged at 6000 × g for 10 min at 4°C. The sediments were then collected and re-suspended in the same volume of CD437 diluted in normal saline at concentrations of 0, 2, 4, and 8 µg/mL. Samples were collected at 0, 2, 4, 8, and 24 h, serially diluted 10-fold with saline, and spread on drug-free plates (Cox et al., 2000). After incubating at 37°C for 24 h, colonies were counted, and the number of viable cells is reported as colony forming units per mL (CFU/mL). The experiments were conducted in triplicate.

Bacterial Growth Curves

A single *E. faecalis* colony was inoculated into 25 mL of BHI medium and grown overnight at 37°C, with shaking at 180 rpm. Bacterial suspension (500 µL of MCF 0.5) was inoculated in to 9.5 mL of BHI medium with CD437 at a concentration of 0.5 × MIC, 1 × MIC, or 2 × MIC, or without CD437, to a final bacterial concentration of ~10⁶ CFU/mL. The cultures were incubated at 37°C, with shaking at 180 rpm, and 200 µL of the suspension was sampled every 2 h to determine the OD at 630 nm. Cultures were continuously monitored for 24 h, and the growth curve was plotted using OD_{630nm} average values over three time points as ordinate and culture time as abscissa. The experiments were conducted in triplicate.

Determination of Minimum Biofilm Inhibitory Concentration

The Minimum Biofilm Inhibitory Concentration (MBIC) is the lowest concentration of an agent that inhibits visible biofilm formation of a microorganism (Xu et al., 2012). The MBIC is measured based on the reduction of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) to a water-soluble orange compound by biofilm cells (Pierce et al., 2008). Overnight culture broth (4 µL) was added to 196 µL of BHI medium containing diluted CD437 in a 96-well plate. A control well with 196 µL of medium and 4 µL of overnight culture broth and a blank control with only 200 µL of BHI medium were used. After incubation of the plate at 37°C for 24 h, the medium was discarded and the plate was washed twice with saline to remove any planktonic cells. For XTT staining (Gomes et al., 2009), XTT was diluted with 1 × PBS (pH = 7.0) to a final concentration of 0.2 mg/mL and mixed with PMS (0.02 mg/mL). The mixture (200 µL) was then added to each well, and after incubation at 37°C for 3 h in dark, OD_{490nm} of sample in each well was measured. The experiments were conducted in triplicate.

Assessment of Biofilm Biomass

The drug dilution process and biofilm culture were as described above. The 96-well plate was washed once with saline to remove planktonic cells, and then 200 µL of 0.25% crystal violet (CV) solution was added. Staining was carried out at room temperature for 15 min and the plate was washed three times with saline to remove excess dye (Stepanovic et al., 2000). During this washing step, precautions were taken to avoid damage to the biofilm. The plate was dried at 50°C for 30 min, and the bound dye was dissolved in each well for 20 min by adding 95% ethanol. The absorbance of the sample was then measured at 570 nm. The experiments were conducted in triplicate.

Assessment of Viable Cells in Biofilm by Colony Count

Mature biofilm was formed by adding 196 µL of BHI medium to a 96-well plate, before adding 4 µL of overnight bacterial culture, and incubated at 37°C for 24 h. After 24 h, the medium was discarded, the plate was washed with saline, and CD437 diluted in BHI medium was added to each well. The plate was incubated at 37°C for 24 h, washed again, and 200 µL of saline was added to

each well. The biofilm was broken up through vigorous pipetting to ensure detachment from wells. The bacterial suspension was then transferred to a new 96-well plate and serial 10-fold dilutions were performed in saline. Five microliters of each dilution was plated on agar plates and the CFU was enumerated after 24 h of incubation at 37°C. The experiment was performed twice with three replicates.

Checkboard Assay

The MIC of the antibacterial drug to be tested was first determined separately. According to the obtained MIC, the drug concentration (generally 6–8 dilutions) was determined, and the highest concentration of drug used was twice the MIC. The dilution of the two drugs was carried out in longitudinal and horizontal rows of the 96-well plate, to ensure that a mixture of different concentrations of the two drugs was obtained in each well. The bacterial inoculation was adjusted to 5 × 10⁵ CFU/mL and incubated at 35°C for 18–24 h (Sueke et al., 2010). The assay was performed in triplicate. The fractional inhibitory concentration index (FICI) was calculated using the following formula:

$$FICI = \frac{MIC(A \text{ combination})}{MIC(A \text{ alone})} + \frac{MIC(B \text{ combination})}{MIC(B \text{ alone})}$$

Judgment criteria: FICI ≤ 0.5 is synergistic; 0.5 < FICI ≤ 1 is additive; 1 < FICI ≤ 4 is irrelevant; and > 4 is antagonistic (Almaaytah et al., 2018).

Assessment of CD437 Effect on Biofilm Morphology

Forty microliters of overnight culture was added to a 6-well cell culture plate (Corning costar, Cambridge, MA, United States) containing 1960 µL of CD437 dissolved in BHI medium and a 18 mm × 18 mm sterile glass cover slide. After 24 h of incubation at 37°C without shaking, the slide was washed once with saline to wash away planktonic cells. Finally, slides were stained with the pre-mixed dyes SYTO9 (green) and PI (red; LIVE/DEAD BacLight Bacterial Viability Kit [L7012], Thermo Fisher Scientific, MA, United States), and then images were captured using a confocal laser scanning microscope (CLSM) (ZEISS LSM800, Jena, Germany). For mature biofilm eradication experiments, 24-h biofilms were treated with or without CD437 for another 24 h. When cover slides were stained with 0.25% CV, biofilms were visualized and photographed using a light microscope (Olympus CX31, Tokyo, Japan).

Human Blood Hemolysis Assay

Blood of a clinically normal person was collected from the Department of Clinical Laboratory of the Third Xiangya Hospital, and then 2 mL of blood sample was centrifuged at 500 × g for 5 min. The supernatant was aspirated and replaced with 50 mL of PBS buffer (pH 7.4) and centrifuged as previously described. This step was performed twice to wash erythrocytes. Erythrocytes of 2–3 individuals were mixed and diluted to 4% with PBS, and then 100 µL of the suspension was added to 100 µL of CD437 of different concentrations prepared in PBS, 0.2%

DMSO (negative control), or 2% Triton X-100 (positive control) in sterile EP (Eppendorf) tubes. The tubes were incubated at 37°C for 1 h, centrifuged at $500 \times g$ for 5 min, and 100 μ L of the supernatant was added to a 96-well plate, before measuring the absorbance at 450 nm. The hemolysis rate was calculated using the following formula:

$$\text{hemolysis(\%)} = 100 \frac{A_{\text{sample}} - A_{0.1\% \text{DMSO}}}{A_{\text{TritonX-100}} - A_{0.1\% \text{DMSO}}}$$

The concentration of CD437 at which 50% hemolysis (HC₅₀) was determined using GraphPad Prism 8 (GraphPad Software Inc., CA, United States). The assay was conducted in triplicate.

Mammalian Cell Culture and Cytotoxicity Test

Human liver cancer cell lines HepG2, Bel-7404, and human umbilical vein endothelial cells (HUVECs) (ATCC, Manassas, VA, United States) were grown in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin antibiotic. Human renal proximal tubular epithelium HK-2 and human colorectal cancer cell line HT-29 (ATCC, Manassas, VA, United States) were grown in DMEM/F12 medium supplemented with 10% FBS and 1% penicillin/streptomycin antibiotic. All the cells were maintained at 37°C with 5% CO₂ under a humidified atmosphere. For the cytotoxicity assay, cell viability was tested using the Cell Counting Kit-8 (CCK-8, DojinDo, Japan). The cells were seeded in 96-well plates with 5,000 cells (100 μ L) per well, allowed to attach for 6–8 h, and then exposed to CD437 at different concentrations (100 μ L), prepared in PBS or 0.1% DMSO (negative control), for 24 h. CCK-8 (10 μ L) was then added to each well and the plates were incubated at 37°C for 1–4 h. The absorbance was measured at 450 nm, and the cytotoxicity was calculated using the following formula:

$$\text{cytotoxicity(\%)} = 100 \left(1 - \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{0.1\% \text{DMSO}} - A_{\text{blank}}} \right)$$

The 50% inhibitory concentration (IC₅₀) value was determined using GraphPad Prism 8 (GraphPad Software Inc., CA, United States). The assay was carried out in triplicate.

qPCR

Briefly, PAO1 were cultured in a 6-well plate and treated with or without CD437 for 24 h, and RNA was isolated using E.Z.N.A. Total RNA Kit II (Omega Bio-tek, Norcross, GA, United States), cDNA was prepared using TransScript All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (Transgene, Beijing, China), qPCR was performed using TransStart Tip Green qPCR SuperMix (Transgene, Beijing, China), primers were used as follows: *PsIA*-forward, CGCTCACGGTGATTATGTTC; *PsIA*-reverse, TACATGAACAACAGCAGGCA; *PeIA*-forward, ACAGCCAGGTAATGGACCTC; and *PeIA*-reverse, AAGCTGTCCAGGGTATCGAG (Kim et al., 2015). All qPCR reactions were run in triplicate using a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories Ltd., Hemel Hempstead, United Kingdom), the following conditions were utilized: 94°C

for 5 s, 40 cycles of 94°C for 5 s, 58°C for 15 s, and 72°C for 10 s, Fold change of each gene was normalized to that of the 16S RNA of *P. aeruginosa*. The untreated samples were used for calibration.

Mouse Peritonitis Model

Specific pathogen-free, 6-week old female ICR mice (SJA Laboratory Animal Co., Ltd., Hunan, China) with a mean weight of 25 g were used in this study. Sterile rat fecal extract (SRFE) was prepared using crushed, dried rat feces by mixing with two volumes of normal saline and autoclaving at 121°C and 15 lb of pressure for 15 min. Then the mixture was centrifuged at $3500 \times g$ at a temperature of 4°C, and the supernatant (100% SRFE) was filtered by 0.22 μ m Millipore Express® PES Membrane Filters. Bacteria for inoculation were grown overnight to stationary phase. The resulting cells were then pelleted at $3500 \times g$ for 10 min at 4°C and resuspended in normal saline, and pelleted again as described above. The washed bacterial was resuspended in 12.5% SRFE (Pai et al., 2003) to obtain the final bacterial concentration of 1×10^8 CFU/ml, and then injected into the mouse abdominal cavity in a final volume of 1 mL. CD437 was dissolved in a 1:1 solution of Kolliphor EL (BASF, Ludwigshafen, Germany) and ethanol and then diluted 1:10 in PBS to a final concentration of 30 mg/kg. At 24 h post-infection, groups of mice ($n = 6$) were treated with 30 mg/kg gentamicin s.c., 30 mg/kg CD437 i.p., or a combination of 30 mg/kg CD437 i.p. and 30 mg/kg gentamicin s.c. every 12 h for 3 days. Control mouse were injected with 200 μ L of 10% Kolliphor EL/ethanol in PBS i.p. every 12 h for 3 days. After euthanizing mice, liver and spleen tissue was homogenized in PBS, The numbers of bacterial in the homogenates were counted by 10-fold serial dilution and spotting on blood agar plates. The bacterial load was counted as CFU/g tissue. This study was carried out in accordance with the Animal Welfare Act and National Institutes of Health guidelines for animal care and use, and all experimental protocols were approved by the IRB of Third Xiangya Hospital, Central South University.

Statistical Analysis

The quantitative data are presented as mean $\pm$ standard deviation. A student's t-test was used for between-group comparisons, and multi-group comparisons were performed using a one-way ANOVA. The statistical analyses were performed using SPSS 21.0 (SPSS Inc., Chicago, IL, United States). The results with P -values of < 0.05 were considered significant.

RESULTS

Bactericidal Effect of CD437 on *E. faecalis* ATCC 29212

The growth curve of *E. faecalis* ATCC 29212 showed that CD437 can effectively inhibit the growth of bacteria. CD437 partially inhibited bacterial growth at a concentration of 0.25 μ g/mL, and the inhibitory effect increased with CD437 concentration. The MIC of CD437 against *E. faecalis* ATCC

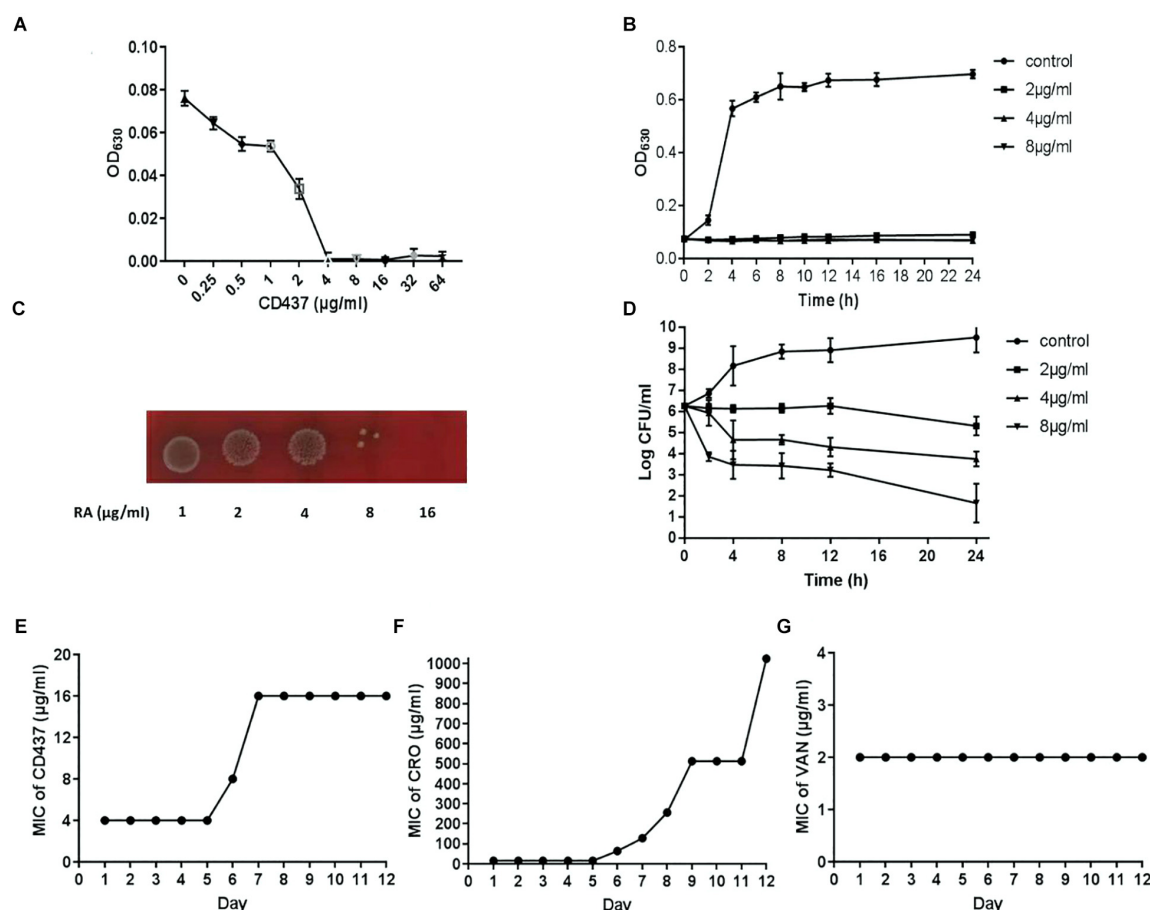


FIGURE 1 | CD437 exerts a potent bactericidal effect on *E. faecalis* ATCC 29212. **(A)** Growth of *E. faecalis* ATCC 29212 after exposure to CD437 in BHI medium for 18 h. OD_{630nm}, optical density at 630 nm. **(B)** The growth curve shows that 2 µg/mL CD437 can significantly inhibit bacterial growth. **(C)** Bacterial cultures from the MIC test were streaked on agar and incubated for 48 h. **(D)** The time-kill curve shows that CD437 has a bactericidal mechanism of action, as evident by the drop in viable cell counts after 4 h. Data are presented as mean ± s.d. The results are representative of three independent experiments. Serial-passage experiments with *E. faecalis* ATCC 29212 selected for increasing resistance to **(E)** CD437, **(F)** ceftriaxone sodium, and **(G)** vancomycin.

29212 was 4 µg/mL (Figure 1A) and the MBC was 16 µg/mL (Figure 1C). The OD_{630nm} value was not detectable until 16 h when the concentration of CD437 was 2 µg/mL, and at concentrations greater than 4 µg/mL, the bacteria did not grow at all (Figure 1B). The number of viable bacteria decreased from 10⁶ CFU/mL to 10⁵ CFU/mL after 4 h of treatment with 4 µg/mL CD437. Additionally, at a concentration of 8 µg/mL, the number of viable bacteria decreased rapidly to 10⁵ CFU/mL after 2 h and gradually to ~10 CFU/mL after 24 h (Figure 1D). The results demonstrated that CD437 exerts bactericidal action in a relatively short time. Next, continuous culture under sub-inhibitory drug concentrations was employed to screen for resistant bacteria. During a passage over 12 consecutive days, the MIC of CD437 for *E. faecalis* ATCC 29212 increased from 4 to 16 µg/mL (Figure 1E), whereas that of ceftriaxone sodium increased from 16 to 1024 µg/mL, a 64-fold increase over 12 consecutive days (Figure 1F). Under the same culture conditions, the MIC of vancomycin did not change (Figure 1G). These results indicate that CD437 has good

bactericidal effect, and the probability of inducing drug-resistant mutations is low.

Effect of CD437 on Biofilm Formation of *E. faecalis* ATCC 29212

Drug resistance can increase significantly with the formation of bacterial biofilm. CV staining can stain not only bacteria, but also biofilm extracellular matrix (including extracellular polysaccharide, protein, and DNA). CV staining showed that with increase in CD437 concentration, the biomass of biofilm decreased gradually, and when the concentration of CD437 was 8 µg/mL, biofilm formation was completely inhibited (Figure 2A). This result was consistent with the results of our XTT assay for detecting the metabolic activity of cells in biofilms (Figure 2B), which indicated that the MBIC was 8 µg/mL. The CLSM results showed that when the concentration of CD437 was two times the MBIC (16 µg/mL), very few bacteria adhered to the slide to form biofilm, and bacteria that did adhere

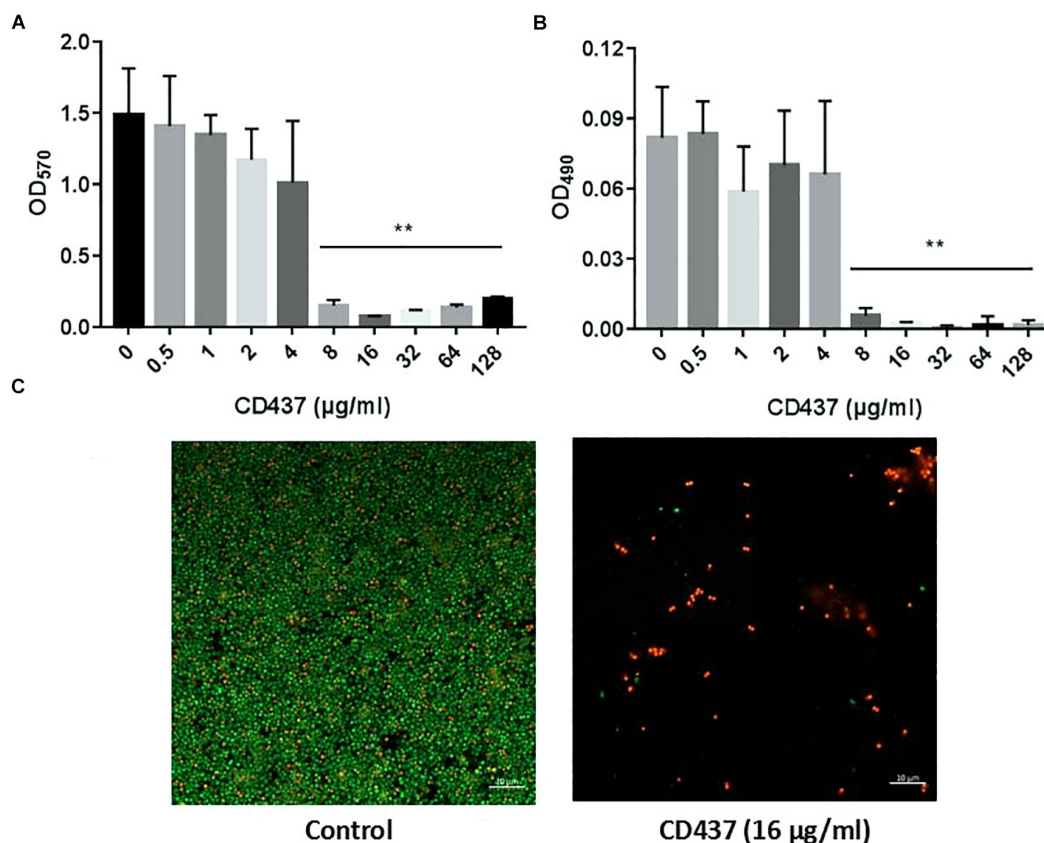


FIGURE 2 | CD437 inhibits the formation of *E. faecalis* ATCC 29212 biofilm. **(A)** Different concentrations of CD437 were co-cultured with bacteria in 96-well plates at 37°C for 24 h. Biofilms were stained with crystal violet and quantified using the absorbance measured at 570 nm. **(B)** Biofilms were treated using the XTT assay and the absorbance was measured at 490 nm. **(C)** The effect of CD437 on *E. faecalis* ATCC 29212 biofilm formation, according to the CLSM. Biofilms were formed on glass slides and stained with SYTO9 (green, live bacteria) and PI (red, dead bacteria). Data are presented as mean $\pm$ s.d. The results are representative of three independent experiments. $**P < 0.01$.

were determined to be dead through staining with PI (red stain) (Figure 2C). CV staining in 96-well plates showed that CD437 could not eradicate mature biofilm, as the biomass did not decrease after treatment (Supplementary Figure S1). The number of viable cells in biofilm was also decreased in a dose-dependent manner (Figure 3A), and the colony count verified this result (Figure 3B). The CLSM showed that CD437 killed some cells in the mature biofilm at a concentration of 4 μ g/mL, and as the concentration of CD437 increased, a greater number of red (dead) cells were observed. At a concentration of 64 μ g/mL, almost all the biofilm cells were dead, and only a small number of green (live) cells were seen (Figure 3C). These results indicate that CD437 is capable of killing bacteria in biofilms, although it cannot eradicate them.

Effect of CD437 and Antibiotic Combination on *E. faecalis* ATCC 29212

To study the effect of CD437 on enterococci infection in combination with several antibiotics, we designed a checkerboard drug combination assay. The results showed that, when CD437 at a concentration of 1 μ g/mL was combined with gentamicin,

the MIC of gentamicin decreased from 8 to 0.25 μ g/mL, a 32-fold decrease (Figure 4A). When CD437 was combined with ceftriaxone sodium, the addition of 2 μ g/mL CD437 reduced the MIC of ceftriaxone sodium from 4 to 1 μ g/mL, a 4-fold decrease (Figure 4B). However, when CD437 was combined with levofloxacin or vancomycin, the MIC of CD437 or the antibiotics did not decrease (Figures 4C,D). When the FICI was calculated, CD437 was found to have a synergistic effect when combined with gentamicin, with an FICI of 0.281. Additionally, CD437 had an additive effect when combined with ceftriaxone sodium, and the FICI was 0.75. The FICI was greater than 1 when CD437 was combined with levofloxacin and vancomycin, indicating that CD437 is ineffective in the presence of ofloxacin and vancomycin (Table 1).

Effect of CD437 on Gram-Positive Cocci and Gram-Negative Bacilli

The MIC of CD437 for four *E. faecalis* clinical strains was 4 μ g/mL, while the MBIC was 8 μ g/mL. The MIC for *S. aureus* RJ-2 was 2 μ g/mL and the MBIC was 4 μ g/mL. The MIC and MBIC for biofilm-positive *S. epidermidis* RP62A were both

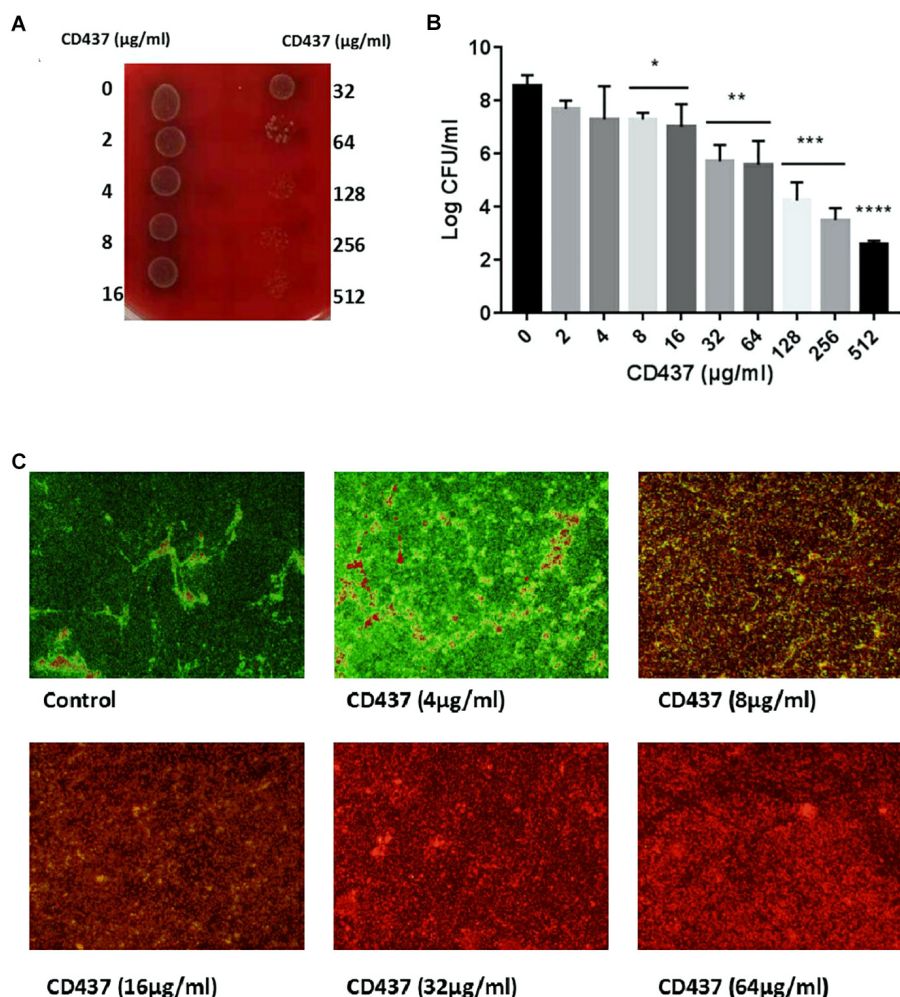
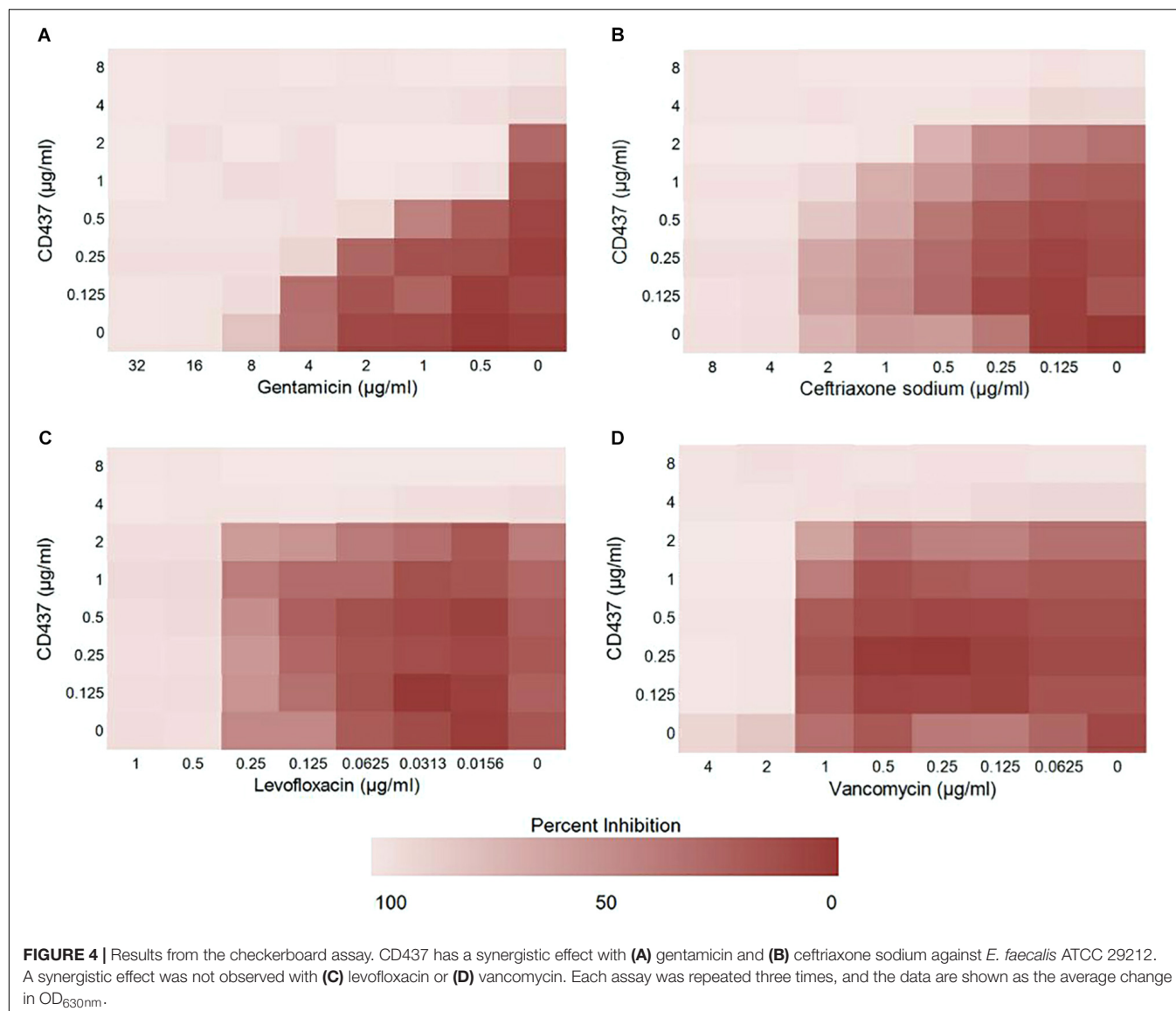


FIGURE 3 | CD437 kills *E. faecalis* ATCC 29212 in mature biofilm. Mature biofilms (24-h-old) were treated with CD437 for another 24 h. **(A)** Biofilms were broken up by vigorous pipetting and detached from wells, and then 5 μ L of bacterial suspension from each well was dropped onto blood agar before incubation at 37°C. **(B)** Live cells in biofilm were enumerated by colony count. **(C)** CD437 killed bacteria in mature biofilm on slides. Representative CLSM images of mature biofilms of *E. faecalis* ATCC 29212 on glass slides treated with CD437 for 24 h. Biofilms were stained with SYTO9 (green, live bacteria) and PI (red, dead bacteria). Images were taken at a magnification of 200 $\times$. Data are presented as mean $\pm$ s.d. ($n = 3$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; and **** $P < 0.0001$.

4 μ g/mL, and the MIC for biofilm-negative *S. epidermidis* ATCC 12228 was 2 μ g/mL, indicating that CD437 had a potent bacteriostatic effect on *Staphylococcus*. CD437 had no effect on *P. aeruginosa* PAO1 (Table 2). The MBIC test showed that CD437 effectively inhibited biofilm formation by *S. epidermidis*, *S. aureus*, and *E. faecalis*, but exerted no effect on gram-negative bacilli (Table 2). However, when PAO1 biofilm present on a glass slide in a 6-well plate was incubated with CD437, biofilm matrix was found to be reduced. The control biofilm produced a viscous extracellular matrix, and when the slides were picked up with a pair of tweezers, wire drawings appeared, but this phenomenon was not observed with PAO1 biofilm treated with CD437. When the slides were then subjected to CV staining, the biofilms of the control group were reticulated and cross-linked, while the CD437-treated biofilm bacteria formed mushroom-like aggregates (Figure 5), and this was more evident

in the CLSM observation of PAO1 (Supplementary Figure S2). In the clinical isolate of *P. aeruginosa*, the above similar phenomena were noticed but their biofilm formation ability was lower than PAO1 and the matrix reduction was not clear as that of PAO1 (Supplementary Figure S3). However, during semi-quantitative CV staining of 96-well plates, there was no significant change in OD_{570nm} (Supplementary Figure S4). Therefore, CD437 did not inhibit the growth and adhesion of *P. aeruginosa*, nor did it reduce the total biomass of *P. aeruginosa* biofilm. There is a research which suggested that PAO1 biofilm matrix thickness was associated with exopolysaccharide, not extracellular DNA, in particular, Pel and Psl (Lee et al., 2017). Pel and Psl polysaccharide production was indirectly measured by qPCR analysis of *pslA* and *pelA*, which are the constituents of the *psl* and *pel* operons, respectively. The results showed that the relative expression levels of *pelA* and *pslA* genes of



CD437-treated PAO1 biofilm bacteria decreased as compared with the control group ($P < 0.05$) (Figure 5B). When CD437 is combined with gentamicin, ceftriaxone sodium, levofloxacin, and ciprofloxacin, it could not reduce the MBIC of these antibiotics to PAO1 (Supplementary Table S1).

Mammalian Cytotoxicity and Hemolytic Activity

The HC_{50} of CD437 for human erythrocytes was 25.95 $\mu\text{g/mL}$, while the IC_{50} value of CD437 for human liver cancer cell lines HepG2 and Bel-7404 was 3.834 and 4.951 $\mu\text{g/mL}$, respectively. The IC_{50} value for human colorectal cancer cell line HT-29 was 10.62 $\mu\text{g/mL}$. The IC_{50} value of CD437 for HUVECs and human renal proximal tubular epithelium HK-2 was 16.48 and 19.52 $\mu\text{g/mL}$, respectively (Figure 6 and Table 3). These results show that CD437 is more toxic against human cancer cells than non-cancerous cells. The IC_{50} value of CD437 against human

non-cancerous cells was greater than the MIC (4 $\mu\text{g/mL}$) and MBIC (8 $\mu\text{g/mL}$) of CD437 against *E. faecalis*.

Evaluation of the Antibacterial Activities in a Mice Peritonitis Model

The *in vivo* efficacy of CD437 were evaluated in a mouse peritonitis model. Six-week-old ICR female mice were intraperitoneally injected with 1×10^8 colony-forming units (CFUs) of *E. faecalis* ATCC 29212 in 12.5% SRFE. As shown in Figure 7, compared to control group, *E. faecalis* abundance decreased approximately 5-fold in liver ($P < 0.01$) by 30 mg/kg CD437 alone while approximately 14-fold decrease in spleen ($P < 0.01$). On the other hand, 30 mg/kg gentamicin alone led to 4-fold decrease in bacterial load in liver ($P < 0.05$), and about 7-fold decrease in spleen ($P < 0.01$). 30 mg/kg CD437 in combination with 30 mg/kg gentamicin resulted in approximately 5-fold decrease in bacterial load in liver

TABLE 1 | Combined effect of CD437 with antibiotics.

Antibacterial agent	Class	MIC _A (ug/mL)		MIC _B (ug/mL)		FICI	Outcome
		Alone	Combination	Alone	Combination		
Gentamicin	Aminoglycoside	8	0.25	4	1	0.281	Synergy
Ceftriaxone sodium	Cephalosporin	16	4	4	2	0.75	Additivity
Levofloxacin	Quinolones	0.5	0.5	4	2	1.5	No interaction
Vancomycin	Glycopeptide	2	1	4	4	1.5	No interaction

MIC_A is the minimum inhibitory concentration of the antibiotic; MIC_B is the minimum inhibitory concentration of CD437; and FICI is the fractional inhibitory concentration index.

TABLE 2 | MIC and MBIC of CD437 for gram-positive cocci and gram-negative bacilli.

Strain	Type	MIC (ug/mL)	MBIC (ug/mL)
<i>S. epidermidis</i> RP62A	ATCC 35984	4	4
<i>S. epidermidis</i> 12228 (BF ⁻)	ATCC 12228	2	/
<i>S. epidermidis</i> 1	Clinical isolate	4	4
<i>S. epidermidis</i> 5	Clinical isolate	2	4
<i>S. epidermidis</i> 10 (BF ⁻)	Clinical isolate	4	/
<i>S. epidermidis</i> 11 (BF ⁻)	Clinical isolate	4	/
<i>S. aureus</i> RJ-2	Clinical isolate (MRSA)	2	4
<i>E. faecalis</i> 1	Clinical isolate	4	8
<i>E. faecalis</i> 9	Clinical isolate	4	8
<i>E. faecalis</i> 11	Clinical isolate	4	8
<i>E. faecalis</i> 13	Clinical isolate	4	8
<i>A. baumannii</i>	ATCC 1195	>64	>64
<i>K. pneumoniae</i>	ATCC 700603	>64	>64
<i>E. coli</i>	ATCC 25922	>64	>64
<i>P. aeruginosa</i> PAO1	ATCC 15692	>64	>64
<i>P. aeruginosa</i> 7	Clinical isolate	>64	>64
<i>P. aeruginosa</i> 14	Clinical isolate	>64	>64
<i>P. Aeruginosa</i> 17	Clinical isolate	>64	>64
<i>P. Aeruginosa</i> 47	Clinical isolate	>64	>64

BF⁻ is negative for biofilm formation.

($P < 0.01$), and around 21-fold decrease in spleen ($P < 0.001$). These results suggested that CD437 is effective *in vivo*.

DISCUSSION

Enterococcus can survive for long periods on environmental surfaces, such as medical equipment, bed rails, and door handles. These bacteria are resistant to heat, chlorine, and some alcoholic agents; additionally, *Enterococcus* often forms biofilm, making treatment difficult.

CD437 is a retinoid-like small molecule originally identified as a selective retinoic acid receptor γ (RAR γ) agonist (Han et al., 2016). It induces cancer cell apoptosis through an unknown mechanism, while exerting no effect on non-cancerous cells. CD437 is toxic to several cancer cell lines derived from primary tumors, including ovarian cancer, non-small cell lung cancer, leukemia, breast cancer, and squamous cell carcinoma

(Schadendorf et al., 1996; Sun et al., 1997; Hsu et al., 1999; Holmes et al., 2003; Rees et al., 2016). The most appealing feature of the use of CD437 as an anti-cancer drug is its selective toxicity against cancer cells and its low toxicity against normal cells (Han et al., 2016). Our data confirmed these results of previous studies. Additionally, A recent study has shown that CD437 can inhibit hepatitis B virus DNA amplification. CD437 directly inhibits DNA polymerase α (Pol α) (Han et al., 2016), and Pol α is vital for intracellular amplification of covalently closed circular DNA (cccDNA) (Tang et al., 2019). These findings indicate that CD437 could have broader therapeutic applications, extending beyond killing cancer cells.

The MIC of CD437 for *E. faecalis* ATCC 29212 and clinical strains of *E. faecalis* was 4 μ g/mL, and the MBIC was 8 μ g/mL, which was lower than the IC₅₀ and HC₅₀ values of CD437. These results suggest that CD437 could be a strong candidate for a new bactericidal drug. Although CD437 cannot eradicate an already formed mature biofilm, it can partially kill some bacteria in the biofilm at a relative low concentration. *E. faecalis* is inherently resistant to several antibiotics and readily accumulates mutations, which further contributes to resistance. In recent years, vancomycin-resistant enterococci have emerged, causing difficulty in treatment. Continuous culture of *E. faecalis* under sub-inhibitory drug concentrations of CD437 showed that CD437 had a relatively low incidence of drug-resistant mutations, compared with the mutation rate when cultured with ceftriaxone sodium.

Combination therapy is often required to treat complex enterococcal infections, in addition to infections of high inoculum and those with biofilm. For non-complex infections, monotherapy is generally sufficient. Endocarditis and meningitis infections caused by *Enterococcus* are usually treated with penicillin or ampicillin in combination with aminoglycoside antibiotics (Mercuro et al., 2018). In this study, the checkerboard assay showed that CD437 had a significant synergistic effect when combined with the aminoglycoside antibiotic gentamicin and had an additive effect when combined with the β -lactam antibiotic ceftriaxone sodium. CD437 alone or in combination with gentamicin also exhibited efficacy in a mice peritonitis model. Gram-positive bacteria contain a single inner phospholipid bilayer, while gram-negative bacteria have two lipid bilayers, and these membranes consist of three primary families of phospholipids: phosphatidylethanolamine (70–80% of total lipids), phosphatidylglycerol (20–25% of total lipids), and cardiolipin (5–10% of total lipids)

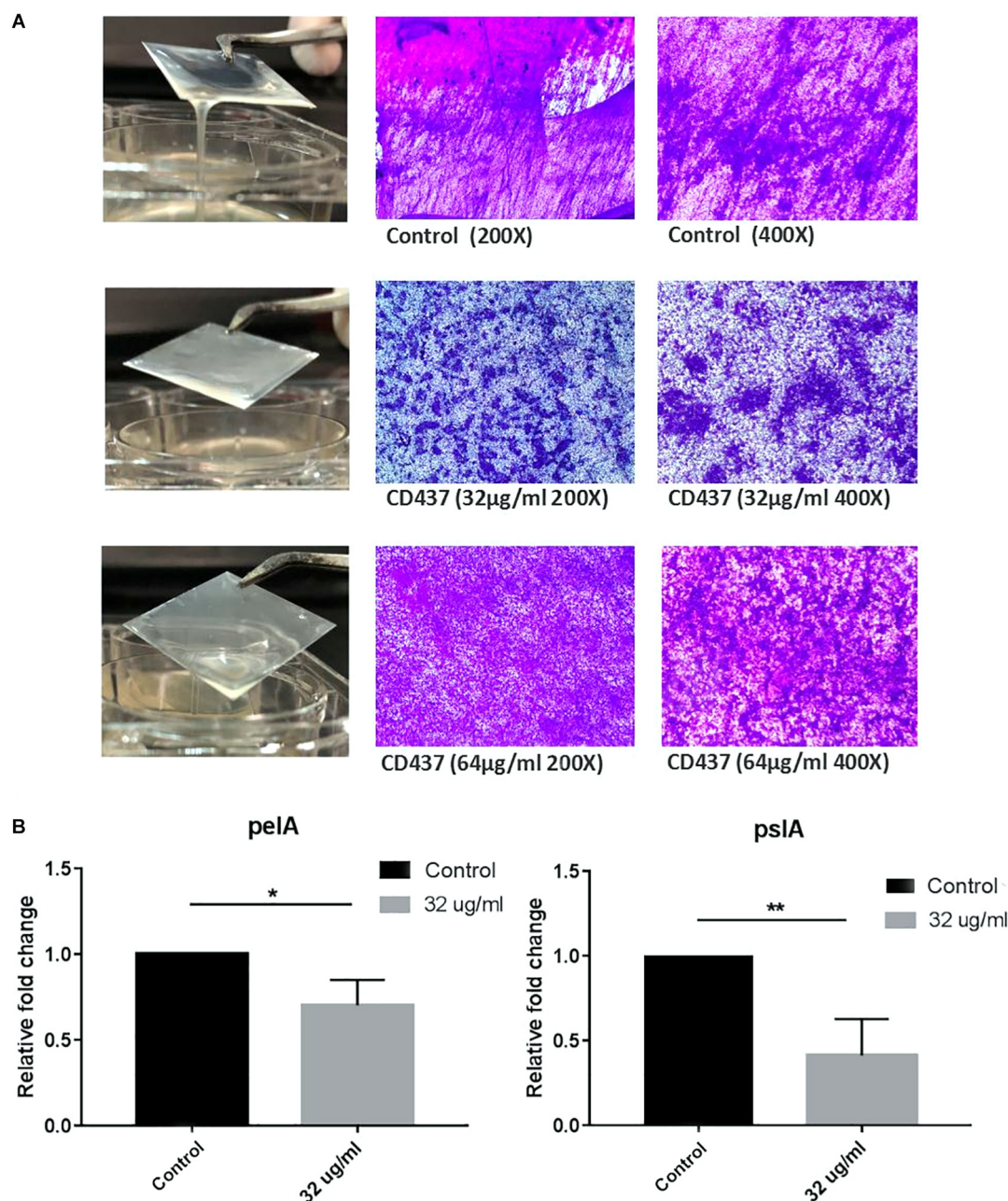


FIGURE 5 | CD437 reduces the formation of PAO1 biofilm matrix, but does not inhibit bacterial adhesion. **(A)** Representative images of 0, 16, and 32 µg/mL of CD437 applied to the PAO1 biofilm. Biofilms were stained with CV. **(B)** Relative expression of *pelA* and *pslA*. PAO1 were cultured with or without CD437 for 24 h, and the transcription of *pelA*, *pslA* was measured by qPCR. * $P < 0.05$; ** $P < 0.01$.

(Auer and Weibel, 2017). CD437 has been reported to kill MRSA cells by destroying the cell membrane lipid bilayer (Kim et al., 2018). As evident above, we speculate that CD437 may kill *E. faecalis* using the same mechanism. Aminoglycoside antibiotics mainly act on the 30S subunit of the bacterial ribosome, which affects bacterial protein synthesis. Therefore, the synergistic effect of CD437 and gentamicin is likely to be the result of increased gentamicin

diffusion through the bacterial cell membrane that has been physically damaged by CD437.

CD437 exhibited no bactericidal effect on gram-negative bacilli, including *K. pneumoniae*, *A. baumannii*, *E. coli*, and *P. aeruginosa* (Table 2). In this study, we confirmed that CD437 has no inhibitory effect on the biomass (CV staining) of *P. aeruginosa* biofilm. One explanation for this result could be that, in the

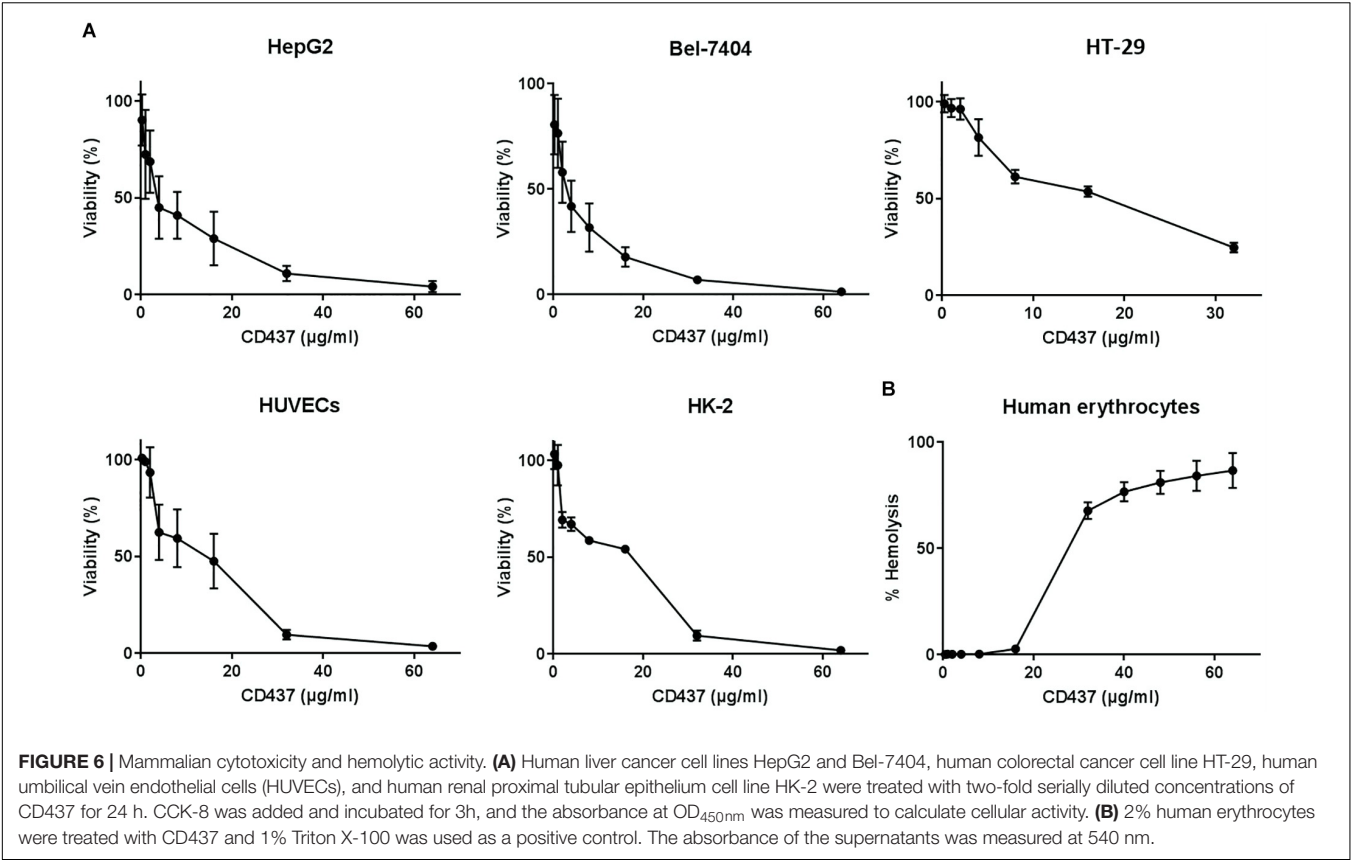
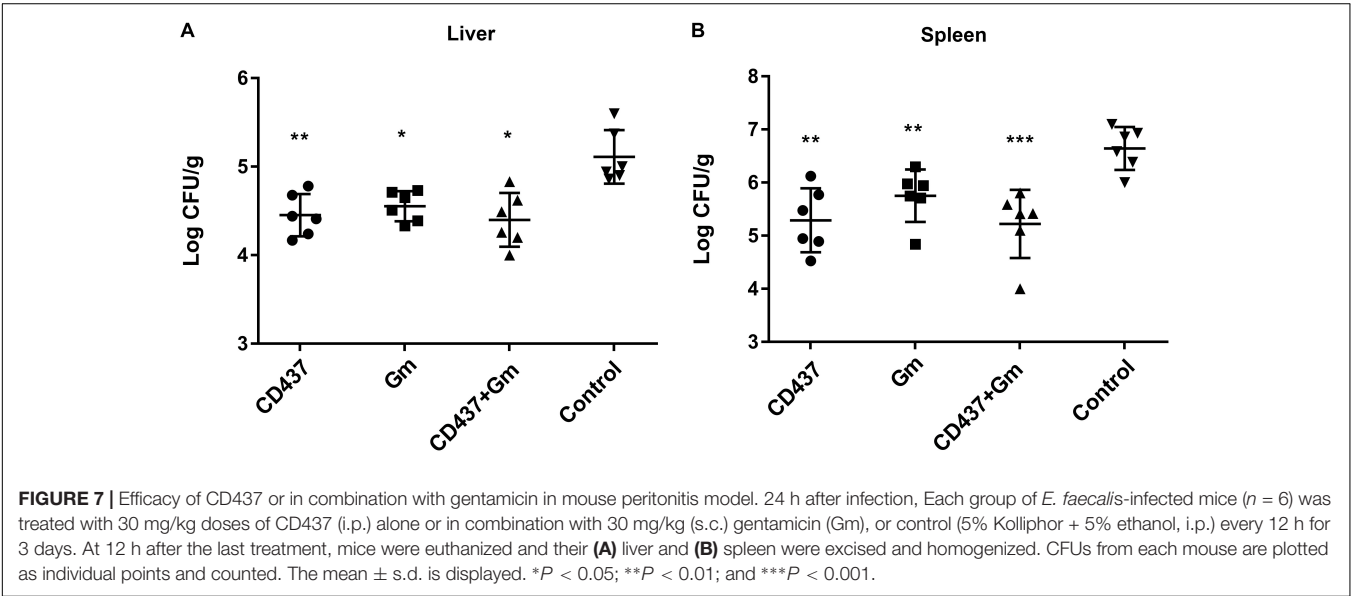


TABLE 3 | IC₅₀ values of CD437 for human cell lines.

Cell lines	Human cancer cell lines			Human normal cell line	
	HepG2	Bel-7404	HT-29	HK-2	HUVECs
IC ₅₀ (ug/mL)	3.834	4.951	10.62	19.52	16.48

HepG2, human liver cancer cell line; Bel-7404, human liver cancer cell line; HT-29, human colorectal cancer cell line; HUVEC, human umbilical vein endothelial cell; HK-2, human renal proximal tubular epithelium. The results represent the average of triplicates.



-well plate semi-quantitative CV staining process, the viscous matrix is easily removed during the washing process. This effect may result in no detectable change in OD_{570nm} between wells. However, in the slide-established biofilm, we found that CD437 inhibited extracellular polymeric substance matrix formation of PAO1, although it did not have an effect on bacterial adhesion and growth. The reduction in matrix is due to the low expression of *pelA* and *pslA*, which are the components of the operons for the synthesis of *pel* and *psl* polysaccharides.

In summary, CD437 has potent bactericidal effect on *E. faecalis*, and it not only inhibits the formation of *E. faecalis* biofilm but also has a killing effect on mature biofilms that are already formed. Additionally, CD437 has bactericidal action against *Staphylococcus* and inhibits the formation of biofilm. While CD437 exhibits no bactericidal effect on *P. aeruginosa*, it reduces the production of its extracellular polymeric substance matrix. Our study suggests that the retinoid compound CD437 has the potential to be further developed as a novel antibacterial drug.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

This study was carried out in accordance with the Animal Welfare Act and the National Institutes of Health guidelines for animal care and use, and all experimental protocols were approved by the IRB of the Third Xiangya Hospital, Central South University (No: 2015-S023). Strains and blood were isolated from clinical samples routinely collected from patients, and the identification of patients was not needed. Therefore, the need for written informed consent was waived and oral informed consent was obtained.

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STANDARD BIOSECURITY AND INSTITUTIONAL SAFETY PROCEDURES

All the biosafety measurements have been adopted and the institutional safety procedures are adhered. The laboratory of our institution has biosafety level 2 (BSL-2) standard where all standards and protocols are adopted as per the guidelines of CLSI.

AUTHOR CONTRIBUTIONS

YW, PS, and FT designed the experiments. FT performed most of the experiments, analyzed the results, and wrote the manuscript. LC and ZL provided the essential reagents and methods. LZ and YL performed the supporting experiments. YW conceived and supervised the study. All the authors read and approved the final manuscript.

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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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Balsacone C, a New Antibiotic Targeting Bacterial Cell Membranes, Inhibits Clinical Isolates of Methicillin-Resistant *Staphylococcus aureus* (MRSA) Without Inducing Resistance

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New options are urgently needed for the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infections. Balsacone C is a new dihydrochalcone extracted from *Populus balsamifera* that has been reported previously as being active against *Staphylococcus aureus*. Here, we evaluate the antibacterial activity of balsacone C against MRSA. Thirty-four (34) MRSA isolates were obtained from hospitalized patients; these isolates were then characterized for their resistance. Most of these MRSA (>85%) were resistant to penicillin, amoxicillin/clavulanic acid, ciprofloxacin, moxifloxacin, levofloxacin, clindamycin, erythromycin, and cefoxitin as well as being sensitive to linezolid, trimethoprim/sulfamethoxazole, rifampicin, and gentamicin. When tested against all MRSA isolates and various gram-positive bacteria, the antibacterial activity of balsacone C produced a MIC of 3–11.6 mg/mL. We observed no resistant isolates of MRSA (against balsacone C) even after 30 passages. Microscopy fluorescence showed that bacteria cell membrane integrity was compromised by low concentrations of balsacone C. Scanning electron microscope (SEM) confirmed balsacone C–provoked changes in the bacterial cell membrane and we find a dose-dependent release of DNA and proteins. This loss of cellular integrity leads to cell death and suggests a low potential for the development of spontaneous resistance.

Keywords: *Populus balsamifera*, buds, balsacone, antibiotic, *Staphylococcus aureus*, MRSA

INTRODUCTION

Infectious diseases are now the second leading global cause of death in the world. Across the globe, bacterial infections kill 700,000 people annually (World Health Organization [WHO], 2016), and by 2050, deaths attributable to antibiotic-resistant infections may even exceed cancer-related deaths (Bagnoli et al., 2017). Methicillin-resistant *Staphylococcus aureus* (MRSA) infections

remain a primary cause of infection-related mortality and represent a major global health care problem. This antibiotic-resistant bacteria was first identified within a health care setting around 1960, and it emerged in the community in the early 1990s (Vestergaard et al., 2019). Methicillin resistance in *Staphylococcus aureus* is due to the acquisition of the mobile genetic element SCCmec (mecA or mecC). This gene codes for a penicillin-binding protein (PBP) that makes the strain resistant to all beta-lactam antibiotics (Bagnoli et al., 2017). Since *S. aureus* has a notorious ability to acquire or develop resistance to antibiotics, it is considered to be a global pandemic threat (CDC, 2013; Sergelidis and Angelidis, 2017). The rapidly increasing limitations of vancomycin and teicoplanin as primary therapies for severe and life-threatening infections have also raised concerns (Gardete and Tomasz, 2014). Within the past 20 years, MRSA has developed reduced susceptibility to vancomycin-intermediate *Staphylococcus aureus* (VISA), and complete resistance has emerged (VRSA) (Bal et al., 2017). Moreover, MRSA resistance to linezolid and daptomycin has also been documented (McGuinness et al., 2017). Novel antibiotics are required urgently to combat this life-threatening pathogen (Laxminarayan et al., 2014; Assis et al., 2017; Subramani et al., 2017).

Over the last 30 years, the gap has widened between the emergence of antibiotic-resistant strains and the development of new antibiotics. Because of this disparity, there is heightened interest in finding new bioactive compounds derived from plants (Subramani et al., 2017). The widespread use of antibiotics derived from fungal or bacterial origin since the 1950s has limited interest in the study of plant compounds as potential antimicrobial agents. Nevertheless, most plants in natural settings respond to fungal and bacterial pathogens by activating low molecular weight antimicrobial compounds (Taylor, 2013). Subramani et al. (2017) list 15 plant-derived compounds and 40 plant extracts that demonstrate antibacterial activities against various multidrug-resistant pathogens, including MRSA.

Several plant species in the Canadian boreal forest show promise as sources of new antibiotics. A new class of potential antibiotics has been recovered recently from buds of *Populus balsamifera* L. (Lavoie et al., 2013). *P. balsamifera* (Salicaceae) is a hardwood tree having a widespread distribution in eastern North America. Buds from this species were used frequently by First Nation populations as treatment for a range of health problems, from the common cold to diabetes (Moerman, 1998). The use of *P. balsamifera* buds within different types of preparations that were applied to wounds, cuts, frostbite, and insect bites suggests that these buds may possess antibacterial properties.

In a previous study, we showed that *P. balsamifera* buds extract had antibacterial activity against a single strain of methicillin-sensitive *Staphylococcus aureus* (MSSA). We also isolated for the first time the Balsacones A, B and C and elucidated their structures. The Balsacone C (BC) (C₂₄H₂₂O₅) is a new dihydrochalcone derivative from *P. balsamifera* buds and was found to be one of the most potent compounds (Lavoie et al., 2013). In the present study, we characterize a suite of clinical MRSA isolates obtained from patients hospitalized at the Chicoutimi Hospital, Saguenay, QC, Canada. We then

evaluate the antibacterial activity of BC (Supplementary Figure S1) against MRSA and various gram-positive and gram-negative bacterial strains. In addition, we assess the propensity of MRSA to develop resistance to BC. Finally, in this work, we investigate the action mechanism responsible for the antibacterial activity of BC and assess its cytotoxicity on human normal cells.

MATERIALS AND METHODS

Cell Culture

We obtained human skin fibroblasts WS1 (ATCC CRL-1502) from the American Type Culture Collection (Manassas, VA, United States). Cells were grown in a humidified atmosphere at 37°C in 5% CO₂, in Dulbecco's Minimum Essential Medium supplemented with 10% fetal calf serum (Hyclone, Logan, UT, United States), 1 × solution of sodium pyruvate, 1 × vitamins, 1 × non-essential amino acids, 100 IU of penicillin and 100 µg/ml streptomycin (Cellgro®, Mediatech, Manassas, VA, United States).

Isolation and Characterization of Methicillin-Resistant *S. aureus* Isolates

Thirty-four (34) isolates of MRSA and one (1) MSSA were obtained from nares (*n* = 31), throat (*n* = 3) and groin pus (*n* = 1) from patients hospitalized at the Chicoutimi Hospital. We identified *S. aureus* using a Slidex Staph-Kit (bioMérieux Vitek, Inc., Hazelwood, MO, United States). Quality control was performed with MRSA isolates ATCC 43300 and *S. aureus* strain ATCC 25923. We determined the *S. aureus* profile using an API Staph test strip having a saline bacterial suspension having a 0.5 McFarland standard (bioMérieux, Durham, NC, United States). API Staph strips were left for 24 h before the results were read. For all isolates, we ran a latex agglutination (Slidex) test to detect methicillin resistance in Staphylococci – based on the production of low-affinity PBP2a, which is encoded by the mecA or mecC gene (data not shown).

Antibiotic Susceptibility Using the Disk Diffusion Method

We produced an antibiogram to confirm the identification of MRSA isolates using a disk diffusion test (Kirby-Bauer) as described by the National Committee for Clinical Laboratory Standards (NCCLS) (Clinical Laboratory Standard Institute [CLSI], 2019). The tested antibiotics included: penicillin (10 units), amoxicillin/clavulanic acid (20/10 µg), ciprofloxacin (5 µg), moxifloxacin (5 µg), levofloxacin (5 µg), clindamycin (15 µg), erythromycin (15 µg), cefoxitin (30 µg), linezolid (30 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), rifampicin (5 µg), gentamicin (10 µg), and vancomycin (30 µg). The cultures were first inoculated on a non-selective plate for 18–24 h. We collected each colony and then transferred the individual colonies to a 5-ml tryptic-soy broth. The inoculum density was standardized with BaSO₄ using 0.5 McFarland standard, and all isolates were incubated at 35°C. We then inoculated the Muller-Hinton agar plates. After 15 min, we

applied the disks having a fixed concentration of antibiotics to the plate surface. Plates were incubated at 35°C for 16–24 h. We measured the growth inhibition zone around each disk, and we related the diameter of the zone to the bacteria susceptibility. The results of the disk diffusion test are expressed as susceptible, intermediate, or resistant. We used *S. aureus* ATCC 25923 as our quality control organism.

Compound and Bacterial Strains

Lavoie et al. (2013) describe the isolation of BC. Purity was confirmed by ¹H-NMR (proton magnetic resonance spectroscopy used to determine the structure of a molecule). Antimicrobial activities of BC were tested against *Escherichia coli* (ATCC 25922), *S. aureus* (ATCC 25923), *Enterobacter aerogenes* (C3032834), *Enterobacter cloacae* (B9040334), *Salmonella typhimurium* (C6162763), *Burkholderia cepacia* (C6101997), *Klebsiella pneumonia* (B8302928), *Staphylococcus epidermidis* (B9030482), *Enterococcus faecalis* (ERV), and *Listeria monocytogenes* (B8222880). All bacterial strains were provided by the Chicoutimi Hospital, Saguenay, QC, Canada. *Streptococcus uberis* (CL) was provided by Collège Laflèche, Trois-Rivières, QC, Canada.

Antibacterial Activity Measurements Using the Microdilution Method

We tested the antibacterial activities of BC using the microdilution method of Banfi et al. (2003). Briefly, 50 µL of growing bacteria were plated in 96-well plates (Costar, Corning Inc.) in nutrient broth (Difco). Increasing concentrations of BC (diluted in methanol) were then added. The final concentration of methanol in the culture medium was maintained at 0.1% (v/v) to avoid solvent toxicity. The negative control was a bacterial suspension without treatment, and the blank consisted of a culture medium only. We tested the bacterial suspension plus solvent to demonstrate the absence of solvent toxicity. Concentration of 3.5×10^5 CFU/mL was used. Microplates were incubated for 24 h at 37°C, and the absorbance was then measured at 540 nm using an automated Varioskan Ascent plate reader. Results are expressed as the concentration at which 100% of bacterial growth is inhibited (MIC).

Induction of Resistance Against BC and Rifampicin

To induce the emergence of drug resistance, we used the broth-dilution procedure of Dalhoff et al. (2005). Briefly, approximately 3.5×10^5 CFU was added to 10 ml nutrient broth. Bacteria were grown overnight in nutrient broth containing different concentrations of the compounds (1–10 mg/L for BC and 0.004– mg/L for rifampicin). For each passage (passage refers to transferring bacteria from a previous culture to a fresh growth medium), we transferred an aliquot (1:20) from the tube containing the highest drug concentration with visible bacterial growth to a second set of serial antibiotic dilutions. After incubating the broths overnight, we repeated the dilution procedure for a total period of 10 passages for rifampicin and 30 passages for BC. After completion of the passages, we

subcultured the bacteria having the highest MIC onto a drug-free nutrient broth. We tested these latter samples for antibiotic susceptibility with microdilution assay. Results are expressed as the concentration inhibiting 50% of bacterial growth (IC₅₀).

Cell Membrane Integrity

To evaluate the integrity of cell membranes, we used the BacLight Live/Dead bacterial viability kit (L-7012; Molecular Probes).

TABLE 1 | Evaluation of antibacterial activity of balsacone C against *Staphylococcus aureus* and the clinical isolates of MRSA and MSSA.

Isolate number	Balsacone C	Isolate number	Balsacone C
	MIC (mg/L)		MIC (mg/L)
ATCC 25923	3.4 ± 0.7	08-U-0202	6.4 ± 0.8
08-U-0185	12 ± 1	08-U-0203	5.1 ± 0.6
08-U-0186	4.1 ± 0.6	08-U-0204	4.7 ± 0.6
08-U-0187	8 ± 1	08-U-0205	3.4 ± 0.5
08-U-0188	4 ± 1	08-U-0206	4 ± 1
08-U-0189	6.7 ± 0.7	08-U-0207	3 ± 1
08-U-0190	6.9 ± 0.4	08-U-0208	7.8 ± 0.4
08-U-0191	6 ± 1	08-U-0209	4.9 ± 0.7
08-U-0192	11 ± 1	08-U-0213	11.6 ± 0.5
08-U-0193	7.4 ± 0.5	08-U-0214	3.9 ± 0.1
08-U-0194	5.6 ± 0.6	08-U-0215	4.4 ± 0.3
08-U-0195	8.6 ± 0.7	08-U-0216	9.4 ± 0.7
08-U-0196	4.2 ± 0.6	08-U-0217	4.6 ± 0.3
08-U-0197	3.4 ± 0.2	08-U-0218	5.4 ± 0.1
08-U-0198	3 ± 1	08-U-0219	3.6 ± 0.4
08-U-0199	7.6 ± 1.0	08-U-0220	3.3 ± 0.6
08-U-0200	10 ± 1	08-U-0221	4.9 ± 0.6
08-U-0201	3.3 ± 0.6	08-U-0222	3.5 ± 0.5

ATCC 25923 is *Staphylococcus aureus*. Data are representative of three different experiments. Main ± standard deviation, n = 3. MIC is defined as the lowest concentration able to inhibit 100% of bacterial growth.

TABLE 2 | Evaluation of antibacterial activity of balsacone C against different bacteria.

Bacteria taxa	Antibacterial activity of balsacone C		
	Strain	Gram	MIC (mg/L)
<i>Enterococcus faecalis</i>	ERV	+	10.9 ± 0.8
<i>Listeria monocytogenes</i>	B8222880	+	20 ± 3
<i>Staphylococcus epidermidis</i>	B9030482	+	27 ± 2
<i>Streptococcus uberis</i>	CL	+	10.1 ± 0.7
<i>Burkholderia cepacia</i>	C6101997	–	32 ± 2
<i>Enterobacter aerogenes</i>	C3032834	–	>100
<i>Enterobacter cloacae</i>	B9040334	–	>100
<i>Escherichia coli</i>	ATCC 25922	–	>100
<i>Klebsiella pneumonia</i>	B8302928	–	>100
<i>Salmonella typhimurium</i>	C6162763	–	>100

Data are representative of three different experiments. Main ± standard deviation, n = 3. MIC is defined as the lowest concentration able to inhibit 100% of bacterial growth.

Briefly, we added approximately 2×10^7 CFU/ml to tubes containing 10 ml of nutrient broth. Untreated and treated samples having BC (IC_{50} : 1.5 mg/L and MIC: 3 mg/L) were grown for 24 h at 37°C and then were centrifuged at $10,000 \times g$ for 10 min. The supernatant was removed, and the pellet was resuspended in 2 ml NaCl (0.85%). We added 1 ml of the sample to 20 ml NaCl (0.85%), incubated the suspension for 1 h at room temperature, and then centrifuged the mixture at $10,000 \times g$ for 10 min (repeated twice). We removed the supernatant and resuspended the pellet in 10 ml NaCl. We added 3 μ L of Syto9 and 3 μ L of propidium iodide (PI) to each sample. Samples were then incubated at room temperature for 15 min. We analyzed the integrity of the bacterial membranes using a fluorescence microscope (Reichert) equipped with a halogen lamp, Neoplan 100 $\times$ /1.25 oil objective and a 1,713 filter cube (fluorescein; 490/510/520 nm) at $1,000\times$ magnification. In the assays, the fluorescent green nucleic acid stain Syto9 passes in living and dead bacterial cells, whereas PI cannot penetrate intact membranes. When the cellular membrane is damaged, PI can penetrate bacteria and cause the cells to appear red (31).

Scanning Electron Microscope

Previous work has shown that membrane damage can be confirmed by combining fluorescence microscopy and scanning electron microscope (SEM) imagery (De Sousa et al., 2012; Nostro et al., 2017). We prepared the samples for SEM following De Sousa et al. (2012) with some modifications. Briefly, untreated and treated samples having BC (1.5 and 3 mg/L) were grown 3 h at 37°C. We removed the samples from the cultures, washed them with PBS and then fixed the samples in phosphate buffers (pH 7.2) containing 2.5% glutaraldehyde for 2 h at room temperature. The fixed cells were collected via centrifugation at $2,000 \times g$ and washed three times with phosphate buffers. The fixed bacteria were dehydrated with ethanol (30–95%). We mounted the dried specimens on aluminum stubs using a conductive carbon cement; the specimens were allowed to dry and were then coated with a

gold film. We observed the samples with an SEM at 20 kV and $25,000\times$ magnification.

Release of Bacterial Intracellular Constituents

Intracellular material released from the cells was quantified as described in Virto et al. (2005) with some modifications. Briefly, we added approximately 8×10^7 CFU/ml to tubes containing 10 ml nutrient broth. Untreated and treated samples having BC (1.5 and 3 mg/L) were grown 3 h at 37°C and were then centrifuged at $2,000 \times g$ for 10 min. We transferred the supernatant in a cuvette and measured the UV absorbance using a spectrophotometer (Multiskan™ GO Spectrophotometer – Thermo Fisher Scientific) – nucleic acids have an absorption peak at 260 nm, proteins at 280 nm. We compared our results with those of untreated control samples.

Acridine Orange/Ethidium Bromide Staining

We evaluated the integrity of WS1 cell membranes using a double staining assay with acridine orange (AO) and ethidium bromide (EB) as described in Ribble et al. (2005). AO (15 mg) and EB (50 mg) were dissolved in 1 ml of 95% ethanol and then added to 49 ml of PBS, gently mixed, aliquoted, and stocked at -20°C . Before use, we diluted the stock solution 1/10 in PBS (pH 7.4). We plated growing WS1 cells (1×10^4 cells) onto a 96-well plate and incubated them for 18 h. After treating the cells with 1.5 and 3 mg/L of BC, we re-incubated them for 14 h. The cells were then washed with PBS and incubated for 5 min with the dual fluorescent staining solution (AO/EB). For our observations, we used Cytation3 (Cell Imaging Multi-Mode Reader) at excitation and emission wavelengths of 530 and 590 nm, respectively.

Calcein-AM Cell Cytotoxicity Assay

We tested the toxicity of BC against the human healthy cell line WS1 as described in Yang et al. (2002). In brief, we plated WS1 cells (1×10^4 cells) onto a 96-well plate and incubated the plates for 18 h. After the incubation period, we treated the cells with 1.5 and 3 mg/L of BC and 0.82 mg/L of doxorubicin. After 24 h of incubation, we removed the media and washed the cells with PBS. We then added 100 μ L of calcein-AM (0.25 mg/L) and incubated the cells for 20 min. WS1 cells were washed twice in PBS, and we observed the cells using Cytation3 (Cell Imaging Multi-Mode Reader) at excitation and emission wavelengths of 490 and 520 nm, respectively. First, we evaluated the effect of BC on WS1 membranes using double labeling with OA and EB (Mihoub et al., 2018). Human healthy cells were incubated in the presence or absence of BC at IC_{50} (1.5 mg/L) and MIC (3 mg/L) concentrations. Beta-hederin, a cytolytic triterpenoid, was used as positive control.

Statistical Analysis

For all analyses, we ran two-way ANOVAs all followed by a post-test Holm-Sidak method using SigmaStat® software (Systat Software Inc., San Jose, CA, United States). Differences were deemed as statistically significant when $P < 0.05$.

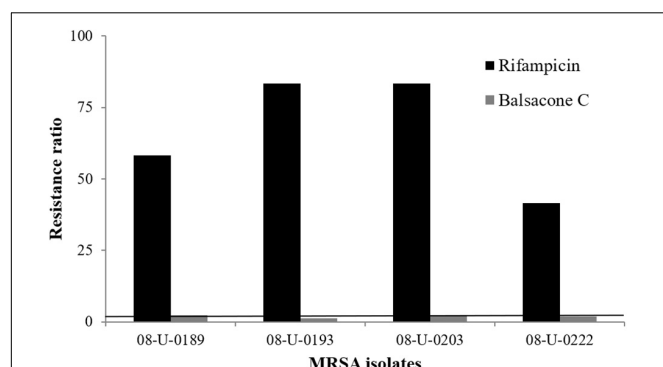


FIGURE 1 | Induced bacterial resistance using rifampicin and balsacone C on four different MRSA isolates. Resistance ratio is IC_{50} of resistant isolate/ IC_{50} of susceptible isolate (>30 fold) after 10 passages for rifampicin and 30 passages for balsacone C. The solid line defines the limits of equal susceptibility (onefold).

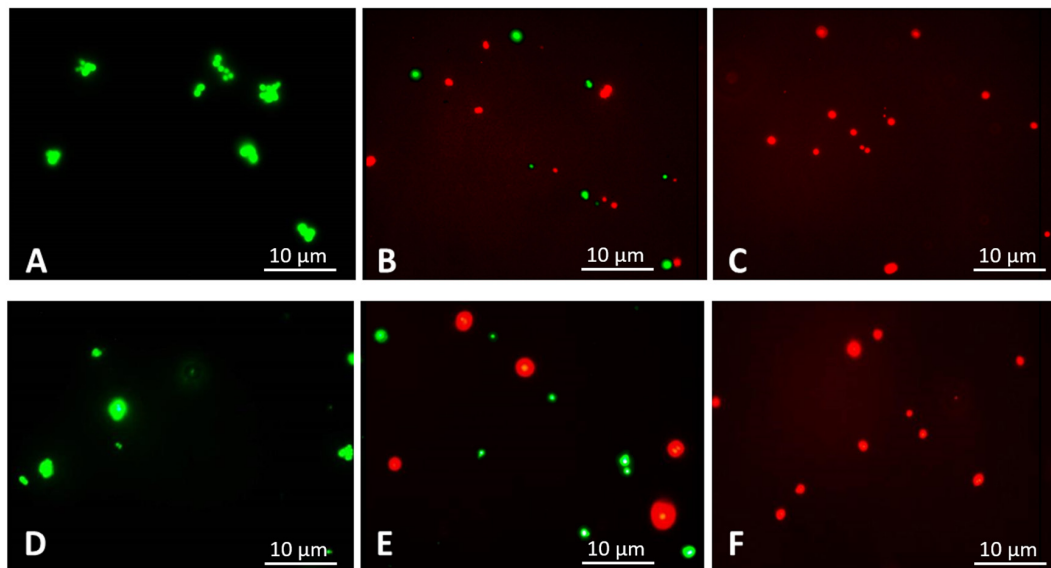


FIGURE 2 | Fluorescence microscopic images, 1,000× magnification. Following 3 h of treatment with balsacone C, *Staphylococcus aureus* and MRSA (08-U-0214) were stained using a LIVE/DEAD BacLight Viability kit. Live cells are indicated by green fluorescence (Syto 9), whereas cells having damaged membranes are indicated by red fluorescence (PI). Untreated *S. aureus* (negative control) (A); *S. aureus* after treatment with 1.5 mg/L balsacone C (B); *S. aureus* after treatment with 3 mg/L BC (C); untreated MRSA (negative control) (D); MRSA after treatment with 1.5 mg/L balsacone C (E); and MRSA after treatment with 3 mg/L balsacone C (F).

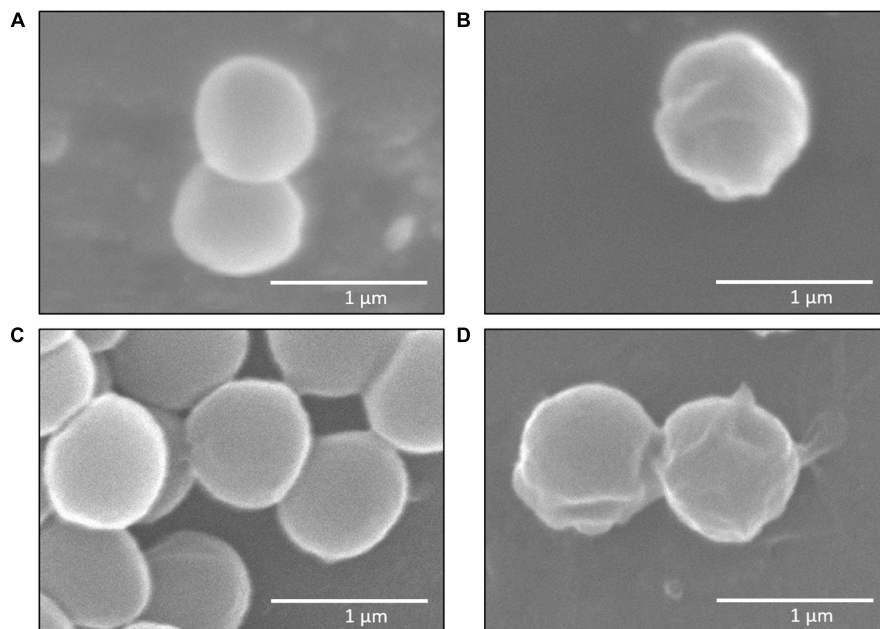


FIGURE 3 | Scanning electron microscope (SEM) images of *Staphylococcus aureus* and MRSA (08-U-0214), 25,000× magnification. Untreated *S. aureus* (negative control) (A); *S. aureus* after treatment with 3 mg/L balsacone C (B); untreated MRSA (negative control) (C); and MRSA after treatment with 3 mg/L balsacone C (D).

RESULTS AND DISCUSSION

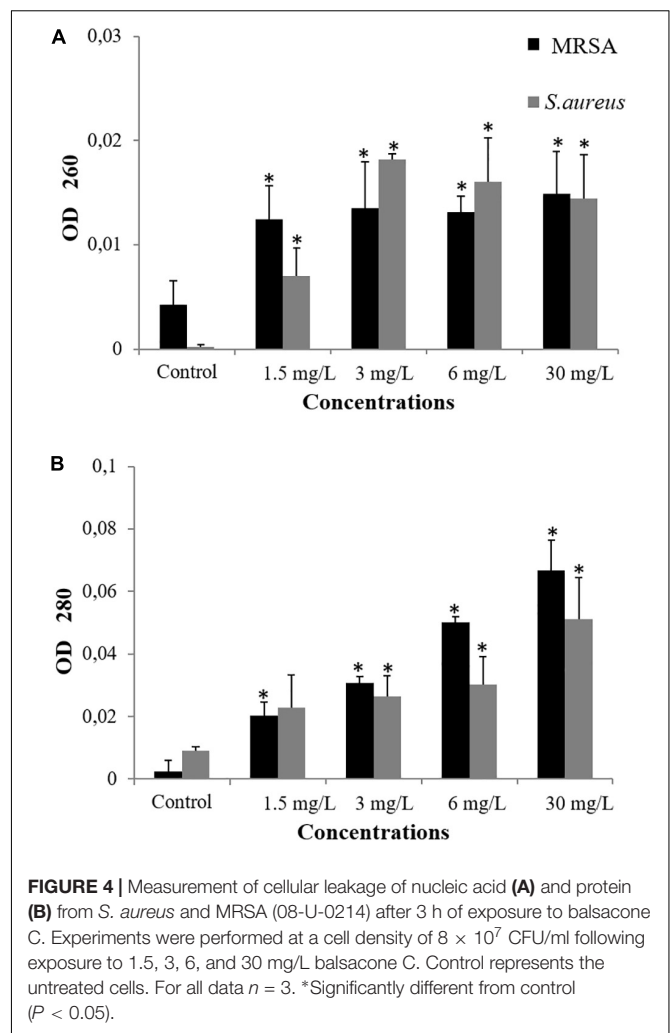
All 35 bacterial clinical isolates were indeed *S. aureus*. Moreover, API Staph identified two different *S. aureus* biotypes including the biotype 6736153 and the biotype 6736113 at a

probability of 97.8 and 86.7%, respectively (Supplementary Table S1). Our control *S. aureus* ATCC 25923 was identified as the biotype 6736153. A latex agglutination test for the PBP2a protein encoding by *mecA* genes also confirmed that thirty-four (34) bacterial isolated were MRSA (data

not shown). An antibiogram of *S. aureus* and all isolates was performed with various classes of antibiotics – they include beta-lactam (penicillin, amoxicillin/clavulanic acid), fluoroquinolone (ciprofloxacin, moxifloxacin, levofloxacin), lincosamide (clindamycin), macrolide (erythromycin), cephalosporin (cefoxitin), oxazolidone (linezolid), sulfonamide (trimethoprim/sulfamethoxazole), rifamycin (rifampicin), aminoglycoside (gentamicin) and glycopeptide (vancomycin). The results indicated that *S. aureus* (ATCC 25923) was sensitive to all antibiotics tested except levofloxacin (**Supplementary Tables S2, S3**). Moreover, with the exception of isolate 08-U-0189, all MRSA were resistant to penicillin, erythromycin, and cefoxitin. Consequently, isolate 08-U-0189 is considered as a MSSA. On the other hand, all isolates were 91.4% resistant to ciprofloxacin, moxifloxacin, levofloxacin, and clindamycin and 88.5% resistant to amoxicillin/clavulanic acid (**Supplementary Table S2**). These antibiogram profiles were similar to the typical phenotype of endemic MRSA isolates as experienced by Chao et al. (2013) in China. Interestingly, three MRSA were sensitive to all fluoroquinolone antibiotics (08-U-0194, 08-U-0204, and 08-U-0209). Moreover, 3% of MRSA were resistant to TMP/SMX and rifampicin, 6% to gentamicin and 34% had intermediate resistance to vancomycin, however, the MIC should be determined to confirm the presence of a VISA (**Supplementary Table S3**). All MRSA were sensitive to linezolid. The most resistant isolate of MRSA (08-U-0214), showed resistance to beta-lactam (penicillin, amoxicillin/clavulanic acid, methicillin), fluoroquinolone (ciprofloxacin, moxifloxacin, levofloxacin), lincosamide (clindamycin), macrolide (erythromycin), cephalosporin (cefoxitin), rifamycin (rifampicin), aminoglycoside (gentamicin), and intermediate to glycopeptide (vancomycin).

The antibacterial activity of BC, was evaluated using a microdilution assay against *S. aureus* (ATCC 25923) and the 35 MRSA and MSSA isolates. BC was active against all bacteria tested producing a MIC of 3–11.7 mg/L (**Table 1**). Furthermore, the most resistant MRSA (08-U-0214) was also sensitive to BC and had a MIC of 3.9 mg/L. Most of the new antibiotic candidates reported in the literature possess a MIC in the same range of BC. For example, Adnani et al. (2017) describe keyicin, a new bis-nitroglycosylated anthracycline, that has an action mechanism different from that of other anthracyclines. Keyicin was active against gram-positive *Bacillus subtilis* with a MIC of 7.97 mg/L and against MRSA with a MIC of 2.01 mg/L. Zhang et al. (2017) obtained MICs of 1.39–5.57 mg/L for two compounds from the Aurachin family that had antimicrobial activity against *S. aureus*, *Streptococcus pyogenes*, and *B. subtilis*. They obtained a MIC of 44.57 mg/L against MRSA, whereas rifampicin and ampicillin had respective MICs of 3.29 and 22.36 mg/L.

We tested BC activity against four gram-positive (*S. epidermidis*, *S. uberis*, *E. faecalis*, and *L. monocytogenes*) and six gram-negative bacteria (*B. cepacia*, *E. aerogenes*, *E. cloacae*, *E. coli*, *K. pneumonia*, and *S. typhimurium*). BC inhibited the bacterial growth of all gram-positive bacteria with a MIC of 10.1–32 mg/L (**Table 2**). In contrast, BC was inactive against the gram-negative taxa with a MIC > 100 mg/L. The exception was for *B. cepacia* with a MIC of 32 mg/L. *B. cepacia* is naturally



resistant to some antibiotic classes including polymyxins, aminoglycosides, trimethoprim, chloramphenicol, quinolones, and beta-lactams (Sousa et al., 2011). The intrinsic multidrug resistance of this bacteria occurs due to the presence of various enzymes and efflux pumps that remove antibiotics from the cell (De Soyza et al., 2008). Rushton et al. (2013) suggested membrane charge/ionization influences antibiotic binding and resistance. Interestingly, these resistance mechanisms of *B. cepacia* are not efficient against BC suggesting an alternative action mechanism is at work. Firstly, we investigated the BC action mechanism using a model of resistance induction described by Dalhoff et al. (2005). Using rifampicin as a positive control, the resistance of one MSSA (08-U-0189) and three MRSA (08-U-0193; 08-U-0203; 08-U-0222) isolates was achieved easily (**Figure 1**). Indeed, the IC₅₀ of rifampicin for all tested isolates was initially lower than 0.012 mg/L, while after ten passages of MRSA in the presence of sub-MIC levels of rifampicin, the isolates were at least 30 × more resistant with an IC₅₀ of 0.41 ± 0.07 to 1.00 ± 0.02 mg/L. Using the same approach, we attempted to induce BC resistance. The IC₅₀ of BC was first 1.4 ± 0.1 to 2.9 ± 0.5 mg/L, however, repeated passages (30) of MRSA in the presence of sub-MIC

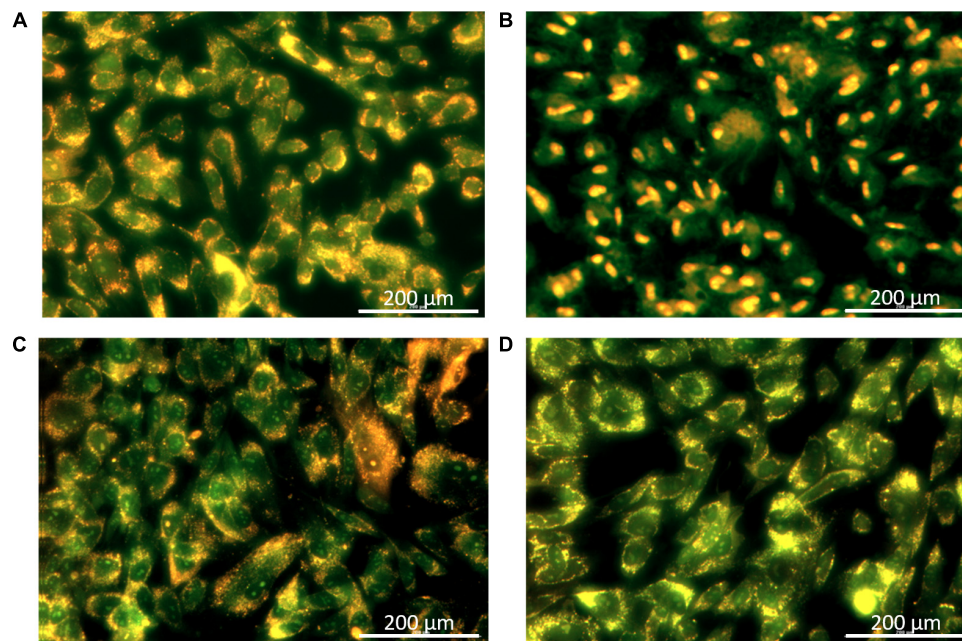


FIGURE 5 | Cellular membrane integrity after treatments with balsacone C; images were captured by a Cytation 3 Cell Imaging Multi-Mode Reader with a 20× objective. WS1 cells were stained with acridine orange/ethidium bromide to visualize intact membranes. Stained control cells (**A**); positive control beta-hederine 7.5 mg/L (**B**); cell treated with 1.5 mg/L balsacone C (**C**); and cell treated with 3 mg/L balsacone C (**D**). Data are representative of three different experiments.

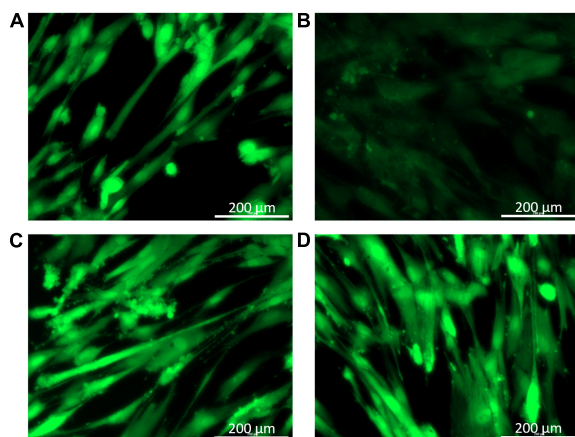


FIGURE 6 | Cell viability after treatments with balsacone C; images were captured by a Cytation 3 Cell Imaging Multi-Mode Reader with a 20× objective. WS1 cells were stained with calcein-AM to visualize living (green) and dead cells. Stained control cells (**A**); positive control doxorubicine 0.55 mg/L (**B**); cell treated with 1.5 mg/L balsacone C (**C**); and cell treated with 3 mg/L balsacone C (**D**). Data are representative of three different experiments.

levels of BC failed to produce resistant bacteria with an IC_{50} of 3.0 ± 0.3 to 3.9 ± 0.4 mg/L after induction.

The rapid bactericidal action of BC suggests that the possible action mechanism might occur via altering the integrity of bacterial cell membranes, as suggested by Ling et al. (2015) with their study on a new antibiotic, the teixobactin. The

effect of BC on the membrane integrity, assessed using the dead/live BacLight bacterial viability assay (Grégori et al., 2001; Stiefel et al., 2015), found untreated *S. aureus* (control) to have no membrane damage (**Figure 2A**). *S. aureus* treated with 1.5 mg/L BC (IC_{50}) generated a mix of green fluorescent (intact membrane) and red fluorescent (damaged membrane) bacteria (**Figure 2B**), and all bacteria had damaged membranes at 3 mg/L (MIC) (**Figure 2C**). We obtained similar results with MRSA (**Figures 2D–F**). Therefore, BC alters bacterial cell membranes.

Structural changes in the membrane, such as an altered fluidity, should lead to a slight modification in the cell surface structure (Alakomi et al., 2006). SEM images of samples having a 3-h exposure to BC at a MIC concentration (3 mg/L) determined if BC affected bacterial cell membrane structures and possibly membrane functions (Sipponen et al., 2009). We observed marked alterations of the cell structure surface of *S. aureus* (**Figure 3B**) and MRSA (**Figure 3D**) compared to the negative control (**Figures 3A,C**). Cell surfaces became irregular, and we observed invagination and structural alterations. These SEM observations of *S. aureus* and MRSA cell surfaces confirm the susceptibility of these bacteria to BC.

Damage induced by antibacterial agents such as BC can provoke the release of intracellular components – these components include small ions, such as potassium and phosphates, and much larger molecules, including DNA, RNA, and proteins (Maillard, 2002; Johnston et al., 2003; Liu et al., 2004; Virto et al., 2005; Lee and Je, 2013; Ukuku et al., 2013; He et al., 2016; McKenzie et al., 2016; Sannasiddappa et al., 2017). To test whether BC provoked

the release of DNA and proteins, we observed the UV-absorbance values at 260 and 280 nm. For treated samples of *S. aureus* and MRSA, absorbance values increased significantly in a dose-dependent manner when compared to untreated cells ($P < 0.006$). Thus, BC appears to induce the disruption of the cellular membrane and cause the release of intracellular constituents such as DNA (**Figure 4A**) and proteins (**Figure 4B**). Furthermore, the BC concentrations that induce cell membrane damages match the observed antibacterial activity.

Some antibiotics are known to target bacterial cell membranes. Epand et al. (2016) reviewed compounds that interact with bacterial cell membranes. They mention that this approach for antibiotics is a complex field that is only beginning to be exploited. Antibiotics that target the bacterial membrane or precursors appear to have a high potential as they show a fast and extensive bactericidal effect, in particular against MRSA and VISA (Bambeke et al., 2008). Membrane-damaging antibiotics can interact directly with the bacterial membrane bilayer, thereby disrupting its function and its physical integrity, which leads to the loss of membrane permeability and the altering of membrane properties. For example, daptomycin induces membrane permeabilization, depolarization, and disruption of multiple cellular processes; telavancin and oritavancin inhibit peptidoglycan biosynthesis by binding to the D-Ala-D-Ala termini to cause membrane permeabilization and depolarization, whereas polymyxin and chlorhexidine cause a collapse of the membrane potential (Kuyyakanond and Quesnel, 1992; Hurdle et al., 2011). Although we observed that BC appears to target bacterial cell membranes, further research is needed to determine the specific target of BC within the bacterial cell.

Balsacone C was found previously weakly cytotoxic against human cells using viability assay (Lavoie et al., 2013). In the present work, the effect of BC on human cellular membrane was assessed on healthy skin fibroblasts, WS1. Beta-hederin, a membrane cell permeabilizer, was used as positive control (Gauthier et al., 2009; Mihoub et al., 2018). In contrast to untreated cells (**Figure 5A**), beta-hederin treated cells are permeable to EB. This produces a red-orange fluorescence located at the nucleus (**Figure 5B**), which indicates membrane alteration (Mihoub et al., 2018). The healthy human cells treated with 1.5 and 3 mg/L (MIC) of BC produced a green-orange fluorescence diffused throughout the cell without nucleus fluorescence. A similar pattern was observed in the untreated cells (**Figures 5C,D**) and confirms that BC does not cause any membrane alteration at both concentrations tested. The viability of WS1 healthy cells, post-treatment with BC, was evaluated using calcein-AM. This hydrophobic probe permeates live cells easily and becomes strongly green fluorescent after cleavage of the acetoxymethyl ester by intracellular esterases. As expected, untreated cells had an intense green fluorescence (**Figure 6A**). Cells treated with BC at 1.5 and 3 mg/L (**Figures 6C,D**) also produced an intense green fluorescence, thereby confirming that BC is not cytotoxic for WS1 human cells. In contrast, the green fluorescence of cells treated with 0.82 mg/L of doxorubicin (**Figure 6B**)

was much weaker. These preliminary results suggest that BC could be used to treat bacterial infections without affecting healthy human cells.

CONCLUSION

New antibiotics are urgently warranted to combat resistant bacteria such as MRSA. Our results indicate that BC induces bacterial cell membrane damage. This damage leads to the loss of membrane integrity and the release of intracellular constituents, followed by cell death after relatively short incubation times. This promising new therapeutic candidate represents a “membrane active agent” mainly used against gram-positive bacteria, such as MRSA. Induction experiments on MRSA and MSSA isolates did not lead to resistance. Future research will focus on improving the structure of balsacone in order to increase its activity and identify the specific target of BC. Moreover, *in vivo* tests on mice models should be performed to determine the best way of administration, the toxicity and the efficacy of BC.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

HC performed the experiments, analyzed the data, and wrote the manuscript. FS, M-EO, MM, and LR performed some of the experiments and revised the manuscript. AP and DG analyzed the data and revised the manuscript. JL conceived the experiments, analyzed the data, and wrote the 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/fmicb.2019.02341/full#supplementary-material>

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Targeting the Sugary Armor of *Klebsiella* Species

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The emergence of multidrug-resistant strains of Gram-negative *Klebsiella* species is an urgent global threat. The World Health Organization has listed *Klebsiella pneumoniae* as one of the global priority pathogens in critical need of next-generation antibiotics. Compared to other Gram-negative pathogens, *K. pneumoniae* accumulates a greater diversity of antimicrobial-resistant genes at a higher frequency. The evolution of a hypervirulent phenotype of *K. pneumoniae* is yet another concern. It has a broad ecological distribution affecting humans, agricultural animals, plants, and aquatic animals. Extracellular polysaccharides of *Klebsiella*, such as lipopolysaccharides, capsular polysaccharides, and exopolysaccharides, play crucial roles in conferring resistance against the host immune response, as well as in colonization, surface adhesion, and for protection against antibiotics and bacteriophages. These extracellular polysaccharides are major virulent determinants and are highly divergent with respect to their antigenic properties. Wzx/Wzy-, ABC-, and synthase-dependent proteinaceous nano-machineries are involved in the biosynthesis, transport, and cell surface expression of these sugar molecules. Although the proteins involved in the biosynthesis and surface expression of these sugar molecules represent potential drug targets, variation in the amino acid sequences of some of these proteins, in combination with diversity in their sugar composition, poses a major challenge to the design of a universal drug for *Klebsiella* infections. This review discusses the challenges in universal *Klebsiella* vaccine and drug development from the perspective of antigen sugar compositions and the proteins involved in extracellular antigen transport.

Keywords: *Klebsiella* species, multidrug resistance, lipopolysaccharide, capsular polysaccharide, exopolysaccharide, complement system, vaccine, antibiotics

INTRODUCTION

Klebsiella species (spp.) are rod-shaped and encapsulated Gram-negative bacteria in the Enterobacteriaceae family (Podschun and Ullmann, 1998; Kenneth and Ryan, 2003; Murray and Baron, 2007; Paczosa and Meccas, 2016). Eleven species have been identified in the *Klebsiella* genus, namely, *Klebsiella pneumoniae* (*K. pneumoniae*) (subsp. *pneumoniae*, subsp. *ozaenae*, subsp. *rhinoscleromatis*), *Klebsiella oxytoca* (*K. oxytoca*), *Klebsiella ornithinolytica* (*K. ornithinolytica*), *Klebsiella planticola* (*K. planticola*), *Klebsiella terrigena* (*K. terrigena*) (Murray and Baron, 2007), *Klebsiella variicola* (*K. variicola*) (Rosenblueth et al., 2004) [subsp. *tropicalensis* (Rodrigues et al., 2019)], *Klebsiella granulomatis* (*K. granulomatis*) (Carter et al., 1999), *Klebsiella aerogenes* (*K. aerogenes*) (Tindall et al., 2017), *Klebsiella africanensis* (*K. africanensis*) (Rodrigues et al., 2019), *Klebsiella grimontii* (*K. grimontii*) (Passet and Brisse, 2018), and *Klebsiella*

quasipneumoniae (*K. quasipneumoniae*) (subsp. *quasipneumoniae* and subsp. *similipneumoniae*) (Brisse et al., 2014). *Klebsiella pneumoniae* (*K. pneumoniae*) (Kp) are further classified into classical (cKp) and hypervirulent (hvKp) strains based on their phenotype and nature of pathogenicity (Shon et al., 2013; Russo et al., 2018). *Klebsiella* spp. are generally found in animal and human gut microbiota (Selden et al., 1971; Taur and Pamer, 2013; Bilinski et al., 2016; Paczosa and Mecsas, 2016). They colonize a wide range of hosts including plants and mammals (Bagley, 1985; Podschun and Ullmann, 1998; Podschun et al., 2001; Wyres and Holt, 2018) and can grow ubiquitously in water and soil (Bagley, 1985; Podschun and Ullmann, 1998; Podschun et al., 2001; Rock et al., 2014).

Klebsiella spp. are generally opportunistic pathogens (Wyres and Holt, 2018) and do not usually affect healthy individuals (Bagley, 1985; Centers For Disease Control Prevention, 2010). Generally, it is immunocompromised individuals, such as patients undergoing chemotherapy, neonates, and the elderly, that are affected by cKp infections. In contrast, hvKp can infect healthy individuals of any age and can infect nearly every site of the body and spread metastatically (Liu et al., 1986; Fang et al., 2007; Russo et al., 2018). *Klebsiella* spp. utilize the following virulence traits to protect themselves from the host immune response (Davies, 2003; Lavender et al., 2004; Mishra et al., 2015; Paczosa and Mecsas, 2016; Hsieh et al., 2019): capsular polysaccharides (CPS), lipopolysaccharides (LPS), siderophores, fimbriae (alternatively, pili), a type VI secretion system, outer-membrane proteins, porins, efflux pumps, an iron transport system, biofilms, and allantoin metabolism. Among these, CPS, LPS, siderophores, and fimbriae are well-characterized virulence factors of *Klebsiella* spp. (Paczosa and Mecsas, 2016). These virulence factors assist *Klebsiella* spp. in evading the innate immune response of the host and to survive in different sites within the host, rather than actively suppressing host immune system components (Domenico et al., 1994; Hsieh et al., 2019). Notably, increased production of CPS and aerobactin (an iron-chelating siderophore) is specific to the hvKp pathotype (Cheng et al., 2010; Russo et al., 2018), as increased production of CPS results in a hypermucoviscous phenotype that has a viscous string length >5 mm (Cheng et al., 2010). Nevertheless, hypermucoviscosity is not specific to the hvKp pathotype, as cKp can also exhibit such a phenotype (Catalan-Najera et al., 2017; Russo et al., 2018). Furthermore, hvKp strains are not always hypermucoviscous (Catalan-Najera et al., 2017; Russo et al., 2018). Thus, the genes involved in the regulation of CPS (Cheng et al., 2010) and aerobactin production are used to distinguish the cKp and hvKp pathotypes (Russo et al., 2018). These are not elaborated here, as it is beyond the scope of this review.

Klebsiella spp. cause a variety of opportunistic nosocomial and community-acquired infections (Podschun and Ullmann, 1998; Tsai et al., 2008; Lin et al., 2010; Paczosa and Mecsas, 2016; Martin and Bachman, 2018; Vading et al., 2018; Juan et al., 2019), such as urinary tract infection (Goldstein et al., 1978; Sewify et al., 2016), soft tissue infection (Goldstein et al., 1978), pneumonia (Lee et al., 1996; Tan et al., 1998), septicemia (Arredondo-Garcia et al., 1992; Al-Anazi et al., 2008), bacteremia (Goldstein et al., 1978; Lin et al., 1997), meningitis (Price

and Sleigh, 1972; Ku et al., 2017; Khaertynov et al., 2018), and pyogenic liver abscesses (Chowdhury and Stein, 1992; Youssef et al., 2012). As *Klebsiella* spp. have acquired resistance against various antimicrobials, they often become a challenge in treating these infections (Bengoechea and Sa Pessoa, 2019). For instance, Kp isolates have continuously accumulated resistance against four important classes of antibiotics, namely, the third-generation cephalosporins, aminoglycosides, fluoroquinolones, and carbapenems (Navon-Venezia et al., 2017; The European Antimicrobial Resistance Surveillance Network, 2018). Multiple drug resistance such as this eventually leads to extremely drug-resistant *Klebsiella* strains (XDR) (Magiorakos et al., 2012; Navon-Venezia et al., 2017).

In general, Kp is a hospital-associated pathogen that is subjected to continuous selective pressure due to continuous exposure to multiple antibiotics. *K. pneumoniae* inactivates a spectrum of beta-lactams through the action of carbapenemases and an extended spectrum of beta-lactamases (ESBL). As a consequence, Kp can become resistant to beta-lactams and thrive in healthcare settings (Hawkey and Jones, 2009; D'andrea et al., 2013; Andrade et al., 2014; Zhang et al., 2016; Feng et al., 2018; Fu et al., 2018). For example, a New Delhi metallo- β -lactamase 1 (NDM-1)-producing Kp strain originating from India has now disseminated across the globe (Yong et al., 2009; Khan et al., 2017). In 2016, a patient infected with NDM-1-producing Kp died due to a lack of treatment options in Nevada (Chen et al., 2017). Colistin, a drug of last resort that has been used against carbapenem-resistant *Enterobacteriaceae*, targets bacterial lipid A. *K. pneumoniae* has developed resistance against colistin through mutations in lipid A modification regulatory genes such as *mgrB* (Cannatelli et al., 2013; Jayol et al., 2014; Olaitan et al., 2014; Poirel et al., 2015; Wright et al., 2015). Although both cKp and hvKp are global pathogens, the former is predominantly found in Western countries, while the latter is observed in the Asia-Pacific Rim (Fazili et al., 2016; Rossi et al., 2018; Russo and Marr, 2019). However, the evolution of hvKp strains with multiple drug resistance (MDR) and extreme drug resistance (XDR) is due to either hvKp acquiring drug-resistant plasmids from cKp (Zhang et al., 2015, 2016; Wei et al., 2016; Feng et al., 2018; Fu et al., 2018; Yao et al., 2018) or cKp acquiring an hvKp virulence plasmid (Gu et al., 2018). Both pose a significant challenge with respect to the treatment of infection.

Klebsiella pneumoniae has evolved several mechanisms to resist antibiotics. In comparison to *Escherichia coli* (*E. coli*), Kp has acquired double the number (more than 400) of antimicrobial-resistant (AMR) genes (Wyres and Holt, 2018). Interestingly, ESBL-producing Kp exhibits carbapenem resistance as a result of alterations in permeability due to loss of porins (Bradford et al., 1997; Martinez-Martinez, 2008; Leavitt et al., 2009) and overexpression of efflux pumps (Van De Klundert et al., 1988). *Klebsiella pneumoniae* has also acquired AMR through horizontal gene transfer enabled by plasmids and a mobile genetic environment (Pendleton et al., 2013). The emergence of plasmids with ESBL genes in Kp is one such example (Wachino et al., 2004; Queenan and Bush, 2007; Woodford et al., 2011; Lee et al., 2016). The translocation of carbapenemase-encoding genes from Kp plasmids onto a

chromosome makes infections almost impossible to control (Lee et al., 2016). Due to chromosomal mutations, Kp has also become resistant to the antimicrobial peptide colistin (Olaitan et al., 2014; Doorduyn et al., 2016; Liu et al., 2016), leaving very few therapeutic options for the treatment of patients infected with Kp. Thus, it has become increasingly challenging to treat Kp infections, as reflected by the increase in the number of severe infections and the scarcity of effective antimicrobials (Paczosa and Meccas, 2016).

As *Klebsiella* spp. are reservoirs for antibiotic-resistant genes (Navon-Venezia et al., 2017; Bengoechea and Sa Pessoa, 2019), they can act as key traffickers of AMR genes to other environmentally and clinically important Gram-negative bacteria. One such example is spread of carbapenem resistance genes from Kp (Sidjabat et al., 2009) strains originated in the United States (Smith Moland et al., 2003) to other Gram-negative bacterial species such as *Salmonella* (Miriagou et al., 2003), *Enterobacter* spp. (Hossain et al., 2004), *Escherichia coli* (Bratu et al., 2007), and *Proteus mirabilis* (Tibbetts et al., 2008). Such examples of interspecies spread have been observed for quite some time. Similar to Kp, other *Klebsiella* species have also acquired resistance against antibiotics. *Klebsiella grimontii* (which is closely related to *Klebsiella oxytoca*) is a newly added species to the *Klebsiella* genus (Passet and Brisse, 2018) and has acquired resistance against carbapenem (Liu et al., 2018). These events prompted the World Health Organization (WHO) to call for a global effort to develop next-generation antibiotics against *Klebsiella* infections (World Health Organization, 2017, 2018).

The rise in multidrug-resistant *Klebsiella* spp. (as well as hvKp strains) and their periodic outbreak and global spread (Navon-Venezia et al., 2017) warrant a new treatment strategy, along with a new set of antibiotics and vaccines for *Klebsiella* infections. Targeting bacterial survival mechanisms (rather than destroying the bacteria) exerts less selective pressure on the bacteria. For example, targeting the sugary armor of *Klebsiella* spp., such as the LPS, CPS, and exopolysaccharide (EPS), would be an efficient alternative strategy. Though structural information and mechanical insights relating to the transport of CPS and LPS onto the bacterial surface through various proteinaceous nanomachines are available (Rahn et al., 1999, 2003; Kos et al., 2009; Ruiz et al., 2009; Shu et al., 2009; Freinkman et al., 2012; Sachdeva et al., 2017; Bi et al., 2018), only a fragmented picture of their utility as potential drug and vaccine targets exists. To this end, this review focuses on targeting the CPS, LPS, and EPS armors of *Klebsiella* spp.

HOST INNATE IMMUNE DEFENSES AGAINST KLEBSIELLA SPECIES

When a pathogen enters a host, it must contend with the mechanical, chemical, and cellular barriers exhibited by the host, and *Klebsiella* spp. is no exception (Zhang et al., 2000). Initially, it has to overcome mechanical barriers such as the epithelia of the skin, mucociliary clearance, the low-pH environment of the genitourinary tract or gastrointestinal tract, etc. Subsequent to this, the pathogen must circumvent the humoral and cellular

innate defenses. Several humoral defenses (opsonic, bactericidal, and bacteriostatic) are used by the host for bacterial clearance (Kabha et al., 1997; Zhang et al., 2000; Ivin et al., 2017). One such humoral defense is the complement system, which is activated in three different pathways (namely, the classical, alternative, and mannose-binding lectin pathways) (Murphy et al., 2012) for the purpose of clearing bacteria. In addition, the pathogen has to deceive antimicrobial peptides, collectins, and cellular components (i.e., neutrophils, monocytes/macrophages, dendritic cells, and innate lymphoid cells) of the innate immune defense to survive and maintain its growth in the host (Murphy et al., 2012). The mechanisms of *Klebsiella* spp. defense against the host are covered in detail in recent reviews (Doorduyn et al., 2016; Paczosa and Meccas, 2016; Bengoechea and Sa Pessoa, 2019).

Once *Klebsiella* spp. overcome the mechanical barriers of the host, a variety of host immune defense pathways are activated by pathogen recognition receptors (PRRs) such as “Toll-like” receptors (TLRs), nucleotide-binding oligomerization domain-like receptors (NLRs), etc. (Takeuchi and Akira, 2010) through the detection of pathogen-associated molecular patterns (PAMPs). As CPS and LPS are major pathogen surface components, many of the PRRs activate these immune response pathways primarily mediated by the detection of LPS and CPS. For instance, upon binding to TLR4, the CPS activates the NF- κ B-mediated inflammatory and immune response pathways (Regueiro et al., 2006, 2009; Yang et al., 2011). Interaction of LPS with TLR4 and MD2 receptors on the host innate immune cells also induces the NF- κ B-mediated inflammatory response (Kawai and Akira, 2010; Maeshima and Fernandez, 2013). The lung collectins SP-A and SP-D, which are soluble PRRs, bind to LPS and facilitate agglutination and phagocytosis by macrophages. The recruitment of the classical complement pathway (following LPS detection) and that of the lectin-mediated complement pathway (upon detection of CPS) are some of the major host strategies for bacterial clearance (Walport, 2001; Ricklin et al., 2010; Holers, 2014; Gomez-Simmonds and Uhlemann, 2017). On detection of LPS, NLR protein family members assemble to form inflammasome, which activates caspase 4/5 in humans and caspase-11 in the mouse. This triggers the activation of non-canonical inflammasome to produce IL1 β and induce bacterial cell death (Hagar et al., 2013; Shi et al., 2014). Opsonophagocytosis mediated by neutrophils and macrophages is also a major bacterial clearance strategy (Domenico et al., 1994; Salo et al., 1995; Regueiro et al., 2006).

HOST IMMUNE EVASION STRATEGIES OF KLEBSIELLA SPP.

Klebsiella spp. make use of several sophisticated stealth immune evasion strategies to escape from the host innate immune response, rather than actively suppressing it. However, recent research indicates that *Klebsiella* spp. have also developed several anti-immune strategies that involve the attack of key regulators and effectors of the host immune system. This makes them formidable pathogens capable of disseminating and growing

across a variety of sites in their hosts (Paczosa and Meccas, 2016; Bengoechea and Sa Pessoa, 2019). To establish in the host, the pathogen has to counteract the host innate immune defenses (Zhang et al., 2000). The surface oligosaccharide molecules (CPS and LPS) are some of the major virulence factors that *Klebsiella* spp. use to protect themselves from the host immune response.

Capsular Polysaccharide

The surface of *Klebsiella* spp. is shielded by a thick layer of CPS fibers that protect the bacteria from the environment (Amako et al., 1988). The polysaccharide capsule assists the bacteria in surviving stressful environmental conditions such as desiccation and exposure to detergents. High-molecular-weight CPS, consisting of linear or branched oligosaccharides, form a shield around the *Klebsiella* spp. cell surface and represent a physical barrier against the complement system, as also seen in *E. coli* (Meri and Pangburn, 1994; Alvarez et al., 2000; Cortes et al., 2002b; Abreu and Barbosa, 2017). This shield plays a crucial role in protecting Kp against innate immune response mechanisms, evading complement deposition and opsonization, reducing recognition, and adhesion by epithelial cells and phagocytes, and abrogating lysis by antimicrobial peptides and complement cascades (Podschun and Ullmann, 1998; Fang et al., 2004; Lin et al., 2004; Pomakova et al., 2012; Paczosa and Meccas, 2016; Martin and Bachman, 2018). Poorly encapsulated Kp strains are readily vulnerable to phagocytosis (Cortes et al., 2002a; De Astorza et al., 2004). As compared to capsular Kp strains, acapsular Kp strains are more easily phagocytosed by innate immune cells (Domenico et al., 1994; Yoshida et al., 2000; Cortes et al., 2002b; Lawlor et al., 2005, 2006). Deletion of the genes responsible for capsule formation in the clinical strains ideally leads to a non-pathogenic bacterium by drastically impairing the virulence of Kp (Cortes et al., 2002b; Lawlor et al., 2006). It has been shown that the thickness of CPS (rather than its chemical composition) determines the extent of protection it confers to *Klebsiella* spp. (De Astorza et al., 2004). Not surprisingly, hvKp exhibits enhanced resistance to a variety of humoral defenses such as complement killing, HBD-1 to HBD-3 [human beta-defensin (HBD)], and to antimicrobial peptides such as neutrophil protein 1 and lactoferrin (Fang et al., 2004).

The capsule type, also known as the K-antigen or K-type, is *Klebsiella* species-specific and is widely used in the serotyping of *Klebsiella* spp. Traditionally, *Klebsiella* spp. are identified as having 77 K-antigens (viz., K1–K81, excluding K75–K78) based on the diversity in their sugar composition, type of glycosidic linkage, and the nature of enantiomeric and epimeric forms (<https://iith.ac.in/K-PAM/>, K-PAM unpublished) (Pan et al., 2015). Recently, additional K-types have been identified based on the CPS locus or K-locus (KL) arrangement. These are known as the KL series (KL1–KL81, KL101–KL149, KL151, KL153–KL155, and KL157–159) (Wyres et al., 2016). It is noteworthy that the KL1–KL81 locus types and the K1–K81 K-types are synonymously used. However, the sugar compositions of the remaining antigens in the KL series are as yet unknown. The variation in the repeating units of different K-antigens leads to varying degrees of detection of *Klebsiella* spp. by the innate immune system (Kabha et al., 1995; Doorduyn et al., 2016).

Among the 134 K-types (including the KL series) identified so far (Wyres et al., 2016), only a few of them are frequently found in the strains isolated from clinical samples (Cryz et al., 1986). Due to the increased production of CPS, hypervirulent Kp strains produce a hypercapsule, which is a hypermucoviscous EPS bacterial coating that may significantly contribute to Kp pathogenicity (Shon et al., 2013). *Klebsiella pneumoniae* strains with a hypercapsule are less sensitive to complement detection and elimination (Pomakova et al., 2012) and also have increased resistance to phagocytosis (Fang et al., 2004; Lin et al., 2004; Pomakova et al., 2012) compared to the classical strains. However, some cKp strains are also found to have a hypermucoviscous coating (Catalan-Najera et al., 2017; Russo et al., 2018). Notably, the presence of fucose in the hypercapsule has been implicated in the evasion of the immune response for the K1 antigen (Wu et al., 2008; Yeh et al., 2010). Although Kp strains possessing K1 and K2 serotypes are often found to be hypervirulent (Fung et al., 2002; Struve et al., 2015), other capsule types such as K5, K20, K47, K54, K57, and K64 are also found in hvKp strains (Yu et al., 2008; Shon et al., 2013; Russo et al., 2018).

Klebsiella spp. K-antigens are negatively charged (as is the case for other Gram-negative bacteria) and consist of up to six monosaccharides in their main chain as well as in the branch: D-mannose, D-glucose, D-galactose, L-fucose, and L-rhamnose. Of particular note is a completely new monosaccharide, 4-deoxy-threo-hex-4-enopyranosyluronic acid, that is found in K38 but is absent in any other K-antigen structure (Jansson et al., 1994). Detailed analyses of *Klebsiella* spp. K-antigen sugar compositions (Table 1) reveal that as with *E. coli* (Kunduru et al., 2016), K-antigens are negatively charged due to the presence of uronic acid and/or pyruvate substitutions (<https://iith.ac.in/K-PAM/>, unpublished work). Additionally, they also have O-acetyl, O-lactose, O-formyl, and glutamate substitutions. The evolution and variability in the sugar composition of CPS are one of the major advantages possessed by *Klebsiella* when evading the host immune response.

Lipopolysaccharide

Pathogenicity factor LPS, also known as endotoxin, is found on the bacterial outer leaflet of the outer membrane and plays an important role in offering protection against cationic antimicrobial peptides (Clements et al., 2007; Llobet et al., 2015) and the complement system in certain serotypes (Merino et al., 1992). *Klebsiella pneumoniae* exploits the versatility of both CPS and LPS to counteract the complement system (Ciurana and Tomas, 1987; Alvarez et al., 2000; Shankar-Sinha et al., 2004; Doorduyn et al., 2016; Adamo and Margarit, 2018). It has been shown that purified LPS from Kp inhibits serum-mediated clearance (Merino et al., 1992). The structure of LPS consists of lipid A, core oligosaccharides, and O-antigens, among which the O-antigen composition is highly variable across different strains of *Klebsiella* spp. (Table 2, http://iith.ac.in/K-PAM/o_antigen.html) (Lugo et al., 2007). Unlike K-antigens, *Klebsiella* spp. has only 11 O-antigens (Clarke et al., 2018). O-antigen

TABLE 1 | Main-chain and side-chain sugar composition of 79 K-antigens of *Klebsiella* spp.

K-antigen	Main chain sugars														K-antigen	side chain sugars														K-antigen	Extra											
	α -L-Fucp	α -L-Fuc-Oac	β -D-Galf	α -D-Galp	α -D-Galp-pyr	α -D-GalpA	β -D-Galp	β -D-Galp-Oac	β -D-Galp-pyr	β -D-GlcpA	β -D-GlcpA-pyr	β -D-GlcpA-Oac	α -D-Manp	α -D-Manp-Oac		β -D-Manp	β -D-Manp-Oac	β -D-Manp-pyr	α -L-Rhap	α -L-Rhap-OAc	α -L-Rhap-pyr	β -L-Rhap	α -D-Galp	α -D-Galp-pyr	α -D-GalpA	β -D-Galp	β -D-Galp-pyr	α -D-Glcp	α -D-GlcpA			β -D-Glcp	β -D-Glcp-pyr	β -D-Glcp-Olac	β -D-Glcp-Ofor	β -D-GlcpA	β -D-GlcpA-Glu	β -D-GlcpA-Olac	α -D-Manp	α -D-Manp-pyr	β -D-Manp	β -D-Manp-pyr
K1	1											1										K1																				
K2										1	1					1						K2						1														
K3						1	1							2								K3															1					
K4								1	1	1				1								K4																				
K5											1		1					1				K5																				
K6	1								1	1								1				K6																				
K7										1		1	1		2							K7	1																			
K8				1			1		1													K8													1							
K9				1																		K9													1							
K10				1			1			1	1			1								K10	1																			
K11				1						1		1										K11		1																		
K12			1	1						1									1			K12					1								1							
K13										1		1					1					K13				1		1														
K14			1								1				1							K14								1										1		
K15					4																	K15						1	1													
K16	1									1												K16					1															
K17										1	1											K17																		1		
K18										1												K18						1												1		
K19										1	1											K19																		1		
K20																1						K20																		1		
K21a																						K21a		1																		
K21b																						K21b			1																	
K22																						K22						1								1						
K23																						K23						1														
K24										1												K24																	1			
K25																						K25								1												
K26																						K26				1	1															
K27																						K27																				
K28																						K28																				
K30																						K30																				
K31																						K31																				

(Continued)

TABLE 1 | Continued

K-antigen	Main chain sugars															side chain sugars																											
	α -L-Fucp	α -L-Fuc-Oac	β -D-Galp	α -D-Galp	α -D-Galp-pyr	α -D-GalpA	β -D-Galp	β -D-Galp-Oac	β -D-Galp-pyr	β -D-GlcA	β -D-GlcA-pyr	β -D-GlcA-Oac	α -D-Manp	α -D-Manp-Oac	β -D-Manp	β -D-Manp-Oac	β -D-Manp-pyr	α -L-Rhap	α -L-Rhap-OAc	α -L-Rhap-pyr	β -L-Rhap	K-antigen	α -D-Galp	α -D-Galp-pyr	α -D-GalpA	β -D-Galp	β -D-Galp-pyr	α -D-GlcA	α -D-GlcA-pyr	β -D-GlcA	β -D-GlcA-pyr	β -D-GlcA-Olac	β -D-GlcA-Ofor	β -D-GlcA-pyr	β -D-GlcA-Glu	β -D-GlcA-Olac	α -D-Manp	α -D-Manp-pyr	β -D-Manp	β -D-Manp-pyr	α -L-Rhap	β -L-Rhap-pyr	Extra
K32			1															1		1	1	K32																					
K33								1							1	1						K33		1				1															
K34						1		1										3				K34																			1		
K35				1				1					2									K35												1									
K36																		3				K36						1					1										
K37										1												K37		1												1							
K38			1					1														K38						1													β -L-Sug		
K39								2			2		2									K39																					
K40				1				1					2					2				K40																					
K41			1	1				1										1				K41		1				1						1									
K43			1										2									K43											1										
K44								1		1								2				K44																					
K45								1		1								3				K45												1									
K46			1					1					1									K46						1												1			
K47																		1				K47											1								1		
K48								1		1								2				K48		1																			
K49			2										1									K49		1																			
K50								1	1				2									K50			1																		
K51			2																			K51			1	1																	
K52																						K52																				-1	
K53													2					1				K53																			1		
K54	1	1						2	2													K54											2										
K55																		1				K55	1					1															
K56			1																			K56																			1		
K57													1									K57															1						
K58	1							1									1					K58	1																				
K59										1							2					K59												1									
K60			1										1									K60					1		2														
K61								1		1			1									K61	1																				

(Continued)

K-antigen	Main chain sugars																K-antigen	side chain sugars													
	α -L-Fucp α -L-Fuc-Oac	β -D-Galp α -D-Manp	α -D-Galp α -D-Galp-pyr	α -D-GalpA β -D-Galp	β -D-Galp-Oac β -D-Galp-pyr	α -D-Glcp α -D-GlcpA	β -D-Glcp β -D-Glcp-Oac	β -D-Glcp-pyr β -D-GlcpA	β -D-GlcpA-pyr β -D-GlcpA-Oac	α -D-Manp α -D-Manp-Oac	β -D-Manp β -D-Manp-Oac	β -D-Manp-pyr	α -L-Rhap α -L-Rhap-OAc	α -L-Rhap-pyr β -L-Rhap	K-antigen	α -D-Galp α -D-Galp-pyr		α -D-GalpA β -D-Galp	β -D-Galp-Oac β -D-Galp-pyr	α -D-Glcp α -D-GlcpA	β -D-Glcp β -D-Glcp-pyr	β -D-Glcp-Olac β -D-Glcp-Ofor	β -D-GlcpA β -D-GlcpA-Glu	β -D-GlcpA-Olac α -D-Manp	α -D-Manp-pyr β -D-Manp	β -D-Manp-pyr α -L-Rhap	β -L-Rhap-pyr Extra				
K62						1				1						K62															
K63	1															K63															
K64						1	1			2						K64				1								1			
K66		1				1				2						K66					1							1			
K67							1			2			1			K67	1						1					1			
K68				1	1					1						K68						1			1						
K69							1				2					K69		1		1											
K70				1		1								1		K70															
K71							1						2		1	K71				1			1				1				
K72													3			K72					1										
K73							1						2		1	K73						1									
K74				1						2						K74		1		1											
K79				1									3			K79			2												
K80				1						2						K80				1							1				
K81				1									4			K81															
K82		1			1		1									K82						1									
K83				1									1			K83	1			1											

Fuc, Fucose; *Gal*, Galactose; *Glc*, Glucose; *Rha*, Rhamnose; *Man*, Mannose; *GalpA*, Galacturonic acid (Pyranose); *Glc pA*, Glucuronic acid (Pyranose); *Oac*, O-acetyl group; *Pyr*, pyruvyl group; β -L-Sug, 4-deoxy-three-hex-4-enopyranosyluronic acid.

is also used in the typing of *Klebsiella* spp. Among the 11 O-antigen types found in *Klebsiella* spp., O1, O2, O3, and O5 are found in clinically imported strains (Hansen et al., 1999; Follador et al., 2016). O-antigens of *Klebsiella* spp. consist of D-galactose, D-galactofuranose, D-mannose, D-ribofuranose, and N-acetyl-D-glucosamine sugars. Their composition varies between different O-antigens, leading to differences in their antigenicity. Like the K-antigen, the O-antigens differ from each other in terms of sugar composition, glycosidic linkage, number of repeating units, and epimeric and enantiomeric forms (<https://iith.ac.in/K-PAM/>, unpublished work) (Follador et al., 2016; Clarke et al., 2018). Unlike the K-antigens, only acetyl group substitution is observed in O-antigens (and only in one of the O-antigens). While *Klebsiella* spp. strains that have truncated O-antigen or lack O-antigen (termed as “rough LPS”) are susceptible to complement system-mediated killing, the full-length O antigen or smooth LPS-containing *Klebsiella* spp. strains are resistant to complement system-mediated killing (Ciurana and Tomas, 1987; Mccallum et al., 1989; Merino et al., 1992). Although the complement-resistant strains activate the complement cascade, they are not susceptible to killing, as O-antigen variability protects the Kp surface molecules (Merino et al., 1992; Alberti et al., 1996; Shankar-Sinha et al., 2004; Merle et al., 2015).

Exopolysaccharide

The extracellular matrix (which is a component of bacterial biofilm) of *Klebsiella* spp. is composed of proteinaceous adhesins, nucleic acids, and EPS (Sutherland, 2001; Branda et al., 2005; Vu et al., 2009). Compared to other surface-attached polysaccharides, little information is available on biofilm-associated EPS, which is yet another virulence factor of Kp (Cescutti et al., 2016). It has been shown that biofilm polysaccharides of Kp to some extent reduce antimicrobial peptide activity by preventing it from reaching the bacterial membrane or by impeding interaction with the membrane (Bellich et al., 2018). Genetic information regarding the biosynthesis of EPS is encoded in specific operons on the bacterial genome and 30 ORFs have been identified for the hetero-capsular EPS K40-type of *Klebsiella* spp. (Pan et al., 2015). In general, EPS contains rare sugars such as L-fucose, L-rhamnose, or uronic acids (Kumar et al., 2007), and *Klebsiella* is no exception. For example, hexasaccharide repeats of *Klebsiella* I-714 EPS have a high L-rhamnose content in addition to D-galactose and D-glucuronic acid (López-Santin, 1995; Roca et al., 2015). The primary structures of EPS extracted from *K. pneumoniae* strain KpTs113 have K24 CPS-repeating units and the *K. pneumoniae* strain KpTs101 is identical to the O1 antigen of LPS. This observation is supported by the finding that CPS and LPS are required for building the mature biofilm architecture (Balestrino et al., 2008; Benincasa et al., 2016; Cescutti et al., 2016). However, the KpMn7 strain has a rare sugar (rhamnose) in the repeating unit and is highly similar (but not identical) to the K24 CPS unit. Given this intriguing finding, further research is warranted on novel EPS structures found in *Klebsiella* spp. (Kubler-Kielb et al., 2013; Bellich et al., 2018).

TABLE 2 | Main-chain and side-chain sugar compositions of 11 O-antigens of *Klebsiella* spp. Note that the number of occurrences of a particular sugar (which varies between 1 and 5) is also indicated.

O-antigens	Main chain sugars						side chain sugars				
	β-D-Ribf	β-D-Galf	β-D-Galf-2OAc & 6O-Ac	α-D-Galp	β-D-Galp	β-D-GlcNAc	α-D-Manp	β-D-Manp	α-L-Rhap	O-antigens	α-D-Galp
O1				1	1					O1	
O2a		1		1						O2a	
O2c		1				1				O2c	
O2aeh		1		1						O2aeh	1
O2afg		1		1						O2afh	1
O3							5			O3	
O4	1			1						O4	
O5							2	1		O5	
O7	1								3	O7	
O8			1	1						O8	
O12						1			1	O12	

The presence of substitution(s) is also indicated next to the corresponding sugar (Clarke et al., 2019). The abbreviations used for sugar molecules are as per **Table 1**. The sugars mentioned for the first time (in this table) are: GlcNAc, N Acetyl Glucosamine; Ribf, Ribofuranose.

EXTRACELLULAR POLYSACCHARIDE BIOSYNTHESIS AND TRANSPORTATION PATHWAYS

The aforementioned extracellular polysaccharide virulence factors are biosynthesized in the cytoplasm and transported through sophisticated proteinaceous nano-machines onto the bacterial surface. In general, bacteria use three different pathways for the transport of extracellular polysaccharides: (i) a Wzx/Wzy-dependent pathway (Rahn et al., 1999; Whitfield, 2006; Kalynych et al., 2014), (ii) an adenosine tri-phosphate (ATP)-binding cassette (ABC) transporter-dependent pathway (Cuthbertson et al., 2010; Greenfield and Whitfield, 2012; Kalynych et al., 2014), and (iii) a synthase-dependent pathway (Whitney and Howell, 2013). In addition to these, a fourth pathway (the dextrase/sucrase-dependent pathway) has also been identified for EPS secretion (Whitney and Howell, 2013; Schmid et al., 2015; Schmid, 2018).

Klebsiella spp. use a Wzx/Wzy-dependent pathway for CPS secretion (Rahn et al., 1999) that is similar to Group 1 CPS surface export in *E. coli* (Rahn et al., 1999; Whitfield and Paiment, 2003; Sachdeva et al., 2017). *Klebsiella* spp. use three independent ABC transporter-dependent pathways for LPS secretion. Although it is known that *E. coli* uses a Wzx/Wzy-dependent pathway for EPS secretion (Reid and Whitfield, 2005), there is no information available regarding EPS secretion in *Klebsiella* spp. Understanding the mechanisms of transport and the structural features of the proteins involved in such transport is essential for the identification of potential antimicrobial targets and the development of novel antimicrobials. As information on the EPS-secretion pathway is not available for *Klebsiella* spp., the following sections are limited to a review of CPS and LPS transportation strategies used by *Klebsiella*. The structures of the proteins involved in *Klebsiella* CPS and LPS export (with the exceptions of LptDE and LptB₂FG) have been obtained by homology modeling using known template structures from other organisms (see Table 3).

The Wzx/Wzy-Dependent Secretion Pathway

The chromosomal *cps* gene cluster harbors genes that are essential for the biosynthesis of sugar precursor molecules, assembly of the repeating unit, flipping of the repeating unit to the periplasmic side, polymerization of the repeating unit, transport of the nascent CPS, and anchorage of CPS onto the surface of *Klebsiella* (Pan et al., 2015). *Klebsiella* spp. utilize a Wzx/Wzy-dependent CPS secretion pathway, which is similar to that for Group 1 capsule production in *E. coli* (Whitfield and Paiment, 2003; Sachdeva et al., 2017). The process of CPS export in *Klebsiella* spp. begins with the biosynthesis of nucleotide sugar precursors corresponding to a particular K-type and the assembly of the repeat unit at the cytoplasmic face. This occurs with the help of sugar-specific glycosyl transferases encoded by genes such as *wbaP*, *wcaN*, *manC*, *rmlA*, *wcaA*, *wcuD*, *wcuM*, *wckA*, and *wclH* (Rahn et al., 1999; Shu et al., 2009; Pan et al., 2015). Subsequently, recognition of the specific CPS-repeating

unit by the flippase Wzx occurs with the first sugar linked to undecaprenol-pyrophosphate (Und-PP), followed by flipping to the periplasmic side. Repeat unit polymerization is facilitated by Wzy copolymerase (Whitfield and Paiment, 2003; Li et al., 2016). Finally, Wza (an outer-membrane translocon), Wzc (a tyrosin autokinase), and Wzb (a phosphatase) synergistically transport CPS onto the bacterial surface and anchor the CPS onto the outer-membrane protein Wzi (Rahn et al., 2003; Whitfield, 2006; Woodward et al., 2010). This CPS export pathway is common to all *Klebsiella* spp. (as they all have *cps* locus genes). Gene sequences of the *cps* locus (specifically *wzi* and *wzc*) are used in the K-typing of *Klebsiella* spp., owing to limitations in conventional K-typing (Brisse et al., 2013; Pan et al., 2013; Wyres et al., 2016). *Klebsiella* spp. *cps* gene sequences (e.g., *wzi*, *wza*, *wzb*, *wzc*, *wbaP*, *wcaJ*, *wzx*, and *wzy*) vary according to their K-antigen composition and are used in genome-based surveillance of *Klebsiella* spp. (Pan et al., 2015; Wyres et al., 2016) (<https://iith.ac.in/K-PAM/>, unpublished work). Notably, a recent study has shown that the arrangement of the genes in the CPS locus is K-type-specific and this finding has been successfully applied to *Klebsiella* spp. K-typing (Pan et al., 2015; Wyres et al., 2016; Wick et al., 2018). It has been found that a *Klebsiella* spp. strain can either contain initialization glycosyl transferase WbaP or WcaJ, but not both (Shu et al., 2009). Sugar composition analysis indicates that the serotypes K1, K2, K4–K8, K11, K13, K14, K16, K17, K22–K25, K28, K30, K31, K33–K35, K37, K39, K44, K45, K48, K54, K55, K58–K61, K64, K67, K69, K71–K73, and K82 have WcaJ and use glucose-Und-PP as an initializing sugar. On the other hand, K3, K9, K10, K12, K15, K18–K21, K26, K27, K32, K36, K38, K40, K41–K43, K46, K47, K49–K53, K56, K57, K62, K63, K66, K68, K70, K74, and K79–K81 use galactose-Und-PP as the initializing sugar and have WbaP in their *cps* gene cluster.

LPS Biosynthesis in the Cytoplasm by ABC-Dependent Pathway

LPS are glycolipids that encompass three structural moieties, namely, lipid A, core oligosaccharides (core-OS), and the O-antigenic polysaccharide (O-PS) (Whitfield and Trent, 2014). The lipid A (the lipid moiety of LPS) is highly conserved and anchors the LPS on the outer leaflet of the outer bacterial membrane. The core-OS is conserved and acts as a linkage between the lipid A and O-PS. The O-PS is highly variable across different *Klebsiella* spp.

Such complexity in the LPS structure leads to a complex biosynthesis pathway that takes place at the cytosolic and periplasmic faces of the inner membrane: (i) biosynthesis of lipid A through the Raetz pathway, (ii) attachment of core-OS to the lipid A, (iii) flipping of the lipid A-core-OS to the periplasmic end, (iv) biosynthesis of O-PS at the cytoplasmic end, (v) flipping of O-PS to the periplasmic region, and (vi) ligation of O-PS to lipid A-core-OS in the periplasmic region (Raetz and Whitfield, 2002; Whitfield and Trent, 2014). Finally, the LPS molecule assembled in the periplasmic region is exported to the bacterial surface wherein lipid A acts as the anchorage point for the LPS (Okuda et al., 2016). The entire process of LPS biosynthesis and surface export involves four different gene

TABLE 3 | Details of the protein structures used in **Figures 1, 2.**

Components of CPS/LPS biosynthesis and transportation pathway	PDB ID (available)	Organism name for which crystal structure is available	Genbank ID of the Klebsiella sequences used for the homology modeling (% sequence identity with available PDB structure)
CPS BIOSYNTHESIS AND TRANSPORTATION PATHWAY (FIGURE 1)			
Wzi	2YNK	<i>Escherichia coli</i>	BAF47011.1 (99.78%)
Wza	2J58	<i>Escherichia coli</i>	BAF47012.1 (99.44%)
Wzb	2WMY	<i>Escherichia coli</i>	BAF4703.1 (99.32%)
Wzc (cytoplasmic domain)	3LA6	<i>Escherichia coli</i> (strain K12)	BAF47029.1 (57.93%)
Wzx	3MKU	<i>Escherichia coli</i>	BAT24471.1 (14.11%)
LPS BIOSYNTHESIS AND TRANSPORTATION PATHWAY (FIGURE 2)			
Wzm	6AN7/6OIH	<i>Aquifex aeolicus</i> (strain VF5)	CZQ25306.1 (34.5%)
Wzt-NBD	6AN5	<i>Aquifex aeolicus</i> (strain VF5)	CZQ25307.1 (46.32%)
Wzt-CBD	2R5O	<i>Escherichia coli</i>	CZQ25307.1 (100%)
MsbA	3B60	<i>Salmonella typhimurium</i>	SSW84925.1 (94.7%)
LptB ₂ FG	5L75	<i>K. pneumoniae</i>	
LptDE	5IV9	<i>K. pneumoniae</i>	

clusters: *lpx*, *waa*, *rfb*, and *lpt*. The gene products of *lpx*, *waa*, and *rfb* are involved in the biosynthesis of lipid A, core-OS, and O-PS, respectively (Regue et al., 2005; Fresno et al., 2007; Okuda et al., 2016). The *lpt* gene products are involved in the transport of the LPS molecule to the extracellular side of *Klebsiella*. The other protein involved in this biosynthesis process is MsbA, which is part of a different gene cluster. Intriguingly, *Klebsiella* spp. LPS biosynthesis and transportation are driven by ATP hydrolysis at three different stages: flipping of the lipid A-core-OS, flipping of O-PS, and transport of LPS from the periplasmic end to the bacterial extracellular region. These steps are outlined below.

Biosynthesis of Kdo₂-lipid A-core-OS

The biosynthesis of lipid A begins in the cytosolic region with the involvement of nine enzymes synthesized from the *lpx* gene cluster (Raetz et al., 2009). The first step is the substitution of an acyl chain to the 3-OH group of uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) (Anderson et al., 1985; Anderson and Raetz, 1987), followed by the release of an acetate group and the addition of the second acyl side chain. Two such monosaccharides are glycosylated, wherein one is phosphorylated (called lipid X) prior to the reaction, following which disaccharide-1-phosphate is again phosphorylated to synthesize Lipid IV_A at the cytoplasmic face of the inner membrane. The matured lipid IV_A is glycosylated with two 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) residues, which are incorporated by WaaA (a product of the *waa* gene cluster) to produce Kdo₂-lipid IV_A. Subsequently, Kdo₂-lipid A is synthesized by the acylation of Kdo₂-lipid IV_A. This entire Raetz pathway takes place in the cytoplasmic end of the inner membrane and is mediated by several glycosyltransferases, along with other enzymes (Raetz and Whitfield, 2002).

In the next step, core-OS is synthesized by extending Kdo₂-lipid A with the help of several glycosyltransferase enzymes (which vary as per the sugar components of different O-antigens). In general, the core-OS is conceptually divided into two regions,

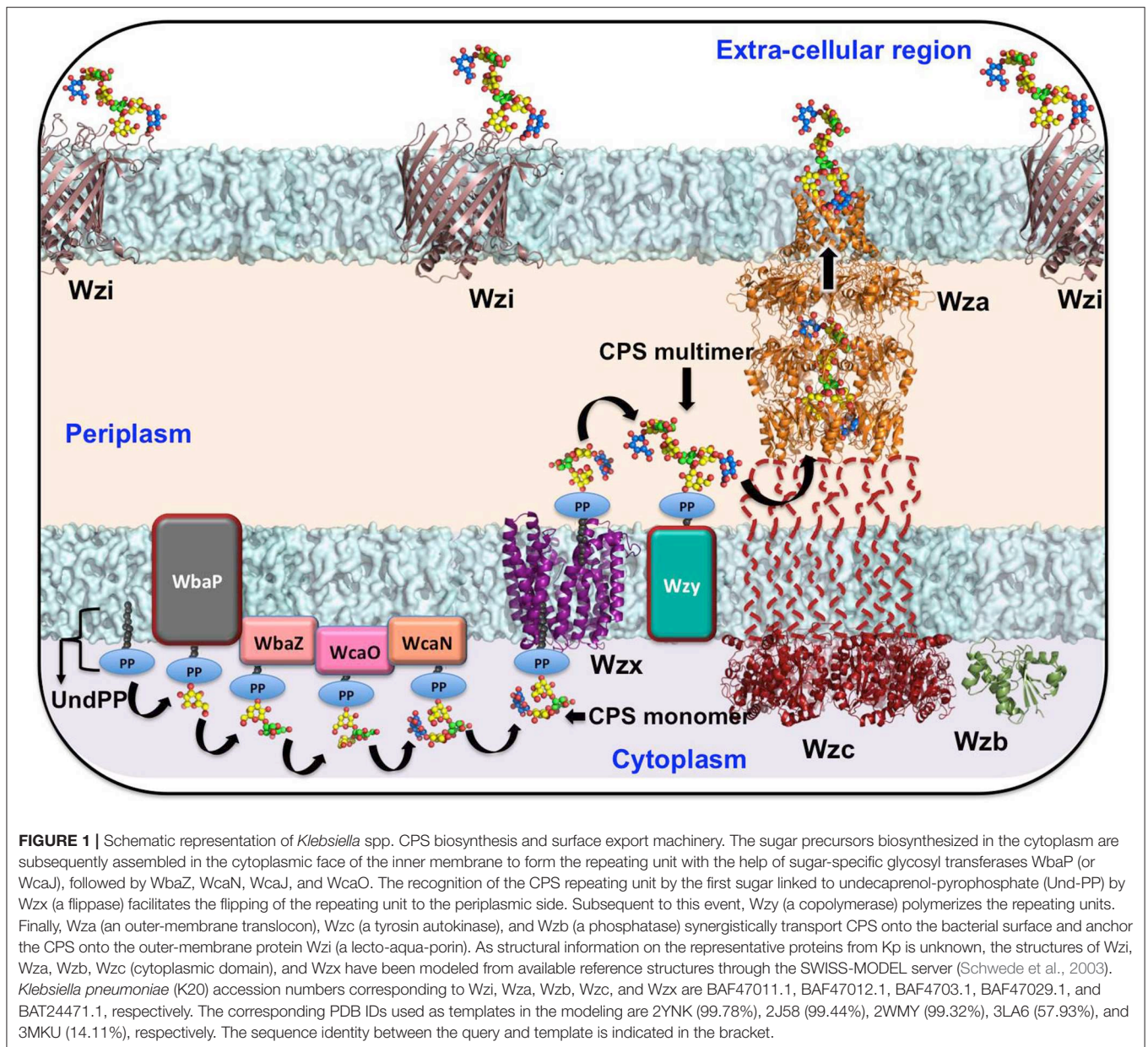
namely, the conserved inner core and the variable outer core. The inner core typically has Kdo₂ and L-glycero-D-manno-heptopyranose (L, D-Hep). The outer core consists of three to six sugars, whose compositions are variable.

Export of Kdo₂-lipid A-core-OS Across the Inner Membrane

The nascent Kdo₂-lipid A-core-OS intermediate is subsequently flipped to the periplasmic end of the inner membrane through an ABC transporter, MsbA. MsbA is a “half” transporter as it contains two different polypeptide chains wherein each chain contains a nucleotide-binding domain (NBD) and a transmembrane domain (TMD). MsbA uses an “outward only” mechanism to flip Kdo₂-lipid A-core across the inner bacterial membrane. In this mechanism, MsbA remains in a resting state with an (inward) open conformation at the cytoplasmic side when ATP is not bound. This inward open form allows Kdo₂-lipid A-core-OS entry. Stable Kdo₂-lipid A-core-OS binding aligns TMD for ATP binding and restricts the opening of TMD. Upon ATP binding, the NBD domain intertwines, and Kdo₂-lipid A-core-OS moves toward the periplasmic side. This coordinated movement of MsbA conformational change and LPS translocation leads to ATP hydrolysis, thus restoring the ground state inward for open confirmation of MsbA. This “outward only” mechanism for Kdo₂-lipid A-core-OS export across the inner membrane is established based on different conformations of MsbA, derived from different Gram-negative bacterial species (Doerrler et al., 2004; Arai et al., 2017; Mi et al., 2017; Ford and Beis, 2019). Upon transport to the periplasmic region, Kdo₂-lipid A-core-OS can undergo environmentally regulated modifications.

O-PS Biosynthesis and Transportation Machinery

Klebsiella spp. O-PS biosynthesis takes place separately at the cytoplasmic end of the inner bacterial membrane. The O-PS has four conceptually different regions: primer, adaptor,



repeating unit, and terminal modification domains (Raetz and Whitfield, 2002). In general, *Klebsiella* spp. have two to five sugars in the O-PS repeating unit that are highly variable for different O-antigens (Clarke et al., 2018). The O-PS repeating unit is assembled on a lipid carrier undecaprenyl phosphate (embedded in the inner membrane) with the help of several glycosyltransferases encoded by *wecA*, *gmlABC*, *wbbMNO*, *wbmV*, *wbmW*, and *wbmX* genes and is transported to the periplasm with the help of an ABC transporter (Clarke et al., 2018). O-PS biosynthesis requires a polyisoprenoid derivative, namely, C₅₅-undecaprenol phosphate (Und-P), which serves as an acceptor for O-PS chain assembly. The reaction begins with the transfer of N-acetylglucosamine (GlcNAc)-1-phosphate onto Und-P. This reaction is facilitated by GlcNAc-1-phosphate

transferase (WecA) and produces Und-PP-GlcNAc, which is the primer region of O-PS. The O-PS is extended on Und-PP-GlcNAc with the help of glycosyltransferases, depending on the sugar composition of the O-PS (Meier-Dieter et al., 1992; Rick et al., 1994; Clarke et al., 1995; Guan et al., 2001; Kos et al., 2009). Depending on the O-antigen, the O-PS biosynthesis *rfb* gene cluster has 6 to 13 genes that are required for O-PS synthesis, of which 6 are essential genes (Clarke and Whitfield, 1992; Clarke et al., 2018). The 5' end of the gene cluster has genes that encode for ABC transporters, and the 3' end of the cluster has genes that produce glycosyltransferases. The adaptor domain, which occurs only once in an O-PS chain and acts as the connection between Und-PP-GlcNAc and the repeat unit domain, is subsequently attached to the growing O-PS. The

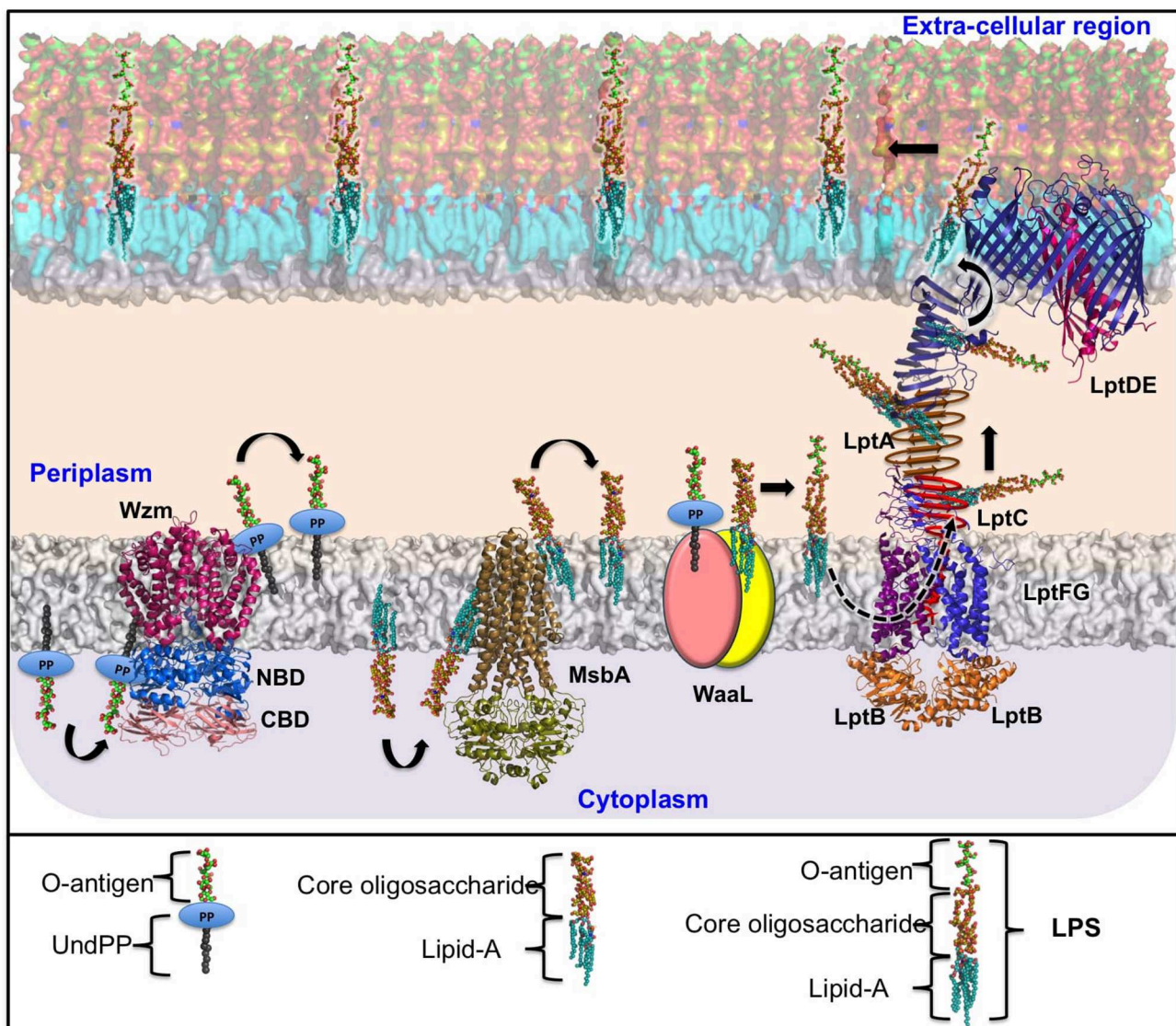


FIGURE 2 | Schematic representation of *Klebsiella* spp. ABC transporter-dependent LPS assembly and transport. The O-antigen repeating unit is synthesized in the cytosol with the help of the corresponding glycosyltransferases and is subsequently polymerized by Wzy and transferred to the periplasm by an ABC transporter (Wzm/Wzt complex). In a similar fashion, the Kdo₂-lipid A-core oligosaccharide biosynthesized in the cytoplasmic region is flipped to the periplasmic region through the ATP-driven MsbA. Following this, the matured O-polysaccharide and lipid A-core-oligosaccharide are ligated by WaaL ligase in the periplasmic region. The completely grown LPS is transported to the bacterial surface through LptA-G assembly as indicated. For the purpose of illustration, the LptB2FG (PDB ID: 5L75) and LptDE (PDB ID: 5IV9) structures are taken directly from Kp, while the Wzm/Wzt complex and MsbA proteins are homology-modeled using structures available in other organisms as templates. The reference PDB IDs for Wzm, Wzt-NBD, and Wzt-CBD are 6AN7 (34.5%), 6AN5 (46.32%), and 2R5O (100%), respectively. The sequence identity between the template and the Kp are given in brackets. The NCBI accession numbers corresponding to the Kp protein sequences are CZQ25306.1 (Wzm) and CZQ25307.1 (Wzt-NBD and Wzt-CBD). LptA and LptC are indicated by schematic representation. The helical and beta-jelly conformation of LptC is shown in red. The beta-jelly conformation of LptA is colored dark gold. As WaaL structural information for any Gram-negative organism is unavailable, the two domains of WaaL are represented in yellow and peach-colored ovals. Note that for the purpose of illustration, O3 has been considered as a case in point. Individual parts of the LPS and O-antigen are annotated separately at the bottom of the figure.

O-PS chain extension takes place by the addition of a repeat-unit domain. The growth of O-PS occurs at the non-reducing end of the polysaccharide chain. Finally, the O-antigen length is regulated either through a covalent modification at the terminal residue of the O-PS (terminal capping/modification) or as a result of the stoichiometry of the Wzm-Wzt ABC transporter

that transfers the Und-PP linked O-PS to the periplasmic end (see below).

O-antigen Transport Through the Wzm/Wzt System

After polymerization, the O-antigens are transported to the periplasmic-leaflet of the inner membrane by an ABC transporter

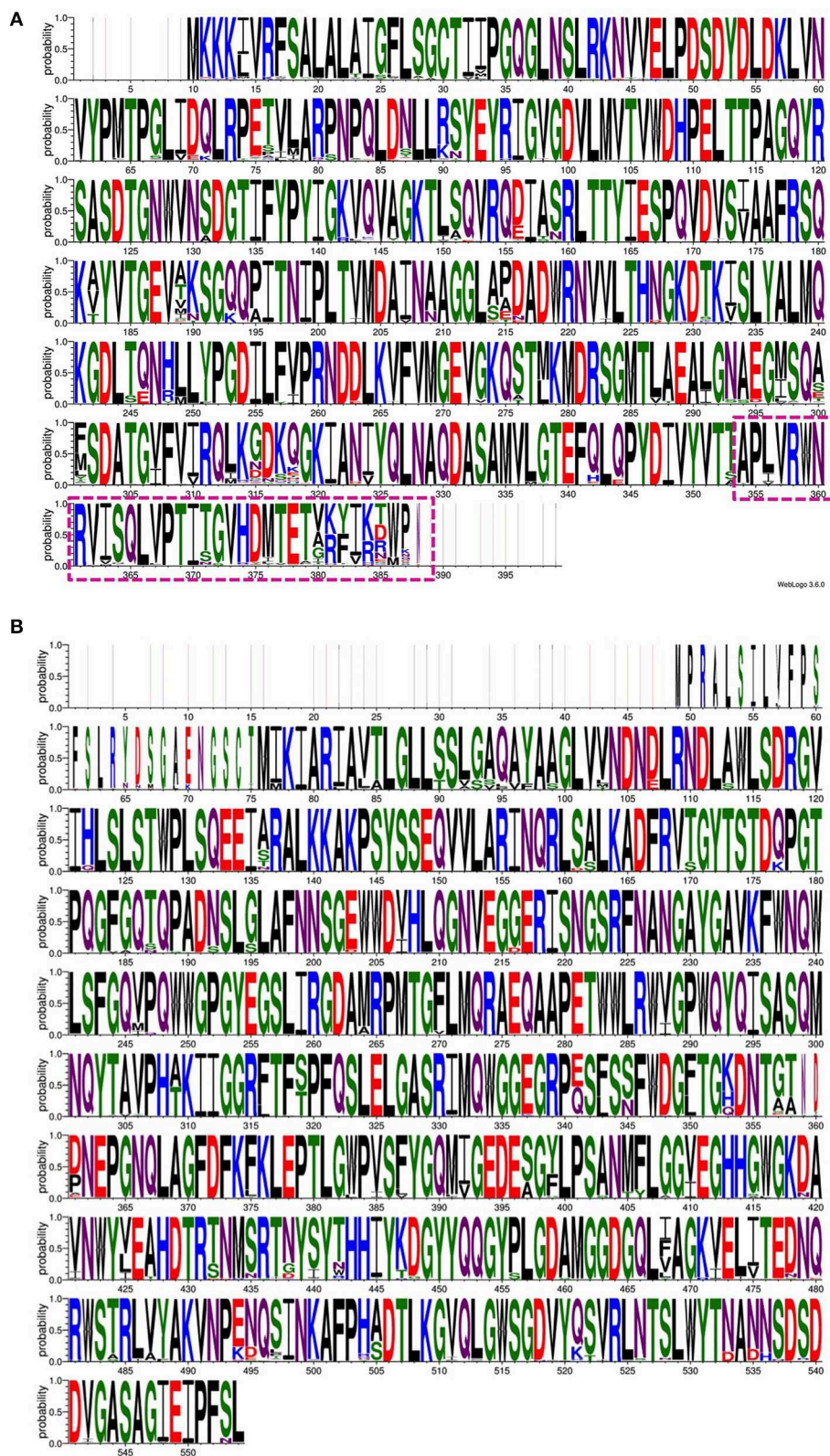


FIGURE 3 | An amino acid sequence logo constructed using the multiple sequence alignment of 139 Wza protein sequences (A) and 138 Wzi protein sequences (B). Note the variation observed in the C-terminal region (transmembrane region) of Wza that faces the extracellular region of the bacterial cell (dash-box) (A). In contrast, Wzi sequences are highly conserved between different serotypes of *Klebsiella* spp. Note that non-redundant sequences that have a defined K-type are used to generate sequence logo.

that has two transmembrane domains (TMDs) (named Wzm) and two nucleotide-binding domains (NBDs) (named Wzt) (Kos et al., 2009). This O-antigen ABC transporter system is common in most of the Gram-negative bacteria. Intriguingly, in some of the *Klebsiella* spp., the O-antigen ABC transporter has an additional carbohydrate-binding domain (CBD) that is fused to the C-terminus of the NBD (Cuthbertson et al., 2005, 2007; Liston et al., 2017). Chemical modifications, such as the addition of a phosphate or methyl group at the non-reducing end of some O-antigens, provide the biosynthesis completion signal, which is recognized by the CBD to accomplish the transport (Liston et al., 2017). O12 is one such antigen that has the CBD, while such a mechanism is absent in the uncapped O-antigen biosynthesis in *Kp* (Bi et al., 2018).

Although structural information pertaining to the *Klebsiella* spp. Wzm/Wzt ABC transporter is unavailable, its homologous structure from *Aquifex aeolicus* has provided insights into the mechanism of O-antigen transport. Wzm/Wzt structures determined from *A. aeolicus* in ATP-free (Bi et al., 2018) and ATP-bound (Caffalette et al., 2019) forms reveal that the formation of a continuous inner transmembrane (TM) channel is wide enough to accommodate an O-antigen chain in the nucleotide-unbound conformation. ATP is seen in the bound conformation at the conserved Walker A, Walker B, and H-loop signature motifs of NDB (Davidson et al., 2008; Locher, 2016). These motifs are conserved between *Klebsiella* spp. and *A. aeolicus* and are essential for the transport of O-antigens across the inner membrane. In the complex form, the NBD adopts a compact structure and interacts with the Wzm dimer. The O-antigen chain bound to the Wzm/Wzt transporter is passed through the TM channel to reach the periplasmic face of the inner membrane, following which the lipid portion of the Und-PP-N-acetamido sugar moiety is inserted into the inner-membrane periplasmic leaflet (onto which the O-antigen is anchored) (Figure 2, left).

LPS Maturation in the Periplasm

The LPS intermediates (Und-PP-linked O-PS and Kdo₂-lipid A-core-OS) that are transported to the periplasm are ligated with the help of WaaL ligase (a product of the *waa* gene cluster) (Regue et al., 2005). The Und-PP-linked O-PS is transferred to Kdo₂-lipid A-core-OS by the formation of a glycosidic bond between the first sugar of the O-PS and the sugar in the outer core.

LPS Transport to the Outer Membrane Through LptA-G

The LPS is transported to the outer membrane through a transport system comprising seven proteins, namely, LptABCDEFG (LptA-G) (Sperandeo et al., 2007; Ruiz et al., 2008; Freinkman et al., 2011, 2012; Villa et al., 2013). All seven of the protein structures of the LPS transport system have been fully characterized (Botos et al., 2016; Dong et al., 2017; Vetterli et al., 2018; Li et al., 2019; Owens et al., 2019). Among these proteins, the LptDE and LptB₂FG complex structures are known for *Klebsiella* spp. (Table 3), while structural information for the remaining components is available for other Gram-negative bacterial species (Vetterli et al., 2018; Li et al., 2019; Owens

et al., 2019). This structural information, combined with existing knowledge of the associated transport mechanisms, has been used here to explain LPS transport in *Klebsiella* spp. Strikingly, the portal for transport of LPS molecules is formed by LptD and LptE, which is connected to a pump-like system formed by the LptB₂FG ABC-transporter through a bridge-like structure consisting of LptA and LptC (Bishop, 2019; Li et al., 2019; Owens et al., 2019). The individual sections of this integrated LPS transporter are discussed below. As the LptA-G transporter is distributed across the inner membrane, periplasmic region, and outer membrane, this nano-machine represents a promising antimicrobial target.

Insertion and Translocation of LPS Into LptB₂FG

LPS is driven across the ABC transporter LptB₂FG (Okuda et al., 2012; Sherman et al., 2014) in a continuous flow from the periplasmic leaflet of the inner membrane to the periplasmic domain of LptC and through the transmembrane helix of LptC (Sperandeo et al., 2008, 2011; Narita and Tokuda, 2009). This is accomplished by utilizing energy derived from the ATP-hydrolysis activity of LptB (Narita and Tokuda, 2009; Sherman et al., 2014). The LptB₂FG complex contains two transmembrane domains (LptF and LptG) and two nucleotide-binding domains (LptB₂) (Ruiz et al., 2008; Narita and Tokuda, 2009). Both LptF and LptG contain a periplasmic β -jelly roll domain that is unique to this ABC transporter (LptB₂FG). LPS passes into LptFG through a lateral opening formed by transmembrane helix 1 (TM1) of LptF and TM5 of LptG through an electrostatic gating mechanism (Dong et al., 2017). The LPS subsequently travels to the periplasmic domain helix (locked in-between TM1 of LptG and TM5 of LptF) of LptC (Okuda et al., 2016) in a stepwise manner (Owens et al., 2019). The soluble periplasmic protein LptA bridges LptC and the N-terminal domain of outer-membrane protein LptD by forming a head-to-tail oligomer (Suits et al., 2008) with a continuous hydrophobic groove (Bowyer et al., 2011; Sperandeo et al., 2011; Grabowicz et al., 2013; Villa et al., 2013). LptA shares a β -jelly roll fold with the periplasmic domain of LptC (Tran et al., 2010) and the N-terminal domain of LptD (Qiao et al., 2014). Strikingly, a β -jelly roll fold arrangement with a similar hydrophobic groove has also been observed in the periplasmic domain of LptF (Dong et al., 2017; Li et al., 2019; Owens et al., 2019), which could explain the transport of LPS to the outer membrane of the bacteria (as mediated by the LptFG complex).

LPS Assembly Onto the Outer Leaflet of the Outer Membrane

The N-terminal domain of the outer-membrane LptD is thought to be very flexible in order to maintain the physical connection and integrity of the LptCAD scaffold (Botos et al., 2016). Soon after the N-terminal domain of LptD accepts the LPS from the periplasmic protein LptA, it undergoes a significant conformational change in such a way as to open up a luminal gate formed by two periplasmic loops of LptE with LptD. The opening of the LptDE lateral gate facilitates LPS transit through the periplasmic hydrophobic groove to the extracellular region (Botos et al., 2016). Subsequent to this, the lipid A section of

LPS is inserted directly into the membrane and facilitates the transition of the polysaccharide fragment through the barrel lumen to the extracellular space (Gu et al., 2015; Botos et al., 2016; Dong et al., 2017).

A THERAPEUTIC PERSPECTIVE FOR COMBATING *KLEBSIELLA* SPP. INFECTIONS

Although antibiotics such as third-generation cephalosporins, aminoglycosides, fluoroquinolones, and carbapenems (Navon-Venezia et al., 2017; The European Antimicrobial Resistance Surveillance Network, 2018) have contributed dramatically to the reduction of morbidity and mortality associated with *Klebsiella* spp. infections, the continued emergence of cKp strains with extreme drug resistance and the newly emerged multidrug-resistant hypervirulent *Klebsiella* strains (Gu et al., 2018) limit current treatment options to eradicate infections (Brisse et al., 2009; Magiorakos et al., 2012; Doorduyn et al., 2016; Navon-Venezia et al., 2017; Martin and Bachman, 2018). Alarming, recent evidence suggests that *Klebsiella* has also evolved mechanisms to actively suppress innate immune responses (Bengoechea and Sa Pessoa, 2019), in addition to other well-known stealthy *Klebsiella* immune evasion strategies. Although many virulence factors are thought to be involved in the counteraction of host defenses by *Klebsiella*, only a few of these are well-studied, including CPS, LPS, fimbriae, and siderophores (Paczosa and Meccas, 2016). As CPS and LPS actively participate in hijacking host defenses to establish infection, targeting these can prevent the growth of *Klebsiella* spp. (rather than killing the pathogen) by imposing less intense selective pressure. Ultimately, this may limit the evolution of resistant strains. Here, the biosynthesis and export of these surface-associated polysaccharides are discussed from the perspective of the treatment of *Klebsiella* infections.

CPS and LPS protect *Klebsiella* spp. from the action of complement cascade and antimicrobial peptides, as well as from engulfment and phagocytosis by host immune cells (Alvarez et al., 2000; Regueiro et al., 2006; Pan et al., 2011). In addition, CPS acts as a physical barrier to protect LPS (Merino et al., 1992; Alvarez et al., 2000). EPS (another surface-associated polysaccharide) is a component of biofilm and has been shown to interfere with the action of antimicrobial peptides of the host immune system (Bellich et al., 2018). Thus, inhibition of the LPS, CPS, and EPS biosynthesis and surface expression would be an effective approach to counteract *Klebsiella* anti-immune strategies. As the EPS secretion pathway and its structural composition in *Klebsiella* spp. is not well-understood, this review discusses the treatment strategies of *Klebsiella* infections from the perspective of CPS and LPS.

CPS and LPS are biosynthesized in cytoplasmic/periplasmic regions of the inner bacterial membrane and are transported to the bacterial surface with the help of sophisticated proteinaceous nano-machines. Thus, blocking the biosynthesis of CPS and LPS or disrupting the assembly of these nano-machineries can block the surface expression of these molecules that offer

protection from the host immune response. For instance, LPS biosynthesis can be targeted in three different stages: lipid A, core-OS, and O-PS biosynthesis. Targeting the components of biosynthesis may prevent the formation of LPS and render *Klebsiella* vulnerable to host defenses. A possibility for novel antibiotic development could involve targeting the lipid A synthesizing enzymes (synthesized by the *lpx* locus), as there are no human homologs for them (Whitfield and Trent, 2014). Indeed, a recent study drawing on multi-omics data from sources including genomics, transcriptomics, structuromics, and metabolic information has listed LpxA, LpxB, LpxC, and LpxD as prioritized non-host homologous protein targets (Ramos et al., 2018). Targeting the LPS export pathway proteins represents yet another strategy. Specifically, the outer-membrane proteins [LptD and LptE (Figure 2)] involved in LPS export represent potential antibiotic targets, given that they are easily accessible (Srinivas et al., 2010; Robinson, 2019). Producing antibodies against these outer-membrane proteins is also of particular clinical interest (Storek et al., 2019). Similarly, Wzm, Wzt, and MsbA could also be targets for the development of novel antimicrobials (Alexander et al., 2018; Ho et al., 2018).

Targeting the proteins that participate in Wzx/Wzy-dependent CPS transport and the surface expression pathway (Figure 1) may interfere with CPS export to the bacterial surface (Sachdeva et al., 2017). For instance, manipulating the function of the aqua-lecto-porin Wzi (Bushell et al., 2013; Sachdeva et al., 2016), as well as capping the extracellular side of Wza (Dong et al., 2006) involved in CPS surface expression through a novel antibiotic, would be potential targets similar to that for *E. coli*. It is worth noting that a similar strategy has been successfully demonstrated in *E. coli* Wza (Kong et al., 2013; Sachdeva et al., 2017). However, the sequence diversity of the surface-exposed region of Wza across various K-types may present a challenge in designing a common antibiotic (boxed region in Figure 3A). In contrast, Wzi is highly conserved and is a potential target for all *Klebsiella* spp. (Figure 3B).

Another approach for the treatment of *Klebsiella* infection involves the development of antibodies targeting CPS and LPS (Szijarto et al., 2017; Diago-Navarro et al., 2018; Kobayashi et al., 2018). Cell surface carbohydrate-based vaccines (Hutter and Lepenies, 2015) can be an effective choice for combating *Klebsiella* infections (Cryz et al., 1985; Cross, 2014; Seeberger et al., 2017; Adamo and Margarit, 2018; Hegerle et al., 2018; Micoli et al., 2018). Glycan epitopes, namely, the antibody-interacting and minimal antigenic determinant of O- or K-antigens, can be used in vaccine development. The heterogeneity and complexity of O- and K-antigens of different *Klebsiella* serotypes may pose a challenge to the development of a polyvalent vaccine against all *Klebsiella* infections. Fortunately, only a few O- and K-antigens are found in clinical isolates; thus, they can be used in the development of a novel immunogenic polyvalent glycoconjugate *Klebsiella* vaccine with the help of improved vaccine technology. Multiple interactions between protein and glycan is essential at different stages of the immune response. Identification of surface saccharide epitope patterns in clinical/hypervirulent strains and their use in the design of a unique synthetic glycan epitope conjugated with an

immunogenic carrier protein may be useful in the development of an effective multivalent glycoconjugate *Klebsiella* vaccine.

Although *Klebsiella* spp. have 12 O-antigens, seroepidemiological investigations have revealed only four *Klebsiella* O serotypes found in clinical isolates (Edelman et al., 1994; Cryz et al., 1995; Trautmann et al., 2004). Thus, *Klebsiella* anti-endotoxin vaccines/antibodies can be developed based on the O-antigen structure of clinical isolates of *Klebsiella* spp. Protection against Kp through anti-LPS antibodies has been successfully demonstrated (Cohen et al., 2017; Pennini et al., 2017; Hegerle et al., 2018). Although thermostable LPS is a strong immune activator, Kp quite often uses modifications of lipid A of LPS in such a way that it is no longer recognized by certain immune receptors such as TLR4 (Llobet et al., 2015). This helps it evade the complement system and to survive within the host during colonization and infection (Llobet et al., 2011, 2015; Kidd et al., 2017; Mills et al., 2017). Modification of the polysaccharide composition of the O-antigen side chain (which is exposed to antibodies) and elongation of the O-antigen has also been documented (Doorduyn et al., 2016). Kp strains with a long O-antigen produce a high-molecular-weight (smooth phenotype) LPS that is less susceptible to serum killing, as compared to strains lacking an O-antigen side chain with a low-molecular-weight (rough phenotype) LPS (Ciurana and Tomas, 1987; Mccallum et al., 1989). For example, D-galactan I to D-galactan III structure modification of the O-antigen is found to improve Kp survival in human serum compared to strains expressing D-galactan I (Szijarto et al., 2016). Similarly, an epidemic multidrug-resistant Kp clone (Tzouveleakis et al., 2013) was found to have a modified O-antigen structure (Wyres et al., 2015; Szijarto et al., 2016). Modification of the glycan structures at the terminal end of the O-antigen has also been shown to alter complement activation in Kp (Tytgat and Lebeer, 2014; Adamo and Margarit, 2018).

CPS could also be exploited to counteract *Klebsiella* anti-immune strategies. Recognition of this possibility has led to the development of a 24-valent CPS-based vaccine for *Klebsiella* (Cryz et al., 1991; Edelman et al., 1994; Campbell et al., 1996; Donta et al., 1996). Although a phase 1 trial of the vaccine has shown it to be immunogenic and non-toxic (Edelman et al., 1994), no further developments have been reported in the last two decades. Similar to LPS, the capsule also undergoes modifications to resist the host complement system (Wyres et al., 2015; Szijarto et al., 2016). This may pose a challenge in developing a vaccine against *Klebsiella* spp. infections. Chemical modifications in K-antigen structures, such as acetylation and deacetylation (Hsu et al., 2016), may also bring about differential effects in CPS antigenicity, representing yet another challenge in the development of vaccines against *Klebsiella* spp. K2-antigen-lacking mannobiose or rhamnobiose produced by a Kp strain

escapes host recognition during the host innate immune response (Sahly et al., 2009). It is worth noting that hvKp strains are frequently found to have K2 antigens.

Use of exogenous cholesterol and bacteriophage depolymerase against *Klebsiella* infections represents yet another promising approach. It has been shown that exogenous cholesterol increases macrophage-mediated phagocytosis by down-regulating the expression of genes responsible for LPS core oligosaccharides production, as well as reducing the anti-phagocytic properties of the Kp capsule (Ares et al., 2019). The discovery that bacteriophage capsule depolymerases can be used against *Klebsiella* capsule types KN1, KN3, KN4, and K56 represents a potential approach for the treatment of Kp infections (Pan et al., 2019).

Significant progress has been made in understanding *Klebsiella* immune evasion strategies. As the CPS and LPS of *Klebsiella* spp. play an important role in hijacking host defenses, targeting these virulence factors may be an efficient strategy against *Klebsiella* infections. The known structural components of *Klebsiella* CPS and LPS export machineries could be useful in the design of novel antibiotics. However, heterogeneity in sugar composition, glycosidic linkage, stereoisomeric forms, and the concomitant variation in the proteins involved in biosynthesis and transport may pose a challenge in the design of antibiotics and vaccines that can be used against diverse *Klebsiella* spp. In addition, the ability of *Klebsiella* spp. to modify components of the CPS and LPS may be another concern. Recent developments in gene sequencing techniques in combination with a metagenomic approach to the investigation of Kp clinical strains help in the design of polyvalent vaccines. A combinatorial therapy involving *Klebsiella* vaccines against surface polysaccharides and antibiotics inhibiting surface antigen assembly may represent the most promising approach.

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TR designed and supervised the entire project. LP and TR wrote the manuscript.

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Review of Antibiotic Resistance, Ecology, Dissemination, and Mitigation in U.S. Broiler Poultry Systems

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Since the onset of land application of poultry litter, transportation of microorganisms, antibiotics, and disinfectants to new locations has occurred. While some studies provide evidence that antimicrobial resistance (AMR), an evolutionary phenomenon, could be influenced by animal production systems, other research suggests AMR originates in the environment from non-anthropogenic sources. In addition, AMR impacts the effective prevention and treatment of poultry illnesses and is increasingly a threat to global public health. Therefore, there is a need to understand the dissemination of AMR genes to the environment, particularly those directly relevant to animal health using the One Health Approach. This review focuses on the potential movement of resistance genes to the soil via land application of poultry litter. Additionally, we highlight impacts of AMR on microbial ecology and explore hypotheses explaining gene movement pathways from U.S. broiler operations to the environment. Current approaches for decreasing antibiotic use in U.S. poultry operations are also described in this review.

Keywords: antibiotic resistant gene determinant, soil microbiome, broiler systems, One Health Approach, environmental dissemination

INTRODUCTION

Antibiotic Use and History in U.S. Broiler Operations

Antimicrobial compounds and antibiotics in U.S. broiler (meat chicken) operations have widely been used to treat and prevent bacterial, protozoal, and fungal pathogens that sicken or kill birds, as well as promote growth (Chapman and Johnson, 2002; McEwen and Fedorka-Cray, 2002; Sneringer et al., 2015). Considering, disease in broiler flocks can account for 20% loss of the

Abbreviations: AMR, antimicrobial resistant; ARG, antibiotic resistant gene; ARGD, antibiotic resistant gene determinant; FDA, Food and Drug Administration; NARMS, National Antimicrobial Resistance Monitoring Systems; WHO, World Health Organization.

gross value of production (Food and Agriculture Organization of the United Nations [FAO], 2013) antibiotics are important tools in poultry production. Continuous improvement in disease management and the establishment of government regulations has lead to staggering increases in poultry production efficiency [e.g., in 1965, 112 rearing days would produce a 1.13 kg chicken with a 4.7 feed conversion ratio (weight/feed intake); whereas current rearing periods are 42 days for a 2.7 kg chicken with a feed conversion ratio of 1.8] (National Chicken Council, 2015). Concurrent with production efficiency increases is consumption, as the average American now consumes 41 kg of broiler meat per year (National Chicken Council, 2018).

The first use of antibiotic drugs in poultry can be traced back to 1946 (Moore et al., 1946) and first resistance was reported in food animals by Starr and Reynolds (1951), with concerns about the development of resistance dating back to 1969 (Dibner and Richards, 2005). After the first cases of antibiotic resistant bacterial diseases in humans, recommendations were made for banning the use of antibiotics as growth promoters if drugs are also prescribed for use in human medicine (e.g., penicillins, tetracyclines, and sulfonamides; Swann et al., 1969). In a survey from 1995 to 2000, there was a substantial decline in the use of antibiotics in U.S. broiler operations (Food and Drug Administration [FDA], 2014). In another report released in 2011, it was estimated that 20–52% of broiler operations used antibiotics for production purposes not related to disease control. This report also found a long-term decline in antibiotic use in broiler production (Sneeringer et al., 2015). More recently, based on a report of antimicrobials sold or distributed for use in food-producing animals from the U.S. Food and Drug Administration (FDA), approximately 3,345,022 kg of antimicrobials were sold and used in the U.S. poultry industry in 2016; with 1,265,420 kg being “medically important” in human medical therapy (Food and Drug Administration [FDA], 2017). Among the most significant action that the FDA Center for Veterinary Medicine has taken, is to transition medically important antimicrobials that are used in the feed or drinking water of food-producing animals to veterinary oversight, and to eliminate the use of these products in animals for production purposes, such as for growth promotion (Guidance for Industry #213; Food and Drug Administration [FDA], 2013).

According to the World Health Organization (WHO), antimicrobial resistance (AMR) is defined by “an increase in the minimum inhibitory concentration of a compound for a previously sensitive strain” (World Health Organization [WHO], 2013). There are four general mechanisms that cause antibiotic resistance: target alteration, drug inactivation, decreased permeability, and increased efflux (Munita and Arias, 2016). It is still uncertain if resistance genes are a result of adaptation through chromosomal mutation (or gene shuffling), or through horizontal gene transfer (or the movement of genetic materials between different organisms), instead of vertical transmission of DNA from parent to offspring (Nesme and Simonet, 2015).

While specific links between antibiotic-use in animal agriculture and human health have been debated (Vaughn and Copeland, 2004), one contributing factor cited

for the decline in antibiotic use is consumer demand for “antibiotic-free” chicken products. There is growing interest in sustainable food production and research is currently being conducted to identify antibiotic alternatives that could support healthy growth and provide defense against pathogenic microbes (Sneeringer et al., 2015; Gadde et al., 2017). Therefore, the broiler industry is now a new leader in management systems that seeks to eliminate the use of antibiotics for the entire broiler lifecycle. A comprehensive review of currently available compounds, their mechanism of action and advantage and disadvantages in applied broiler production is available from Gadde et al. (2017). A brief list of sample types, susceptibility to antibiotics, and mechanism of resistant can be found in **Table 1**.

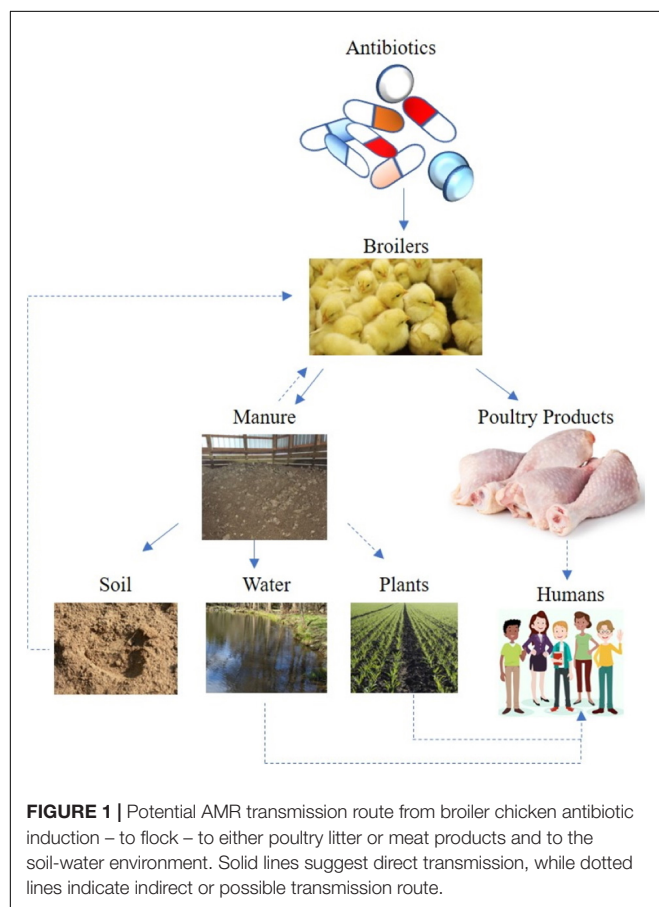
Finally, the U.S. is the world's largest poultry producer with over 9 billion broilers produced annually, with roughly 45% of broilers being produced in 4 mid-south states (Arkansas, North Carolina, Georgia, and Alabama). Poultry litter is a combination of bedding material, poultry excreta, spilled feed and feathers and is produced in significant quantities. By some estimates, nearly 13 million Mg (14 million tons) of broiler litter is produced on U.S. poultry farms annually (Moore et al., 1995; Gollehon et al., 2001). Consequently, large volumes of manure are produced in areas of concentrated poultry production, which serve as a valuable source of nutrients, but are also as possible sources of AMR bacterial populations in the environment (Thanner et al., 2016). Approximately 30–80% of the veterinary antibiotics administered to animals are excreted in manure and urine (Sarmah et al., 2006). Therefore, poultry litter-amended soil may serve as a non-point source for antibiotics that enter surface and ground waters via runoff and leaching. The goal of this review is to provide an update on the development and fate of antibiotic resistance genes (ARG) and bacteria in U.S. broiler poultry operations, and explore hypotheses explaining gene movement pathways to the environment. In the next section, resistance transmission and factors contributing to its development in poultry operations will be discussed as it relates to the soil microbiome.

Reservoirs and Transmission of AMR Bacteria and Genes From Farm-to-Field

Soils are an immense reservoir of microbiological diversity, considering a gram of soil may contain 10^6 – 10^9 bacterial cells of 10^3 – 10^6 different bacterial species (Girvan et al., 2003; Torsvik, 2011). Therefore, it is no surprise that the majority of antimicrobial compounds used in animal healthcare were originally isolated from the soil; namely bioactive compounds synthesized by bacteria (e.g., *Streptomyces* spp.) or fungi (Waksman and Woodruff, 1942). Consequently, the complex ecology of AMR can only be properly assessed by taking environmental reservoirs into account (**Figure 1**). In contrast to the strict clinical definition of resistance, which characterizes resistance phenotypes in isolated bacterial strains, the environmental resistome includes all ARG in the environmental, including ARG precursor genes and cryptic resistance genes (Nesme and Simonet, 2015). Recent research has identified ARG in diverse environmental samples ranging from pristine environments to agricultural soils (Demanèche et al., 2008; Allen et al., 2009; Cook et al., 2014).

TABLE 1 | Sample sources, susceptibility to antibiotics, and mechanisms of potential AMR gene transfer to the environment.

Sample sources	Susceptibility to antibiotics	Mechanisms of AMR gene transfer	References
Poultry fecal waste	The study indicated that poultry samples showed a high prevalence of CTX-M cluster 9 and bla _{TEM} .	Horizontal transfer of ARGs by Bacteriophages	Colomer-Lluch et al., 2011
Composted poultry manure	Poultry manure applications increased AMR genes in the rhizosphere, root endophyte, and phyllosphere, suggesting poultry manure may have an impact on lettuce resistomes.	No mechanism reported.	Zhang et al., 2019
Poultry litter	50% of these isolates were susceptible to ampicillin, 57% to erythromycin, 25% to tetracycline, 4% to chloramphenicol, 40% to kanamycin, 75% to streptomycin, 54% to tobramycin, and 4% to rifampicin.	Transformation and conjugation was reported as a mechanism for horizontal gene transfer between bacteria in poultry litter.	Sridevi Dhanarani et al., 2009
Poultry litter and soil	Out of the 13 antibiotics tested for <i>E. coli</i> , high (>70%) and similar (in the range of 10–15%) resistance against 7 antibiotics was observed in samples from both litter and agricultural soils where poultry litter applied.	No mechanism reported.	Bhushan et al., 2017
Poultry litter	The 86% of litter isolates (163 isolates in total) were resistant to more than one antibiotic.	No mechanism reported.	Furtula et al., 2013



For these reasons, soil is a predominant reservoir for ARG determinants (ARGD, or determinants of resistance; Van Goethem et al., 2018). For example, AMR genes have been recovered from 30,000 years old permafrost samples, which suggests AMR is an ancient phenomenon, existing before antibiotic usages (D'Costa et al., 2011). Laboratory work also demonstrates that antibiotic resistant strains are very stable

even in the absence of antibiotic selection pressure (Gibreel et al., 2005). Consequently, AMR development by pathogenic bacteria and/or commensal (or “friendly”) bacteria is a complex interaction and an evolutionary phenomenon.

Current research has focused on tracking the direction of gene transfer from environment to poultry and has important implications for future antibiotic resistance management and microbial ecology (Cook et al., 2014; Nesme and Simonet, 2015). Three research studies indicate it is probable that lateral resistance gene transfer is the primary pathway of gene acquisition from different environments, including that from soils to pathogenic bacteria genomes (Allen et al., 2009; Forsberg et al., 2012; Nesme et al., 2014). For example, Forsberg et al. (2012) evaluated resistant bacteria via functional metagenomic methods and determined that substantial amounts of resistance genes are shared between the soil and the gut microbiome and can transfer resistance to a previously susceptible *Escherichia coli* host. A shared resistome was also observed (Allen et al., 2009; Nesme et al., 2014) with metagenomics sequencing. These studies continue to emphasize the importance of environmental reservoirs of AMR in the emergence of novel clinical resistance (Nesme and Simonet, 2015).

While some research has not distinguished the direction of transfer (either through gene acquisition or through modulation), studies have shown that commensal (non-pathogenic) and pathogenic microorganisms share resistance genes with soil communities. Specifically, contact of antimicrobial compounds may stimulate bacterial stress response, which can result in increased mutation rates in co-dispersed bacteria, with co-selection amplifying this effect; thus allowing clustering of ARG (Yong-Guan et al., 2017). For example, DNA element class 1 integrons, which are assemblies of gene cassettes that allow bacteria to adapt and evolve through the expression of new genes, can capture and integrate foreign genes from the environment. This has played an important role in spreading antibiotic resistance from non-pathogenic bacteria to pathogenic bacteria in the environment (Yong-Guan et al., 2017). Next generation sequencing now indicates that a derivative of class 1 integrons can be found in every gram of feces

and agricultural animals, with up to 10^{23} copies being released into the environment every day (Gillings, 2017). This is one example of the abundance and distribution of resistant genes, although more research is needed to identify anthropogenic AMR genes in the environment relative to baseline levels (Durso et al., 2016).

The pathogenic bacteria pathway from the animal through the environment is complicated and more longitudinal studies are needed to follow AMR genes through agricultural systems (Yang et al., 2019). These complex transmission routes of AMR bacteria and genes within animals and the environment make it difficult to identify the AMR reservoir and which reservoir poses an animal health. The current approach for assessing the reservoir of AMR bacteria and genes is to identify the indicator bacteria and analyze the level of AMR gene in farm animals (Thanner et al., 2016). With this research, descriptive metadata are needed that describes specific environments, which may reflect survivability and gene transfer. For example, the Terra Genome project includes soil information needed to evaluate terrestrial DNA which includes: (1) site description, (2) sampling description, (3) climate, (4) soil classification, and (5) soil analysis (Cole et al., 2010). These metadata should be important for pathogen viability, easy and inexpensive to obtain, and collected by established and standard methods. These metadata may provide a better understanding and potential mitigation strategies to minimize AMR dissemination.

Sources of AMR Genes in the Environment

The role of the environment as a transmission route for bacterial pathogens has long been recognized, often associated with fecal contamination of water or organic fertilizer applications (Bengtsson-Palme et al., 2018). Depending on antibiotic properties, significant (e.g., up to 90%) amounts of veterinary antibiotics pass un-degraded through the animal gut to manure (Sarmah et al., 2006; Berendsen et al., 2015). Bacterial pathogens can be introduced to a flock via many routes, including feed, water, air, insects and other pests (Trampel et al., 2014; Mouttoutu et al., 2017). Once introduced into a flock, pathogenic bacteria are excreted in the manure, and can survive in the litter (Chen and Jiang, 2014). Therefore, antibiotics, resistance genes, and microorganisms can be transferred from manure to soil (Cook et al., 2014; He et al., 2014). Following land application of poultry litter, antibiotics migrate from soil through runoff, leaching, and particle adsorbed runoff (Kay et al., 2004; Leal et al., 2013; Sun et al., 2013), potentially ending up in soil, surface water, and groundwater (He et al., 2014; **Figure 1**). Measuring antibiotics in a complex matrix, such as soil, is subject to technical limitations, and studies measuring veterinary pharmaceuticals in soil and water are reviewed elsewhere (Thiele-Bruhn, 2003; Dinh et al., 2011; Aga et al., 2016).

Several research efforts have been conducted for testing sources of AMR pathogenic bacteria and the transmission route from the chicken flock, to processing, and the larger environment (Berndtson et al., 1996; Berrang et al., 2001; Posch et al., 2006;

Cook et al., 2014). Numerous routes have been suggested for the introduction of AMR pathogens into the chicken flock, such as horizontal gene transfer from an environmental source to the chicken flock (Krauland et al., 2009), or vertical transmission from breeder to progeny chicks (Pearson et al., 1996). Feed and water can also serve as potential reservoirs and transmit AMR pathogens from the environment to the chicken flock (Byrd et al., 1998; Perez-Boto et al., 2010). Although it is thought that cross-contamination of meat products can occur during the slaughter process (Berndtson et al., 1996; Berrang et al., 2001), there is limited information related to the transmission route from one part of contaminated meat to the whole retail product.

Mechanisms of AMR Gene Transfer

Even though it has been suggested that there is a relationship between antibiotic usage in agricultural animals and AMR emergence, it does not mean that the usage of antibiotics is the only explanation for AMR prevalence. For example, AMR genes are carried by mobile genetic elements and can be transferred among distantly related bacteria from different phyla (Musovic et al., 2006). Plasmids and transposons can serve as the vehicle in horizontal gene transfer. Transposons, coding for antibiotic resistance, are able to cut AMR genes from one bacterial chromosome or a plasmid, and subsequently insert AMR genes into another chromosome or plasmid in other bacteria by the process of conjugation. Through this process, multiple AMR genes can be transferred among different bacteria; thus resulting in multi-drug resistance. Without antibiotic exposure, AMR genes may be able to persist long-term, such as VanA glycopeptide-resistant *Enterococci* (Johnsen et al., 2002). For example, van-resistant *Enterococci* can reportedly be stable after 1,000 generations in serial transfer broth cultures and gnotobiotic mice without antibiotic selection. The administration of antibiotics to farm animals, as a stressor to select AMR genes, is only one explanation and AMR can be driven by acquisition of mobile genes that existed in bacteria and evolved over time in the environment.

Poultry Manure as a Reservoir for Resistant Genes

Approximately 6.9 kg per 1000 kg live weight per day is produced for a typical broiler operation, or 0.6–1.8 Mg per 1,000 broilers per flock (dry weight basis; American Society of Agricultural and Biological Engineers [ASABE] (2005); Moore, 2011), and there is concern that its land application may transport AMR microorganisms and genes to the environment, along with excreted drug residues from birds given antimicrobials, and residual disinfectants used in cleaning. For these reasons, AMR bacteria may be able to spread to the environment by application of litter to soils, which could possibly contribute to an increased frequency of horizontal gene transfer in the soil environment. Land application of poultry litter may also increase the diversity and dissemination of novel gene fragments among soil bacterial populations (Heuer et al., 2009). Research from Binh et al. (2009) indicated that the clinically relevant AMR gene, *aadA* (encoding resistance to streptomycin and

spectinomycin), was introduced via poultry manure into soil. Cook et al. (2014) also evaluated AMR genes in land applied poultry litter and found that litter-borne AMR bacteria flourish following applications.

Typical antibiotic concentrations in manure range from 1 to 10 mg kg⁻¹ (Kumar et al., 2005; Dolliver et al., 2007; Heuer et al., 2011), whereas soil and water concentrations range from trace to µg kg⁻¹ or µg L⁻¹, respectively (Thiele-Bruhn, 2003). In a comparison of indoor and free-range production systems, He et al. (2014) found that ARG in soil were positively correlated with antibiotic, metal, and nutrient concentrations. These data also suggest that both direct selection and co-selection mechanisms contribute to the suite of AMR genes detected. In the following section, authors discuss current approaches for decreasing the likelihood of AMR genes in U.S. broiler operations, as well as mitigation strategies for reducing AMR development.

Current Approaches for Decreasing AMR in Poultry Operations in the U.S.

The development and transmission of AMR determinants in microbial communities in poultry gastrointestinal tracts or on poultry products is a complex phenomenon fueled by a plethora of biotic and abiotic factors. Current approaches for decreasing AMR in poultry operations consist of coordinated multidisciplinary strategies aimed at developing new drugs and antibiotic alternatives and management approaches, and reducing total antibiotic usage (Food and Drug Administration [FDA], 2013). A brief description of each approach is provided below.

Antibiotic Alternatives-Prebiotics, Probiotics, and Antimicrobial Compounds

Development of new antimicrobial drugs is a very labor intensive, time consuming and costly pursuit. More than 20 classes of antibiotics were identified from 1930 to 1962 (Coates et al., 2002). Since then, however, only a few classes of antibiotics have been approved (Butler and Buss, 2006). Other antimicrobial compounds such as antimicrobial peptides, peptidomimetics (Mojsoska and Jenssen, 2015), and virulence inhibitors (Mühlen and Dersch, 2015; Muhs et al., 2017) are being investigated for their efficacy against poultry pathogenic bacteria. Although found to be effective, their application in the industry would require significant industry-level testing and standardization.

Research is also currently being conducted to identify potent antibiotic alternatives that could provide both growth promotion for poultry and defense against microorganisms (Ricke, 2015; Upadhyaya et al., 2015a,b; Ricke, 2018). Prebiotics, probiotics, and antimicrobial compounds are the three major groups that are added to poultry water to reduce pathogenic bacteria colonization in the gut and subsequent contamination of poultry products. The efficacy of antibiotic alternatives on reducing *Campylobacter* colonization has been summarized in this review (Table 2). Prebiotics are defined as substrates that are selectively utilized by host microorganisms conferring

a health benefit (Gibson et al., 2017). These beneficial effects could be through nutritional supplementation of beneficial microorganisms and/or through imparting resistance to pathogenic bacteria colonization. Fructans and galactans are examples of popular prebiotics with several research investigations highlighting their effect in enriching beneficial gut bacteria such as *Lactobacillus* and/or *Bifidobacterium* spp. (Bajury et al., 2017). With advances in microbiome research, our understanding of gut microbiota composition and substances that modify the microbiota has improved. This has expanded the prebiotic concept to include new compounds such as yeast-based products (Park et al., 2017) and dietary fibers (Ricke, 2015, 2018).

Probiotics are live microorganisms, which when administered at adequate dosages, confer a health benefit on the host (World Health Organization [WHO], 2011). The major mechanisms by which probiotics act include competitive exclusion, improving barrier function, immunomodulation, and metabolic effects such as quorum sensing mitigation and virulence modulation in pathogenic bacteria (Oelschlaeger, 2010). In addition to their applications in human nutrition, probiotics are increasingly being supplemented in poultry feed for their health benefits. The commonly used genera include *Bifidobacterium*, *Bacillus*, *Lactobacillus*, and *Lactococcus*. Several probiotics have been reported to decrease the colonization of *Campylobacter* in the gastrointestinal track of broilers (Eeckhaut et al., 2016). This ability of probiotics to reduce poultry associated foodborne pathogenic bacteria could be due to their ability to promote beneficial gut bacteria that may exclude pathogens. For example, probiotic strains of human origin-*Lactobacillus rhamnosus* GG, *Propionibacterium freudenreichii* spp. *shermanii* JS, and *Lactococcus lactis* spp. *lactis* were found to attach to chicken intestinal mucus thereby reducing the binding and colonization of *Campylobacter* (Ganan et al., 2013). In addition to *Campylobacter*, several probiotic candidates have shown efficacy in reducing colonization of *Salmonella* spp. *in vitro* (Muyyarikkandy and Amalaradjou, 2017; Nair and Kollanoor Johny, 2017) and in poultry (Higgins et al., 2008).

Another management practice that could reduce the dissemination of AMR genes is the use of plant extracts. Plant-derived compounds represent a relatively safe, effective, and environmentally friendly source of antimicrobials. Plant extracts have been used in many cultures as food preservatives and dietary supplements for reducing spoilage and promoting growth. Due to their low cost, non-toxic nature, and antimicrobial efficacy, several phytochemicals are promoted as in-feed or in-water (nanoemulsion forms) supplements for reducing poultry pathogenic bacteria colonization. Extensive research in the last 2 decades has identified a plethora of compounds with antimicrobial efficacy (Gracia et al., 2016; Guyard-Nicodeme et al., 2016). Compounds such as caprylic acid (obtained from coconut oil), trans-cinnamaldehyde (from cinnamon bark), carvacrol (from oregano oil), and eugenol (from clove oil) have found to be effective in controlling *Salmonella* and *Campylobacter* in poultry (Kollanoor Johny et al., 2010, 2012; Arsi et al., 2014; Upadhyaya et al., 2015a,b). Medium chain fatty

TABLE 2 | The efficacy of antibiotic alternatives (phyto chemicals, probiotics, and probiotics and prebiotics) on reducing *Campylobacter* colonization and counts in broilers.

Phyto chemicals	Solis de los Santos et al. (2008; 2009; 2010) demonstrated that in feed supplementation of Caprylic acid, a medium chain fatty acid consistently reduced <i>Campylobacter</i> colonization in broiler chickens. Kollanoor Johny et al. (2010) previously reported the <i>in vitro</i> ability of thymol and carvacrol to inhibit both <i>Campylobacter jejuni</i> and <i>Salmonella</i> Enteritidis in chicken cecal contents. Results from Kollanoor Johny et al. (2012) suggest that Transcinnamaldehyde and Eugenol supplemented through feed could reduce <i>Salmonella</i> Enteritidis colonization in market-age chickens. Upadhyaya et al. (2013) revealed that antimicrobial wash with Eugenol or carvacrol rapidly inactivated <i>S. Enteritidis</i> on eggs to below detection limit at 32°C. Arsi et al. (2014) reported that in feed supplementation of plant extracts such as thymol or carvacrol reduced <i>Campylobacter</i> colonization in broiler chickens. Wagle et al. (2017a) demonstrated that supplementation of β-resorcylic acid in poultry feed for 14 days at 0.5 and 1% reduced <i>Campylobacter</i> populations in cecal contents by ~ 2.5 and 1.7 Log CFU/g, respectively. Use of select doses of β-resorcylic acid showed significant reduction of <i>C. jejuni</i> on chicken skin and meat samples (Wagle et al., 2017b).
Probiotics	Aguiar et al. (2013) selected isolates for enhanced motility and the results from this study demonstrated that motility-enhanced isolates are more efficacious than unenhanced isolates in reducing <i>Campylobacter</i> colonization in broiler chickens. Arsi et al. (2015a) screened 116 isolates of probiotic strains and reported that six out of 116 strains reduced <i>Campylobacter</i> counts by at least 1–2 log <i>in vivo</i>.
Probiotics and prebiotics	In a separate study, Arsi et al. (2015b) demonstrated that prebiotics did not consistently reduced <i>Campylobacter</i>. However, prebiotics (MOS) did significantly decrease the <i>Campylobacter</i> load when used in combination with <i>Probiotics</i> spp. (Arsi et al., 2015b).

acids emulsion (caproic, caprylic, capric, and lauric acids) also reduce *Campylobacter* survival in drinking water and feed (Solis de los Santos et al., 2008, 2009; Solís de los Santos et al., 2010). Similar anti-*Campylobacter* efficacy has been reported with the addition of monocaprin emulsion (Thormar et al., 2006) thyme, and pine oil (Ozogul et al., 2015) in poultry feed. Research is still needed on how the use of these compounds may disrupt ARGD moment and fate in the environment.

Management Approaches for Controlling AMR Development

Identifying feasible management practices is one of the objectives of the USDA Action Plan to control AMR development in animal agriculture (United States Department of Agriculture [USDA], 2014). Establishing good farm practices, maintaining proper hygiene, controlling vectors that transmit poultry pathogens, reducing stress in poultry during housing and transport, and identification of Critical Control Points during processing that contribute to AMR development are some of the key areas that hold promise and require detailed investigations. Scientifically, validated studies are required to test the effect of aforementioned factors on AMR development in poultry production and develop appropriate recommendations for the industry.

There is some evidence to suggest organic practices may also reduce the spread of AMR genes to the environment (Rothrock et al., 2016). For example, when comparing the numbers of infected hens from conventional and organic farms, hens from organically grown farms were less infected by *Campylobacter* than from conventional grown farms (Lestari et al., 2009; Kassem et al., 2017). *Campylobacter* isolated from organically grown hens exhibited significantly lower resistance to three antibiotics: ciprofloxacin, erythromycin, and tylosin (Kassem et al., 2017). However, the study from Noormohamed and Fakhr indicated that multidrug resistance existed in both organic and

conventional farms (Noormohamed and Fakhr, 2014). Lestari et al. (2009) also provided differences of AMR patterns between conventional and organic chicken. Among 126 *Salmonella* isolates from conventionally and organically raised chicken carcasses obtained from retail stores in Louisiana, *Salmonella* isolates from organic chicken samples were susceptible to 11 of the antimicrobials, while isolates from conventional chickens were only susceptible to 4 antimicrobials (Lestari et al., 2009). However, it is still too early to conclude that organic chickens are less likely to harbor AMR than conventionally raised chickens.

CONCLUDING REMARKS

Antibiotic resistance is a common ecological feature in soil, as is antibiotic production, therefore, AMR genes are ubiquitous and represent a reservoir of transferable genetic material. In addition, resistance is an advantageous trait for microorganisms surviving stressful environmental conditions. Only since the 1970s has it been realized that soils receiving poultry litter may be a major reservoir and transmission route for ARG. Thanks to advances in molecular biology, bioinformatics, and sequence data throughput, much more data are available on resistance genes, as well as the complex matrix that is soil and poultry litter. However, untangling the complexity of microbial ecology and environmental factors (e.g., particle size, pH, water availability, vegetation cover etc.) as it relates to transfer (transformation, conjugation or transduction) of genetic resistance is a multifaceted issue and widely considered a key challenge facing agriculture.

A major challenge facing microbiologists is to track dissemination of resistance genes in poultry production systems and identify reservoirs of resistance genes. Understanding factors that drive selection and dissemination of environmental antibiotic resistance, as well as mitigation strategies that

will reduce the environmental dissemination of ARG. Future improvements in monitoring AMR movement in surface water from land-applied poultry litter will be critical to prevent the spread of resistance genes in the environment.

The pathogenic bacteria pathway from animals through the environment is complicated and more research is needed to follow AMR genes through these systems using the One Health Approach. Finally, owing to consumer demand for antibiotic-free meat products, much research has been done on promising antibiotic replacements (e.g., prebiotics, probiotics, and antimicrobial compounds). However, further research is needed on their efficacy and influence on AMR gene movement from farm-field.

AUTHOR CONTRIBUTIONS

YY, AA, KC, CW, and AU assisted in writing sections of the manuscript, as well as conceived of the outline and overall

direction of this manuscript. PO, SR, JD, and PM provided final edits and direction on the current state of the literature, as well as guidance on soil and poultry microbiology.

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